Paradoxical Effect of Isoniazid on the Activity of Rifampin-Pyrazinamide Combination in a Mouse Model of Tuberculosis

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To investigate the antagonism between isoniazid (INH) and rifampin (rifampicin) (RIF)-pyrazinamide (PZA) combination observed in Mycobacterium tuberculosis-infected mice, extensive pharmacokinetic studies of INH were performed and followed by experiments to assess the impact of increasing doses of INH on the antimicrobial activity of RIF-PZA combination. INH at 6.25 mg/kg of body weight produced a maximum concentration of drug in serum ($C_{\text{max}}$) value of 4 μg/ml and an area under the concentration-time curve from 0 to 24 h ($\text{AUC}_{0–24}$) value of 4.9 μg · h/ml, the former being close to the $C_{\text{max}}$ value observed after the standard 5-mg/kg dose in humans. INH at 25 mg/kg produced a $C_{\text{max}}$ value of 22 μg/ml and an $\text{AUC}_{0–24}$ value of 29 μg · h/ml, the latter being close to the AUC observed after a 5-mg/kg dose of INH in humans with the slow acetylation phenotype. Beginning 2 weeks after aerosol infection with M. tuberculosis, mice were treated for 8 weeks with INH at twofold-increasing doses, ranging from 1.56 to 50 mg/kg, either alone or in combination with RIF-PZA. Given alone, INH exhibited dose-dependent activity. Combined with RIF-PZA, INH exhibited dose-dependent antagonism of RIF-PZA activity. To determine the individual components of RIF-PZA combination with which INH was antagonistic, mice were treated for 8 weeks with RIF alone, PZA alone, RIF-PZA, and INH at 3.125, 12.5, or 50 mg/kg either alone or combined with RIF or PZA. Addition of INH to RIF had additive activity, whereas addition of INH to PZA resulted in a negative interaction. Finally, a 10-mg/kg dose of INH in mice may best represent the 5-mg/kg dose in humans and decrease the antagonism of INH with RIF-PZA.

The addition of rifampin (rifampicin) (RIF) and pyrazinamide (PZA) to the standard treatment of tuberculosis (TB), based on the combination of streptomycin, isoniazid (INH), and para-aminosalicylic acid, shortened the duration of treatment necessary to prevent relapse and led to the current 6-month short-course regimen (5, 6, 10). The decisive treatment-shortening effect of RIF and PZA is also observed in the murine model of TB (2). However, in the murine model, the combination of RIF and PZA has consistently been found to be more effective than the three-drug combination of RIF, PZA, and INH (9, 13, 14, 17), suggesting that INH has a negative impact on the activity of RIF-PZA combination. Whether this phenomenon also occurs in humans is unknown because the risk of the emergence of drug resistance has always mandated the inclusion of INH in RIF-PZA-containing regimens studied in clinical trials.

Even though the apparent antagonism between INH and RIF-PZA combination has been described to occur in the murine model for quite some time, it is not yet determined whether it is an artifact of the murine model related to the relatively high daily dose of INH given to mice compared to the dose given to humans. The daily dose of INH in mice is usually 25 mg/kg of body weight (3) to compensate for the rapid inactivation of INH in mice and still match the area under the serum concentration-time curve (AUC) observed with the standard 5-mg/kg dose used in humans. Therefore, it is possible that the high peak serum concentrations of INH produced by the 25-mg/kg dose used in mice (10) result in an adverse interaction with RIF and/or PZA in the mouse that does not occur with the INH concentrations produced by standard doses used in humans. To test this hypothesis, we performed pharmacokinetic studies of INH administered at doses relevant to human doses and then conducted an experiment in which we assessed the antimicrobial activity of increasing by twofold the doses of INH, ranging from 1.56 to 50 mg/kg, given either alone or in combination with RIF-PZA. After observing antagonism even with low, nearly inactive doses of INH, we then investigated whether antagonism was observed when INH was combined with RIF or PZA individually.

