Investigation of elimination rate, persistent subpopulation removal and relapse rates of *Mycobacterium tuberculosis* by combinations of first-line drugs in a modified Cornell mouse model

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Running title: Rifampicin, isoniazid and pyrazinamide in a modified Cornell model

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ABSTRACT

Currently, the most effective tuberculosis control method resides in case-finding and 6 months chemotherapy. There is a need to improve our understanding about drug interactions, combination activities and the ability to remove persistent bacteria in the current regimens, particularly in relation to relapse. We aimed to investigate the therapeutic effects of three main components, rifampicin (RMP), isoniazid (INH), and pyrazinamide (PZA), in current drug regimens using a modified version of the Cornell mouse model. We evaluated the post-treatment levels of persistent *Mycobacterium tuberculosis* in the organs of mice using culture filtrate derived from *M. tuberculosis* strain H37Rv. When RMP was combined with INH, PZA or INH-PZA, significant additive activities were observed compared to each of the single drug treatments. However, the combination of INH and PZA showed a less significant additive effect than either of the drugs used on their own. Apparent culture negativity of mouse organs was achieved at 14 weeks of treatment with RMP-INH, RMP-PZA and RMP-INH-PZA but not with INH-PZA, when conventional tests, namely culture on solid agar and in liquid broth indicated that the organs were bacteria negative. The relapse rates for RMP-containing regimens were not significantly different to a 100% relapse rate at the numbers of mice examined in this study. In parallel, we examined the organs for the presence of culture filtrate-dependent persistent bacilli after 14 weeks of treatment. Culture filtrate treatment of the organs revealed persistent *M. tuberculosis*. Modelling of mycobacterial elimination rates and evaluation of culture-filtrate dependent organisms showed promise as surrogate methods for efficient factorial evaluation of drug combinations in tuberculosis in mouse models and should be further evaluated against relapse. The presence of culture filtrate-dependent persistent *M. tuberculosis* is the likely cause of disease relapse in this modified Cornell mouse model.

Key words: *Mycobacterium tuberculosis*, isoniazid, rifampicin, pyrazinamide, persistent bacilli, Cornell model

INTRODUCTION

Tuberculosis (TB) remains a major killer worldwide and is responsible for approximately two million deaths annually (1). The main obstacle for successful disease control resides in the ability of *M. tuberculosis* to persist in the host despite host immune responses and chemotherapy. Prolonged multi-drug antimicrobial therapy is necessary to achieve a cure, which leads to poor patient compliance, high relapse rates (7 - 13%) and the emergence of drug-resistance (2). Although short course TB therapy has been in clinical use for nearly four decades, the drug interactions and the ability to remove persistent bacteria with the current regimens have not been clearly demonstrated. Previous work in the murine Cornell model has shown that after 7 weeks of intensive treatment with isoniazid (INH) and pyrazinamide (PZA) to induce a latent infection, the follow-up treatment with rifampicin (RMP) alone, RMP-INH, RMP-PZA or RMP-INH-PZA exhibited very similar anti-tuberculosis activities (3). However, another study found that when mice were treated with INH-RMP-PZA, INH-RMP or RMP-PZA for 6 months, the RMP-PZA treated group demonstrated significantly lower relapse rates than the INH-RMP-PZA or INH-RMP groups (4). This study suggested that INH antagonised the actions of RMP-PZA (4) because INH in the regimen significantly reduced the Cmax and the area under the serum concentration-time curve of RMP in the mice (4) leading to higher relapse rates. The antagonism between INH and RMP-PZA was due to a negative interaction between INH and PZA in the combination and the effect was INH dose dependent (5). It was not clear what interaction INH has with each of the components in the regimens. To provide greater clarity, it is important to identify and evaluate the level of persistent bacilli after chemotherapy. This information is of clinical importance since combination therapy involving RMP-INH-PZA is commonly employed. Using appropriate

drug-combinations has the potential to maximise therapeutic effects whilst minimising side effects of multiple drug therapy. Furthermore, evaluation of post-treatment persister levels may serve as a biomarker to predict relapse rate (6). In this study, we examined the therapeutic effects of each of the components singly, in two-drug and three-drug combinations using a modified Cornell mouse model. We evaluated persistent *M. tuberculosis* using culture filtrate which was shown by others (7) to contain resuscitation promoting factors (RPF) in mouse organs from a population of mice of which a sample had apparently culture negative organs after long-term chemotherapy.

MATERIALS AND METHODS

Bacterium and growth condition. *M. tuberculosis* strain H37Rv was mouse-passaged and grown in 7H9 medium supplemented with 10% albumin dextrose complex (ADC; Becton and Dickinson, UK) and containing 0.05% Tween 80 at 37°C without disturbance for 15 days. The culture was subsequently frozen at -70°C for storage. To determine the viable counts prior to infection, colony forming unit (CFU) counting was performed prior to freezing and once again after thawing. CFU counts were carried out by plating serial 10-fold dilutions of the cultures on 7H11 agar medium supplemented with oleic albumin dextrose complex (OADC, Becton and Dickinson, UK). Colonies were counted after incubation of the plates at 37°C for 3 to 4 weeks and viability was expressed as Log CFU/ml. The cultures were subsequently diluted in phosphate-buffered saline (PBS) and used for inoculations in mice.

Modified Cornell mouse model. Rifampicin, isoniazid and pyrazinamide were tested singly or in double (RMP-INH, RMP-PZA and INH-PZA) or triple (RMP-INH-PZA) combinations using a modified Cornell mouse model which was based on the model previously established

in Cornell University (8, 9). The model was conducted using the experimental design and procedure described below.

(i) Infection of mice. Female BALB/c mice (6 to 8 weeks old) were obtained from Harlan UK Ltd. A total of 364 mice was infected intravenously via the tail vein with 1.2×10^5 CFU of mouse-passaged *M. tuberculosis* strain H37Rv per mouse as described previously (8, 10, 11). The animal husbandry guidelines and all animal experiments were performed according to the Animals Scientific Procedures Act, 1986 (an Act of the Parliament of the United Kingdom 1986 c. 14) (Home Office Project licence Number 70/7077) with approval from St George's, University of London ethics committee.

(ii) Chemotherapy. As shown in Table 1, mice were randomly allocated into eight groups. Control group consisted of infected and untreated mice; 4 of these were sacrificed at 2 hours after infection (D0) and 4 were killed at the beginning of treatment, D14 and D21 days after infection. The treatment groups were as follows: single drug treatment group, each contained 16 mice receiving RMP, INH or PZA, respectively, for 8 weeks. Combination groups, each contained 76 mice were administrated with RMP-INH, RMP-PZA, INH-PZA or RMP-INH-PZA, respectively, for 14 weeks. Single drug therapy started 14 days after infection, when a large bacterial load in the organs (the mean CFU counts reached 10^7 per lung or spleen) had been achieved with visible symptoms of disease. Combination therapy started at 21 days after infection. All groups were treated by daily oral administration (0.2 ml) for 5 days per week at the dosages of RMP 10 mg/kg, INH 25 mg/kg or PZA 150 mg/kg. The drug suspensions were prepared freshly for the daily dosage. In the combination containing RMP, RMP was administered 1 hour before the other drugs to avoid drug to drug interactions (4). Immediately after termination of 14 weeks of chemotherapy, the remaining mice were administered 0.5 mg/mouse of hydrocortisone acetate by daily oral administration for 8

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weeks to suppress host immune response. CFU counts from lungs and spleens were performed to determine disease relapse.

(iii) Assessment of infection and treatment efficacy. As seen in Table 1, to examine *M. tuberculosis* infection and baseline CFU counts before initiation of chemotherapy, 4 untreated control mice were sacrificed at 2 hours, day 14 and day 21 after infection, respectively. For assessment of treatment efficacy, 4 mice were sacrificed at the 2, 4, 6 and 8 weeks post treatment for single drug treatment to monitor CFU counts. For combination therapy, a sample of 8 mice was sacrificed at 2, 4, 6 and 8 weeks and 10 mice were used at 11 and 14 weeks of treatment (Table 1). Lungs and spleens from mice were removed rapidly after sacrifice and a sterile autopsy was performed. The organs were transferred into 2 ml tubes each containing 1 ml sterile distilled water and 2 mm diameter glass beads. Lungs and spleens of mice were homogenised using a reciprocal shaker (Thermo Hybaid Ltd) for 40 seconds at 6.5 speed. CFU counts from each lung and spleen were performed using serial dilutions of the homogenates. At 14th week treatment, the entire organ homogenates (the total volume of each organ homogenate was approximately 1.5 ml including the organ and 1 ml of water) from the 10 mice were aliquoted equally into three tubes which were used 1. CFU counting by addition of the homogenate to 2 ml of sterile distilled water following by plating out the entire organ homogenate suspension on 6 selective 7H11 agar plates, 2. culturing in 5 ml of selective Kirchner liquid medium by the addition of polymyxin B 200 U/ml, carbenicillin 100 mg/L, trimethoprim 20 mg/L and amphotericin B 10 mg/L (Selectatab, Mast Diagnostica GmbH) for 4 weeks with subsequent sub-culturing of the entire culture onto Löwenstein-Jensen slopes for a further 4 weeks and 3. resuscitation of persistent bacteria. Culture negative organs were defined as no colonies grown on 7H11 agar plates and no growth in selective Kirchner liquid medium following inoculation on Löwenstein-Jensen slopes.