MATERIALS AND METHODS

Antimicrobials. INH and RIF were purchased from Sigma (St. Louis, MO), and PZA was purchased from Fisher Scientific International (Suwanee, GA). Stock solutions were prepared weekly in distilled water, as previously described (18). All antibiotic solutions were stored at 4°C.

Pharmacokinetics of INH. To determine the single-dose pharmacokinetic profiles of INH in mice, 63 female BALB/c mice, at 6 to 7 weeks old (Charles River, Wilmington, MA), received a single oral dose of INH at 1.56 mg/kg, 6.25 mg/kg, or 25 mg/kg by gavage. Three mice from each group were anesthetized with isoflurane and exsanguinated by cardiac puncturing at 15 and 30 min and 1, 2, 4, 6, and 8 h after dosing. The experiment was performed in triplicate. Serum samples were separated and stored at −80°C before being shipped overnight on dry ice to the Infectious Disease Pharmacokinetics Laboratory, National Jewish Medical and Research Center, Denver, CO. Drug concentrations in the mouse serum samples were determined using validated high-performance liquid chromatography methods. Serum concentration data were entered into a WinNonlin®

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‡ Published ahead of print on 20 July 2009.
worksheet (WinNonlin version 4.0, 2002; Pharsight, Mountain View, CA) and analyzed using standard noncompartamental techniques in order to determine the relevant pharmacokinetic parameters.

**Bacterial strain.** Mycobacterium tuberculosis H37Rv was passaged in mice, frozen in 1-ml aliquots, and stored at −80°C before use. For each infection, an aliquot was thawed and subcultured in Middlebrook 7H9 broth supplemented with 10% oleic acid-albumin-dextrose-catalase (Difco, Detroit, MI) and 0.05% Tween 80 (Sigma).

**Aerosol infection.** In all experiments, female BALB/c mice (Charles River, Wilmington, MA) aged 4 to 6 weeks old were infected by the aerosol route using the inhalation exposure system (Glas-Col Inc., Terre Haute, IN) and a log-phase broth culture (Difco 7H9 with 10% oleic acid-albumin-dextrose-catalase and Tween 80) with an optical density at 600 nm of approximately 1.0. The mice were randomized into treatment groups (five mice per group per time point) after aerosol infection. Untreated mice were routinely killed (i) on the day of infection to determine the numbers of CFU implanted in the lungs and (ii) on the day of treatment initiation to determine the pretreatment CFU count. Quantitative lung cultures were performed on selective 7H11 plates (Becton-Dickinson, Sparks, MD), as described previously (18). All procedures involving animals were approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

**Drug treatment.** In all experiments, antibiotics were administered once daily, 5 days per week, for 2 weeks at doses by gavage. Treatment was initiated 14 days after infection (D0), and the drugs were administered at the following doses: RIF at 10 mg/kg, PZA at 150 mg/kg, and INH at doubling doses from 1.56 to 50 mg/kg. The doses of RIF and PZA were selected to match the AUC value obtained with recommended human dosages (9). RIF was given 1 h prior to administration of the other drugs to avoid any adverse pharmacokinetic interaction (2, 3, 9).

In the first experiment testing the impact of increasing doses of INH on the activity of RIF-PZA combination, 549 infected mice were treated for 8 weeks with INH at doubling doses ranging from 1.56 to 50 mg/kg either alone or in combination with RIF-PZA (RIF-PZA + INH). A control group was treated with RIF-PZA only. The treatment was continued for 20 weeks, with groups receiving RIF-PZA + 6.25 mg/kg INH, RIF-PZA + 25 mg/kg INH, or RIF-PZA, although PZA was discontinued after the 8-week initial phase.

In the second experiment testing the impact of increasing doses of INH on the activity of the two-drug combinations of INH-RIF and INH-PZA, 215 infected mice were treated for 8 weeks with INH at 3.125, 12.5, and 50 mg/kg either alone or in combination with RIF or with PZA. Control groups included mice treated with RIF alone, PZA alone or RIF-PZA combination.

**Assessment of treatment efficacy.** Treatment efficacy was assessed on the basis of the number of lung CFU. To assess bacterial activity, five mice from each treatment group were sacrificed after 1, 2, 4, and 8 weeks of treatment in the first experiment and after 2, 4, and 8 weeks in the second experiment for quantitative lung CFU counting. Lungs were removed under aseptic conditions, placed in phosphate-buffered saline, homogenized, and plated on selective 7H11 plates (Becton-Dickinson, Sparks, MD), as previously described (18). Plates were incubated for 4 weeks at 37°C in a 5% CO₂ environment before CFU counts were determined.

**Detection of INH resistance.** All mice treated with 1.56 to 30 mg/kg INH alone were evaluated after 8 weeks of treatment to determine if selection of INH-resistant mutants had occurred. Aliquots (0.5 ml) of lung homogenate were plated undiluted on selective 7H11 plates containing 0.2 µg/ml of INH. When an increased proportion of resistant mutants compared to the pretreatment value was detected in a treatment group, the extent of INH resistance was determined for two mice per group by suspending the colonies isolated on INH-containing plates in phosphate-buffered saline and inoculating them on 7H11 plates with INH concentrations of 0, 0.1, 0.2, 1, 10, or 25 µg/ml.

Similarly, in the second experiment, mice were evaluated for selection of INH resistance after 8 weeks of treatment with 3.125 or 12.5 mg/kg INH alone. This was done by plating 0.5 ml of the undiluted lung homogenate onto selective 7H11 plates containing 1 or 10 µg/ml of INH.

**Mutation analysis.** Representative INH-resistant colonies (about 10) from each of the groups treated with INH doses ranging from 3.125 to 50 mg/kg were first screened for catalase activity using the catalase drop method (20). All those that were negative for catalase activity were not evaluated further, as absence of catalase activity was assumed to indicate mutations occurred in the katG gene. INH-resistant mutants with questionable or detectable catalase activity were subjected to mutation analysis. The entire katG gene was amplified by PCR using four overlapping primer sets. The inhA promoter region was also amplified using specific primers. The resulting PCR products were then sequenced to determine the presence of mutations.

**RESULTS**

**Pharmacokinetics of INH.** Results are displayed in Fig. 1 in comparison with human data (19). The lowest dose of INH in mice, 1.56 mg/kg, produced a maximum concentration of drug in serum ($C_{max}$) value of 0.8 µg/ml and an AUC from 0 to 24 h ($AUC_{0-24}$) value of 0.9 µg·h/ml. The 6.25-mg/kg dose of INH in mice produced a $C_{max}$ value of 4 µg/ml similar to the $C_{max}$ of 4.37 µg/ml observed in humans after a 5-mg/kg dose (19). The $AUC_{0-24}$ value obtained was 4.9 µg·h/ml, much lower than the $AUC_{0-24}$ of 22 µg·h/ml observed in humans with the slow acetylator phenotype but just below the AUC0–24 value of 6.20 µg·h/ml observed in rapid acetylators. The 25-mg/kg dose of INH in mice, long considered the human equivalent dose, produced a $C_{max}$ value of about 22 µg/ml, five times greater than that observed in humans after a 5-mg/kg dose, and an $AUC_{0-24}$ value of about 29 µg·h/ml, similar to the AUC in slow acetyling humans. Thus, a 6.25-mg/kg dose of INH in mice matches the human dose on the basis of $C_{max}$ but produces a lower $AUC_{0-24}$, while the 25-mg/kg dose of INH in mice significantly overshoots the $C_{max}$ but matches the AUC0–24 for slow acetylators.