Selection of RMP- and INH-resistant mutants in mice. At 4th, 6th and 8th week post treatment, mouse lung and spleen homogenates were plated on 7H11 plates containing RMP or INH concentration at two fold serial dilution from 1 to 64 mg/L. Colonies from the plates containing MIC value higher than 4 folds were picked and regrown in 7H9 medium. MIC was retested on RMP or INH containing 7H11 agar plates.

Resuscitation of *M. tuberculosis* in mouse lungs and spleens. For resuscitation of *M. tuberculosis* grown in mouse organs, culture filtrates containing RPFs were used as described previously (6, 7). *M. tuberculosis* H37Rv was grown in 7H9 medium for 15 to 20 days until an optical density of 1 to 1.5 was reached. The cultures were harvested by centrifugation at 3000 g for 15 minutes and sterilised by filtration with 0.2 µm filter (Sartorius) twice. The sterilised culture filtrates were made selective by addition of polymyxin B 200000 U/L, carbenicillin 100 mg/L, trimethoprim 20 mg/L and amphotericin B 10 mg/L (Selectatab, Mast Diagnostica GmbH) and immediately used for broth counting of the most probable number (MPN) of the bacilli (7). Broth counting of lungs and spleens after 14 weeks of combination therapy was performed as serial 10-fold dilutions in triplicate in which 0.5 ml of tissue homogenates were added to 4.5 ml of the culture filtrates. At 10-day intervals over a 2-month period of incubation at 37°C, the broth cultures were examined for visible turbidity changes. Growth of *M. tuberculosis* in turbid tubes was confirmed by colonial morphology on 7H11 agar plates. The MPN of viable bacilli was then estimated from the patterns of positive and negative tubes (7). The absence of microorganisms other than mycobacteria from turbid tubes was confirmed by plating on blood agar medium (Oxoid) and Sabouraud dextrose agar (Oxoid). In order to assess the sterility of culture filtrates free of *M. tuberculosis*, tubes containing culture filtrates were incubated at 37°C for 2 months to ensure the absence of *M. tuberculosis* in the culture filtrates.

Statistical analysis. A simple model for monoexponential bacterial growth and elimination (12) (Fig 1) was fitted to the profiles of CFU vs. time obtained experimentally. As simultaneously occurring exponential replication and death rates cannot be differentiated with this type of data, a "knet" exponential rate constant was estimated separately before treatment began ("knet_no_drug" where it would take a net positive value) and during treatment ("knet_with_drug" where it would take negative value). During therapy, knet is a 1st order elimination rate constant which can be interpreted as the slope of the modelled line fit through the logarithmic-transform of the data (with units in these data of wk^{-1}). Parameter estimation was carried out with nonlinear regression using the nonlinear least squares optimisation function "lsqnonlin" as part of the "pracma" package in the R statistical software language, with an objective function weighted by 1/(predicted value)^2. Standard errors of parameter estimates were calculated using the method outlined by Landaw et al. (13) with the Jacobian of model parameter sensitivities estimated using a numerical central difference method. The datasets comprised from multiple individual subject animals were treated as a naïve pool for data analysis purposes (14) rather than using the average of the data at each time-point. The significance of differences between model parameter estimates under different therapies was examined with pairwise Z-tests incorporating a Bonferroni correction of 21, where P values <0.002 would be considered significant. The significance of differences between the relapse rates was determined with pairwise Fisher's exact tests with a Bonferroni correction of 6, with P values <0.008 considered significant.

RESULTS

Survival of mice. During treatment, 4 mice died in the group of RMP-INH (1 at 9 weeks, 1 at 10 weeks and 2 at 12 weeks, 2 mice died in RMP-PZA (1 at 10 weeks and 1 at 12 weeks) and 3 mice died in the group of INH-PZA (1 at 9 weeks, 1 at 10 weeks and 1 at 13 weeks). The reason for the death was unknown but was most likely due to natural causes such as

tumour development or neurological disorders and was unrelated to tuberculosis or treatment. As the time of death was uncertain and also not at the sampling time point, organ bacterial counts were not determined from these animals. No mortality was observed during the course of single drug and RMP-INH-PZA treatments.

Treatment with RMP, INH and PZA singly and in two drug or three drug combination in a modified Cornell mouse model. We investigated the effect of RMP, INH and PZA singly and in double and triple combinations on the rate of bacterial eradication and relapse in a modified Cornell mouse model. The single dose of the drugs was tested in the animals for 8 weeks and terminated before resistant strain emergence (15). As shown in Table 2, Table 3 and Fig 2, RMP at 10 mg/kg, INH at 25 mg/kg or PZA at 150 mg/kg exhibited modest rates of bacterial eradication in both lungs and spleens showing 99% kill (2-log reduction) at around 8 weeks. The exponential rate constants (logarithmic base 10) for net bacterial elimination during treatment ($k_{net\_with\_drug}$) for RMP, INH and PZA were -0.21, -0.27 and -0.26 for lungs and -0.31, -0.29 and -0.26 for spleens (Table 4), respectively. Notably, the drop in CFU counts in both lungs and spleens during the first 2 weeks of treatment with the singly dosed drugs was minimal, though over the complete time course of therapy a clear monoexponential decline in CFU counts was observed. No RMP or INH resistant strains were isolated from 4 to 8 weeks of treatment. In addition, there was no significant difference in activities amongst each of the single drug treatments (Table S1 and S2 in the supplemental material). Interestingly, treatment with RMP combined with INH (Fig. 2A and 2E) or PZA (Fig. 2B and 2F) accelerated the rate of bacterial eradication showing 99% kill (Table 2 and Table 3) at 4 weeks of treatment for RMP-INH and at about 3 weeks for RMP-PZA with the estimation of $k_{net\_with\_drug}$ at -0.53 and -0.51 for lungs and -5.2 and -0.43 for spleens (Table 4), respectively. All the combined therapies were significantly more effective than the single therapy (Table S1 and S2 in the supplemental material). As seen in Table 2, Table 3, Fig. 2C

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and Fig. 2G, 99% kill with the RMP-INH-PZA combination was achieved at about 3 weeks for both lungs and spleens showing a similar elimination rate constant (-0.51 for lung and -0.48 for spleen) to RMP-INH or RMP-PZA (Table 4). There was no significant difference in efficacies amongst these RMP containing regimens against *M. tuberculosis* in this mouse model (Table S1 and S2 in the supplemental material). All the RMP containing combinations achieved undetectable *M. tuberculosis* CFU counts (Table 2 and Table 3) and negative broth growth in selective Kirchner liquid medium in murine lungs and spleens at 14 weeks of treatment. However, when INH was combined with PZA (Fig. 2D and 2H), there was no noticeably increased initial kill compared to each of the single drugs until 4 weeks of treatment followed by a reduction of CFU count showing a 99% kill at 5.6 weeks post treatment (Table 2) for lungs and 4 weeks for spleens (Table 3). This was reflected in the estimates for $k_{net\_with\_drug}$ for the INH and PZA combination, which was -0.42 and -0.44 for lungs and spleens, respectively (Table 4). Although the INH and PZA combinations failed to achieve undetectable *M. tuberculosis* CFU counts in murine lungs after 14 weeks of treatment (Fig. 2D and 2H), the difference in efficacies between the single drug treatment and the combination was significant (Table S1 and S2 in the supplemental material).

Relapse rate of treatment with RMP-INH, RMP-PZA and RMP-INH-PZA in the modified Cornell mouse model. After 8 weeks of immunosuppression with high dosage steroid, disease relapse rates for the treatments with double and triple regimens were determined by the percentage of mice that developed positive *M. tuberculosis* CFU counts in lungs, spleens or both. The organ relapse proportions for the four regimens are shown in Table 5. The treatment with the regimens of RMP-INH, RMP-PZA and RMP-INH-PZA yielded similar relapse rates at 85, 77.3 and 87.5%, respectively. These relapse rates were not significantly different amongst the three drug regimens or to a 100% relapse rate ($P>0.002$ for Fishers exact test including Bonferroni correction for multiple pairwise tests). The INH

and PZA combination was not able to produce negative organ CFU count at the termination of the 14 week treatment (Table 2 and Table 3).