**First experiment.** Mice were infected by aerosol with M. tuberculosis in four successive runs. The day after infection (D-13), mean lung log10 CFU counts were 4.61 ± 0.06, 4.61 ± 0.09, 4.44 ± 0.03, and 4.51 ± 0.14 for mice infected in runs 1 to 4, respectively (mean, 4.54 ± 0.10). Fourteen days after infection, on D0, the mean lung log10 CFU counts had increased to 8.02 ± 0.13, 8.12 ± 0.16, 7.84 ± 0.19, and 7.61 ± 0.23 for mice infected in runs 1 to 4, respectively. The mean lung log10 CFU count at the time of treatment initiation was therefore 7.90 ± 0.25.

All untreated mice died within three weeks of infection. All mice treated with INH alone survived and responded well to the treatment (Table 1 and Fig. 2A), except for mice receiving 1.56 mg/kg INH, which experienced an overall modest decrease in the log10 CFU counts, indicating that this dose prevented death but only just contained the infection. The other groups receiving INH exhibited dose-dependent reductions in log10 CFU counts, with significant differences in mean CFU counts among the mice treated with 3.125, 6.25, and 12.5 mg/kg INH ($P < 0.05$). Beyond 12.5 mg/kg INH, the additional benefit of increasing INH doses was less evident ($P > 0.05$).

The opposite effect of increasing doses was observed when INH was added to RIF-PZA combination (Table 1; Fig. 2B). In mice treated with RIF-PZA only, the reduction in log10 lung CFU counts was limited at week 2 of treatment and was dramatic beyond, as follows: 2 log10 CFU observed at week 4 and an additional 3 log10 CFU observed at week 8. As early as week 4 of treatment, a trend was clearly evident: the addition of increasing doses of INH to RIF-PZA combination was associated with decreasing activity. At week 8 of treatment, the...
increase in lung CFU counts compared to those of RIF-PZA was 0.6 log_{10} CFU ($P$ < 0.05), with the addition of 1.56 mg/kg INH, a dose having only weak inhibitory activity when given alone; it was 0.85 ($P$ < 0.01) and 1.2 ($P$ < 0.001) log_{10} CFU, with the addition of 3.125 and 6.25 mg/kg INH, respectively, and was 1.5 to 2.0 log_{10} CFU ($P$ < 0.001) with the addition of 12.5, 25, and 50 mg/kg INH.

Mice treated during the first 8 weeks with RIF-PZA alone, and RIF-PZA combined with either 6.25 mg/kg INH, which recapitulates the 5-mg/kg dose in human rapid acetylators, or 25 mg/kg INH, which produces an AUC similar to that observed with the 5-mg/kg dose in human slow acetylators, albeit with a significantly higher $C_{\text{max}}$ value, were kept on treatment up to the 20th week to determine the impact of the INH dose during the continuation phase of treatment. Mice received the same drug doses as during the first 8 weeks of treatment, but PZA was withdrawn as usual after the initial 8 weeks (1). As shown in Table 1, the mean CFU count in mice receiving 8

![FIG. 1. Serum concentration-time curves for mice treated with three different doses of isoniazid compared with human data (dashed lines) from the work of Peloquin et al. (19). Conc., concentration.](image)