Determination of persisters after treatment with four drug regimens. In order to determine the effect of the four combination regimens on the post-treatment level of persisters, we analysed lung and spleen homogenates at 14 weeks post-treatment using *M. tuberculosis* culture filtrate resuscitation (6). As shown in Table 6, at 14 weeks post-treatment, although CFU counts and growth in Kirchner liquid medium were negative for the drug regimens INH-RMP, RMP-PZA and INH-RMP-PZA, there were significant amounts of culture filtrate-dependent persisters present in lungs and spleens (1.89 log cells/lung and 2.09 log cells/spleen for RMP-PZA, 2 log cells/lung and 2.18 log cells/spleen for INH-RMP and 1.94 log cells/lung and 2.12 log cells/spleen for INH-RMP-PZA). After INH-PZA treatment, there were 4 log culture filtrate-resuscitated bacilli in both lungs and spleens. If we exclude CFU count positive bacilli, there were still 4-log culture filtrate-dependent persisters in the organs of INH-PZA treated mice.

DISCUSSION

In this study, we re-evaluated the current TB treatment regimen and studied the drug interactions by comparing the bacterial elimination rates, the number of culture filtrate-dependent bacteria present at treatment completion and relapse rates with different therapies in a mouse tuberculosis treatment model based on the model established at Cornell University over a half century ago (8, 9). This model enables us to determine anti-TB activities of combination regimens and, importantly, to measure relapse rates. It is characterized by the inoculation of a large number of bacteria intravenously to initiate an infection and the treatment of the disease once the infection has been established (2 to 3 weeks post infection). In this model, an intensive treatment is able to render mouse organs culture-negative on agar plates and in broth culture lacking culture filtrate, but fails to prevent relapse (10, 11).

However, these apparently culture-negative organs contained viable bacteria that could be cultivated by supplementing broth media with culture filtrate (6) containing RPFs (7). Significantly, we found that when RMP was combined with INH, PZA or INH-PZA, significant additive activities were observed compared to each of the single drug treatments. However, the combination of INH and PZA showed a less significant additive effect to either of the single drug treatments. The combination regimens of RMP-INH, RMP-PZA and RMP-INH-PZA exhibited equivalent treatment efficacies with very similar relapse rates which could not actually be differentiated from a 100% relapse rate, while INH-PZA failed to render organ culture negative after 14 weeks of treatment. Rifampicin-containing regimens reduced the number of culture filtrate-dependent persisters to a greater extent than INH-PZA, but did not eliminate them from mouse organs by the end of 14 weeks of treatment.

In humans, the key for treatment success depends on the bactericidal drugs INH and RMP which rapidly kill actively replicating bacilli in cavities and control disease progression (16) within the first two months of chemotherapy. This is defined by negative acid fast staining in sputum. In fact, bactericidal drugs such as INH exhibit bactericidal activity during the first 2 days of monochemotherapy (17). The need for prolonged treatment is due to the emergence of persistent bacilli which may arise in the heterogeneity of host environments (18). These persistent tubercle bacilli are undetectable by the traditional microbiological methods and become profoundly tolerant to bactericidal drugs (10). Sterilizing drugs such as PZA and RMP contribute to shortening of the treatment duration (18). However, in our study, comparing elimination rate constants for monotherapies in mice, there was no significant difference between RMP, INH or PZA. There was no superior bactericidal activity of INH, which contrasts with the effect of INH in humans. This indicates that treatment profiles are different between mice and humans.

Synergistic drug interactions have not been demonstrated in the treatment of TB in mice. It is generally accepted that more than a 2 log kill compared to the single drug defines a synergistic combination (19). Here we showed that enhanced bactericidal activities were achieved when RMP was combined with INH or PZA. Estimates of the elimination rate constant for all the combinations were significantly faster ($P < 0.0001$) than all single drugs (Table S1 and S2 in the supplemental material) showing 99% kill of the bacilli (a 2 log kill) achieved 4 to 5 weeks earlier than monotherapies. The activities of the combinations namely RMP-INH, RMP-PZA and RMP-INH-PZA shown by the value of the exponential elimination rate constant (Table 4) demonstrated significant additive interactions on the original scale. It is interesting therefore that the INH-PZA combination showed less enhanced effect than the singly dosed drugs at the earlier stage of treatment when there was a large number of actively growing organisms (10) and its increased efficacy compared to the monotherapies was more apparent after 6 weeks of treatment. This was in agreement with the previous findings that INH and PZA combination was more efficacious than the single drug in the reduction of organ bacterial counts and prevention of relapse rates in mice (8, 20) and in humans (21-23). Efficacy of all RMP containing regimens (INH-RMP, RMP-PZA and INH-RMP-PZA) in mouse tuberculosis treatment was very similar ($P>0.05$) as shown by the similarity of the elimination rate constants, which confirmed previous findings (3, 4) while INH-PZA therapy was less effective than other combination therapies ($P < 0.001$) (5). At the end of 14 weeks of treatment, lungs and spleens of mice treated with RMP/INH, RMP/PZA or RMP/INH/PZA became CFU count and broth count negative, conversely, the INH and PZA combination failed to achieve culture negativity in the mouse organs. After 8-weeks of steroid treatment, tubercle bacilli were found in the organs of mice treated with RMP/INH, RMP/PZA or RMP/INH/PZA. Although the elimination rates of the rifampicin containing regimens (RMP-INH, RMP-PZA and RMP-INH-PZA) displayed significant differences to

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INH/PZA (the latter regimen having failed to achieve culture negativity), their relapse rates could not be differentiated from a 100% relapse rate at the numbers of mice examined in this study. This is attributable to the presence of persistent bacteria in the RMP-containing regiments which could only be resuscitated by culture filtrate (Table 6). This observation coincided with the previous finding that early bactericidal activities of certain novel drug regimens were not necessarily predictive of a sterilizing effect (24) which may be attributed to the inability of the drug regimens to eliminate the persistent bacilli which were undetectable using our traditional microbiological methods. Recently, we showed that faster elimination rates derived from high dose RMP treatment led to elimination of persistent bacteria and this contributed to a shortened chemotherapy and a reduced relapse rate (6). It is not known if the elimination rate of culture filtrate-dependent bacteria is likely a surrogate measure of the sterilizing activity of the regimens as this has not been determined. RMP-containing regimens resulted in faster elimination rates than INH-PZA against plate-cultivable and reduced culture filtrate-dependent sub-populations at 14 weeks of treatment. Clearly further study is required to demonstrate if elimination rate of culture filtrate-dependent bacteria is a better surrogate for sterilizing effect.

The major caveat of this study was the relatively short period of chemotherapy in which INH-PZA failed to achieve CFU count negative mouse organs, this made it difficult to compare relapse of all the treatment regimens. It is likely that a difference in the sterilizing activity of these regimens would emerge with longer durations of treatment. Future work aiming to use a larger number of mice and longer treatment duration would illustrate more clearly the relationship between elimination rate and relapse amongst different drug regimens.

Bacterial population dynamics in infected animals is expected to be complex and related to the density and composition of the infecting population. In this study, the route of infection was systemic which was performed according to the previously established method (8, 9).

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Previous studies showed that intravenous infection of *M. tuberculosis* in mice led to slower disease progression in lungs (25) in spite of a high level of systemic immunity. However, low-dose aerosol infection resulted in substantially more virulent of *M. tuberculosis* in mouse lungs (25). In aerosol infected mice, a low number of bacilli was seeded in the lung and these then multiply into larger populations (25) presumably with smaller sub-populations of persistent organisms. It has been shown that slower bactericidal rates of combination regimens were found in intravenously infected mice with a higher relapse rate than aerosol infected animals (26). The difference might be due to different immune responses produced between intravenous and aerosol infected animals. It is not known if different routes of infection affect the level of culture filtrate-dependent persisters. Future work will be conducted to compare persistent *M. tuberculosis* levels in mice using respiratory and systemic infections.

It has been shown that antagonism occurred between INH and the combination RMP-PZA in the treatment of tuberculosis in mice (4). The authors suggested that the antagonistic effect was partially derived from the interaction of INH with RMP as addition of INH significantly reduced the Cmax and AUC of RMP (4). There was also a negative interaction between INH and PZA against *M. tuberculosis* (5) in mice when higher dose of INH was used. In contrast, a separate study showed that the RMP-PZA was less effective than RMP-INH-PZA combination in mouse models with both aerosol and intravenous infections indicating that inclusion of INH in the regimen showed no negative interaction to RMP-PZA (26). Observation of CFU counts over time with RMP-INH, RMP-PZA and RMP-INH-PZA, RMP-PZA treatment showed increased reduction in CFU counts compared to RMP-INH and RMP-INH-PZA especially in week 2, 4 and 6 of treatment (Fig. 2), indicating that INH was slightly antagonistic. However, our data demonstrated that this antagonistic effect when INH is added to the RMP-PZA regimen was not significant based on comparison of the

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elimination rate constants estimated from the profiles of bacterial elimination over time; the knet_with_drug was -0.51 for RMP-PZA and -0.51 for RMP-INH-PZA (significance of difference p>0.002). We also observed that the INH-PZA combination was not antagonistic against *M. tuberculosis* compared to the activities of each single drug. The differences in drug interaction of the current regimens seen from different studies may be attributable to different experimental conditions such as *M. tuberculosis* strains, mouse species, routes of infection and length of treatment used by different research groups (26). Importantly, our demonstration of RMP containing regimens being superior to a RMP-free regimen against *M. tuberculosis* in the modified Cornell mouse model indicated the essential role RMP plays in the current regimen to treat tuberculosis disease. However, the relationship between elimination rate, MPN counts and relapse rates requires further evaluation across a broader range of (possibly non-RMP containing) regimens.