### TABLE 1. Lung CFU counts from the first experiment

<table>
<thead>
<tr>
<th>Drug regimen</th>
<th>Mean lung log_{10} CFU counts ± SD at the indicated time points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>7.90 ± 0.25</td>
</tr>
<tr>
<td>INH_{1.56}</td>
<td>8.10 ± 0.17</td>
</tr>
<tr>
<td>INH_{2.5}</td>
<td>7.50 ± 0.19</td>
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<tr>
<td>INH_{3.125}</td>
<td>7.44 ± 0.22</td>
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<td>INH_{5}</td>
<td>7.25 ± 0.09</td>
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<tr>
<td>INH_{50}</td>
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<td>INH_{6.25}</td>
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<tr>
<td>INH_{25}</td>
<td>7.41 ± 0.27</td>
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<tr>
<td>2RIF-PZA/4RIF</td>
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<tr>
<td>RIF-PZA+INH_{1.56}</td>
<td>7.44 ± 0.08</td>
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<tr>
<td>RIF-PZA+INH_{12.5}</td>
<td>7.34 ± 0.15</td>
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<tr>
<td>2RIF-PZA+INH_{12.5}/4RIF+INH_{25}</td>
<td>7.33 ± 0.33</td>
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<tr>
<td>RIF-PZA+INH_{12.5}</td>
<td>7.33 ± 0.33</td>
</tr>
<tr>
<td>2RIF-PZA+INH_{12.5}/4RIF+INH_{25}</td>
<td>7.33 ± 0.28</td>
</tr>
<tr>
<td>RIF-PZA+INH_{50}</td>
<td>7.50 ± 0.17</td>
</tr>
</tbody>
</table>

*Drugs and doses: rifampin (RIF), 10 mg/kg; pyrazinamide (PZA), 150 mg/kg; isoniazid (INH), dose in mg/kg indicated by subscripted numbers. The number preceding the regimen indicates the duration of the treatment in months. For example, 2RIF-PZA+INH_{12.5}/4RIF+INH_{25} indicates the standard regimen of 2 months of RIF-PZA+INH, followed by 4 months of RIF+INH, with a dose of 25 mg/kg INH throughout.*

*W, week.*

* A total of 2/5 mice had no detectable numbers of CFU, and the remaining had 1 CFU each.

* A total of 4/5 mice had no detectable numbers of CFU, and the remaining had 1 CFU.
weeks of RIF-PZA followed by RIF alone decreased by 0.7 log_{10} CFU between the 8- and 12-week time points. In mice treated with 8 weeks of RIF-PZA+6.25 or 25 mg/kg INH followed with RIF+6.25 or 25 mg/kg INH, the mean CFU counts decreased by nearly 2 log_{10} CFU between the 8- and 12-week time points. This suggests that the addition of INH at 6.25 or 25 mg/kg strongly and equally increased the antimicrobial activity of RIF. After 16 weeks of treatment, all mice were culture negative or nearly culture negative. After 20 weeks of treatment, they were all culture negative.

Second experiment. Having shown that INH antagonizes the activity of RIF-PZA combination in proportion to the size of its dose, we investigated the effect of increasing doses (3.125, 12.5, and 50 mg/kg) of INH on the activity of RIF and PZA individually. In brief, we tested the activity of INH alone, RIF alone at 10 mg/kg, and PZA alone at 150 mg/kg and the impact of increasing doses of INH on the activity of RIF and PZA separately.

Mice were aerosol infected with M. tuberculosis in two successive runs. The day after infection (D-13), the mean lung log_{10} CFU counts were 3.90 ± 0.09 and 3.90 ± 0.14 for mice infected in runs 1 and 2, respectively (mean, 3.90 ± 0.11; P = 0.95). Fourteen days after infection, at treatment initiation (D0), the mean lung log_{10} CFU counts had increased to 7.05 ± 0.16 and 7.13 ± 0.16 for mice infected in runs 1 and 2, respectively (mean, 7.09 ± 0.16; P = 0.45).

Ten of fifteen untreated control mice died 4 weeks after infection. The remaining five mice were moribund and were sacrificed. The mean log_{10} CFU count obtained in these mice was 8.05 ± 0.17. None of the treated mice died, even those treated with PZA alone, despite experiencing an initial increase in their lung CFU counts to 7.62 ± 0.07. All other treated mice experienced decreases in the lung CFU counts.

In positive-control mice treated with INH alone, the mean lung log_{10} CFU counts declined over the 8 weeks of the experiment in a dose-dependent fashion similar to that observed in the first experiment (Table 2 and Fig. 3). Treatment with RIF alone for 2 weeks resulted in an insignificant reduction of 0.2 log_{10} CFU during the first 2 weeks, and underscoring that, at this dose, RIF is not a potent bactericidal drug against actively multiplying bacilli. But the limited activity over the first 2 weeks was followed by a greater bactericidal effect, resulting in a significant reduction of 2.3 log_{10} CFU between week 2 and week 8. For PZA alone, after the limited increase in lung CFU count in the first 2 weeks, an even more dramatic bactericidal activity ensued, resulting in an overall reduction of 3.2 log_{10} CFU between week 2 and week 8. Thus, both PZA and RIF exhibited bactericidal activity only after bacterial multiplication had been arrested in concert with the host immune response.

In the lungs of mice treated with the combination regimens, the addition of increasing doses of INH to RIF (RIF + INH) resulted in increasing bactericidal activity proportional to the INH dose size (Fig. 3A). It is remarkable that for each dose of INH tested, RIF-PZA combination resulted in a greater reduction in lung CFU counts than the single drugs tested. The addition of INH resulted in a greater bactericidal effect, with a significant reduction of 1.8 log_{10} CFU between week 2 and week 8. This suggests that the addition of INH at its dose, we investigated the effect of increasing doses (3.125, 12.5, and 50 mg/kg) of INH on the activity of RIF and PZA individually. In brief, we tested the activity of INH alone, RIF alone at 10 mg/kg, and PZA alone at 150 mg/kg and the impact of increasing doses of INH on the activity of RIF and PZA separately.

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### Table 2. Lung CFU counts from the second experiment

<table>
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<th>Drug regimen</th>
<th>Mean log_{10} CFU counts ± SD at the indicated time points</th>
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<td>Controls</td>
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<td>Untreated</td>
<td>7.09 ± 0.16</td>
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<td>INH_{12.5}</td>
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<td>INH_{2.5}</td>
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<td>INH_{0.125}</td>
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<tr>
<td>RIF</td>
<td>6.90 ± 0.08</td>
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<tr>
<td>PZA</td>
<td>7.62 ± 0.07</td>
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</table>

For Tests:

<table>
<thead>
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<th>Drug regimen</th>
<th>Mean log_{10} CFU counts ± SD at the indicated time points</th>
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</thead>
<tbody>
<tr>
<td>RIF-PZA</td>
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<td>RIF + INH_{12.5}</td>
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<td>RIF + INH_{0.125}</td>
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<td>PZA + INH_{12.5}</td>
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<td>PZA + INH_{0.125}</td>
<td>6.45 ± 0.31</td>
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*Drugs and doses: rifampin (RIF), 10 mg/kg; pyrazinamide (PZA), 150 mg/kg; isoniazid (INH), dose in mg/kg indicated by subscripted numbers.

\*W, week.

\*CFU counts from the remaining 5/13 control mice.
INH and at each time point, the combination RIF+INH was much more active than RIF alone as well as INH alone. Only RIF-PZA combination, which benefits from the synergistic interaction of RIF and PZA (Fig. 4), was more active than any RIF+INH combination at the week 4 and week 8 time points, for a total reduction of 6 log\textsubscript{10} CFU, similar to that seen in the first experiment.

The findings are much more confounding when increasing doses of INH are combined with PZA (Fig. 3B). Initially, after 2 weeks of treatment, all PZA+INH combinations were more active than PZA alone but not more active than INH alone, likely because the initial driver of the regimen effect is the potent bactericidal activity of INH. After 4 weeks of treatment, the combination of 3.125 mg/kg INH with PZA was slightly beneficial, but the combinations of 12.5 or 50 mg/kg INH with PZA were not more active than PZA alone or INH alone. After 8 weeks of treatment, the combinations of 12.5 and 50 mg/kg INH with PZA were detrimental, and even the combination of 3.125 mg/kg INH and PZA was not clearly beneficial.

**Selection of INH-resistant mutants.** During both experiments, the proportion of INH-resistant CFU isolated after 8 weeks of treatment with INH alone increased with increasing the INH dose size. The prevalence of resistant mutants at week 8 ranged from 5.0 \times 10^{-6} to 2.4 \times 10^{-5}, only 0- to 10-fold higher than that at treatment initiation (2.6 \times 10^{-6}), in mice treated with 1.56 mg/kg INH. In mice treated with 3.125 mg/kg INH, the prevalence of resistant mutants was 1.0 \times 10^{-5} to 2.5 \times 10^{-4}, about 10 times higher than in mice treated with 1.56 mg/kg, a likely consequence of the more intensive selection pressure of INH at this dose. In mice treated with 6.25 mg/kg INH, there again was a prevalence of resistant mutants (2.0 \times 10^{-4} to 4.6 \times 10^{-3}) 10 times higher than in mice treated with 3.125 mg/kg. The prevalence of INH-resistant mutants increased to 10^{-2} in mice treated with 25 and 50 mg/kg INH, suggesting that doses of INH above 6.25 mg/kg did not exert much more pressure on the selection of INH-resistant mutants than the 6.25-mg/kg dose. However, the level of INH resistance correlated with the dose size, as mice treated with 25 and 50 mg/kg INH harbored mutants resistant to INH concentrations as high as 25 \mu g/ml. These highly resistant mutants were all catalase negative. Mutants isolated from mice treated with INH doses of <12.5 mg/kg had lower levels of resistance, and a majority of them retained catalase activity.

**Mutation analysis of INH-resistant mutants isolated in mice treated with INH alone.** All INH-resistant colonies from mice treated with 25 and 50 mg/kg INH showed an absence of catalase activity, indicating a probable deletion or other mutation in the katG gene. No further characterization was done for these mutants. Of the 10 INH-resistant colonies screened from mice treated with 12.5 mg/kg INH, all were catalase negative except for one which showed doubtful catalase activity. Mutat-

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**FIG. 3.** Change in lung CFU counts in mice treated with increasing doses of isoniazid (H) given alone or in combination with rifampin (R) (A) or pyrazinamide (Z) (B).

**FIG. 4.** Comparative activities of 10 mg/kg rifampin (R) alone, 150 mg/kg pyrazinamide (Z) alone, and rifampin-pyrazinamide (RZ) in the lungs of mice treated 5 days a week.
tion analysis showed a single point mutation (M1A) in the start codon of the katG gene. The INH MIC against this strain was >8 μg/ml. Of the 10 INH-resistant colonies screened from mice treated with 6.25 mg/kg INH, 2 had weak or doubtful catalase activity. Both strains had single mutations in the katG gene, as follows: W149R (MIC 4 to 8 μg/ml) and D418Y (MIC not tested). Finally, among the 16 INH-resistant colonies screened from mice treated with 3.125 mg/kg INH, 7 had detectable catalase activity. One had doubtful catalase activity but lacked mutations in the katG gene or the inhA promoter region. The remaining six resistant mutants had good catalase activity, similar to that of wild-type H37Rv. These mutants had no mutation in the katG gene; however, all had a −8 T→A mutation in the promoter region of inhA. The INH MIC against these strains was 0.25 to 0.5 μg/ml. Thus, although it was not performed systematically, our overall mutation analysis reveals that higher doses of INH resulted in selection of mutants with absent or deficient catalase production and mutations in the katG gene, while the lowest dose of 3.125 mg/kg INH selected for mutants with low-level INH resistance caused by a mutation in the inhA promoter region.

**DISCUSSION**

The key result of the present work is to demonstrate that the antagonism between the combination of RIF-PZA and INH is dependent on the dose of INH. The higher the dose of INH alone given, the better the antimicrobial effect is. But the higher the dose of INH given in combination with RIF-PZA, the worse the antimicrobial effect is. The results suggest that this paradoxical effect of INH is unlikely to be simply an artifact of drug administration in the murine model, because it is observed even when serum concentrations of INH in mice are entirely within the range produced by standard human doses. For example, the antagonism is clearly evident in mice treated with INH at 6.25 mg/kg, for which the C max is 4 μg/ml, similar to the C max value observed in humans receiving 5 mg/kg. It is also clearly evident in mice treated with INH at 25 mg/kg, for which the AUC 0–24 is 29 mg · h/ml, close to the AUC observed in human slow acetylators of INH receiving 5 mg/kg. Therefore, it appears as a general phenomenon.

One may wonder about the clinical importance of this paradoxical phenomenon. The simplest and perhaps most reasonable response is to consider that the antagonism is the price to pay for having an excellent combined drug regimen that prevents the selection of drug-resistant mutants. In other words, INH has such a crucial role in the early bactericidal activity of the combination regimen and in preventing the emergence of resistance to companion drugs that one could continue to overlook its antagonistic effects. However, one can now imagine replacing INH during the initial phase with impunity by substituting another highly bactericidal drug, like moxifloxacin, which exhibits no antagonism with RIF-PZA combination (4, 17). At the end of the initial phase, when PZA is withdrawn, then INH could again be prescribed in combination with RIF because, paradoxically, INH and RIF also have a clear additive effect. Alternatively, INH and moxifloxacin could be used together with RIF-PZA for the first 1 to 2 weeks before dropping the INH, only to restart it again during the continuation phase. However, such drug juggling is not really suited to field implementation, and the results of the present work might well have no direct clinical implication but help in understanding the role of each antibiotic in the basic drug combination of INH+RIF-PZA. Clearly, there is antagonism between INH and RIF-PZA combination, but this antagonism does not result from antagonism between INH and RIF because these drugs have additive effects. Our data suggest that the antagonism involves INH and PZA, both structural analogues of nicotinamide (7, 16), and may be the subject of a direct antimicrobial interaction. In the past, McCune et al. (15) also raised a similar hypothesis because INH-PZA combination was less potent than INH alone during the initial 2 weeks of treatment. However, if it exists, such an antagonism subsequently disappeared, and the combination of INH and PZA was much more potent than INH alone in achieving culture conversion and preventing relapses in mice (8, 11) and also in humans (5, 6, 12). Other authors (8, 11) arrived at the same findings. As the mechanism of action of each of these drugs is still under discussion (22, 23), it is difficult to speculate about potential mechanisms of this antagonism. Further work is evidently needed to clarify these issues.

In addition to providing a better understanding of the mechanisms involved in the current drug regimen for tuberculosis, the present study has a practical implication for the experimental chemotherapy of TB in the mouse model. Until now, mice have been treated with 25 mg/kg of INH once daily for historical reasons (11). With this dosage, the C max value is about six times higher and the AUC is slightly greater than that obtained in human slow acetylators given 5 mg/kg of INH. With a daily dosage of 6.25 mg/kg INH, the C max is close to that obtained in humans given 5 mg/kg of INH, though obtained earlier and slightly higher. Because the half-life in mice is very short, the AUC, however, is close to that obtained in human fast acetylators given 5 mg/kg of INH. As the antagonism between INH and RIF-PZA combination is dose dependent, it seems reasonable to reduce the daily dose in mice to 10 mg/kg and, hence, have a C max close to the human C max and an AUC intermediate between that obtained in fast and slow acetylators given 5 mg/kg INH. Such a compromise will have little effect on the antagonism of INH and PZA against these strains was 0.25 to 0.5 μg/ml. Thus, although it was not performed systematically, our overall mutation analysis reveals that higher doses of INH resulted in selection of mutants with absent or deficient catalase production and mutations in the katG gene, while the lowest dose of 3.125 mg/kg INH selected for mutants with low-level INH resistance caused by a mutation in the inhA promoter region.

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