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Figure legend

Figure 1. A simple mathematical model for exponential growth and decline of bacteria

Figure 2. Treatment profiles of *M. tuberculosis* H37Rv with RMP, INH and PZA singly or in combination in the modified Cornell mouse model. The results of a single experiment are shown with viability expressed as log CFU counts per lung or per spleen. Mice were infected intravenously at week -2 or -3 and the infection was allowed to progress for 2 or 3 weeks prior to treatment with RMP, INH and PZA singly or in combination indicated as a solid arrow for 14 weeks (time weeks 0 – 14). At week 2, 4, 6, 8, 11 and 14 of post treatment, CFU counts in the organs from each treatment group were estimated**.** Steroid treatment was started immediately after the termination of 14 weeks of antibiotic treatment as indicated with an empty arrow. A. treatment with RMP, INH and RMP-INH in lungs. B, treatment with RMP, PZA and RMP-PZA in lungs. C. treatment with RMP, INH, PZA and RMP-INH-PZA in lungs. D. treatment with INH, PZA and INH-PZA in lungs. E. treatment with RMP, INH and RMP-INH in spleens. F, treatment with RMP, PZA and RMP-PZA in spleens. G. treatment with RMP, INH, PZA and RMP-INH-PZA in spleens. H. treatment with INH, PZA and INH-PZA in spleens.

Differential equation:

$$\frac{d\,Bacteria}{dT} = k_{growth} * Bacteria - k_{death} * Bacteria$$
$$= (k_{growth} - k_{death}) * Bacteria$$
$$= k_{net} * Bacteria$$

Analytical function of time:

$$Bacteria(t) = Bacteria_{initial} \times 10^{\wedge(knet \cdot t)}$$

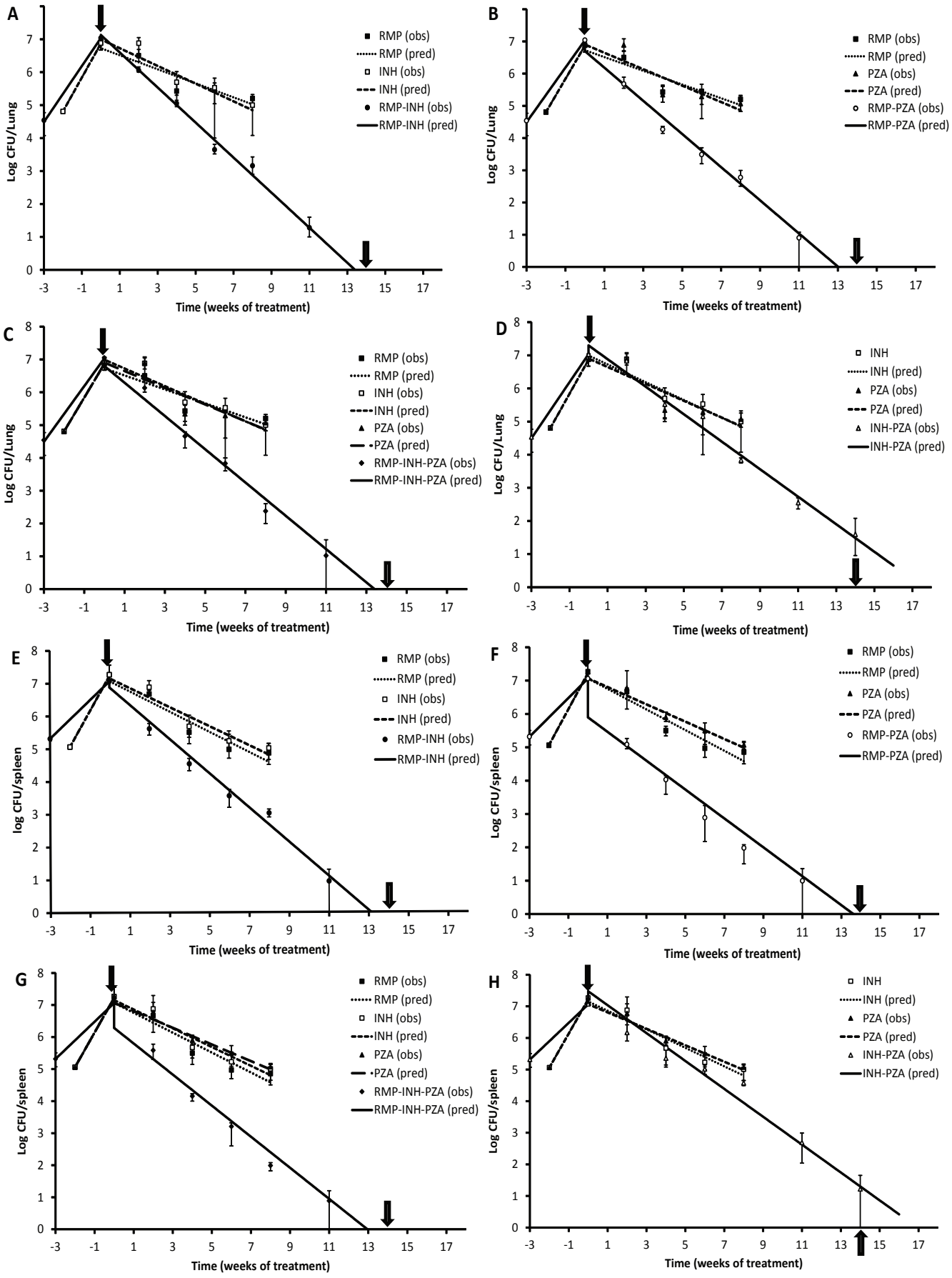

TABLE 1. Mouse tuberculosis experimental design

Treatment groups[a]	Total No. of mice[b]	D0	D14	D21	2W	4W	6W	8W	11W	14W	22W[c]
Control	12	4	4	4							
RMP	16				4	4	4	4			
INH	16				4	4	4	4			
PZA	16				4	4	4	4			
RMP-INH	76				8	8	8	8	10	10	24
RMP-PZA	76				8	8	8	8	10	10	24
INH-PZA	76				8	8	8	8	10	10	24
RMP-INH-PZA	76				8	8	8	8	10	10	24

[a] Mice were intravenously infected at day 0. Treatment commenced at 14 days after infection for single drug therapy and 21 days for combination therapy. Dosages for each drug were as follows: RMP 10 mg/kg, INH 25 mg/kg and PZA 150 mg/kg.
[b] Total mice were infected and treated excluding natural death of the mice during the course of treatment
[c] 8 weeks of hydrocortisone treatment post 14 weeks of treatment

TABLE 2. Bactericidal and sterilising activities of experimental regimens against *M. tuberculosis* in mouse lungs

Time of infection and treatment	Mean Log CFU per lung ± SD							
	Control	RMP	INH	PZA	RMP-INH	RMP-PZA	INH-PZA	RMP-INH-PZA
D0[a]	4.38 ± 0.04							
D14[b]	6.86 ± 0.13							
D21[c]	7.04 ± 0.01							
W2[d]		6.48 ± 0.14	6.83 ± 0.25	6.87 ± 0.13	6.05 ± 0.07	5.66 ± 0.13	6.84 ± 0.04	6.10 ± 0.16
W4		5.40 ± 0.15	5.57 ± 0.37	5.32 ± 0.15	5.05 ± 0.07	4.26 ± 0.08	5.46 ± 0.24	4.63 ± 0.17
W6		5.37 ± 0.29	5.27 ± 0.70	5.19 ± 0.35	3.64 ± 0.12	3.46 ± 0.18	5.16 ± 0.04	3.81 ± 0.14
W8		5.18 ± 0.13	4.89 ± 0.40	5.05 ± 0.15	3.12 ± 0.21	2.73 ± 0.22	3.83 ± 0.07	2.32 ± 0.24
W11					1.20 ± 0.27	0.77 ± 0.48	2.54 ± 0.12	0.63 ± 0.70
W14[e]					0	0	1.82 ± 0.42	0

a. 2 hours post-infection. b. 14 days post-infection. e. 21 days post-infection. d. week 2 post-treatment. e. CFU counts were derived from one third of tissue homogenate and limit detection was 3 CFU/lung.

TABLE 3. Bactericidal and sterilising activities of experimental regimens against *M. tuberculosis* in mouse spleens

Time of infection and treatment	Mean Log CFU per spleen ± SD							
	Control	RMP	INH	PZA	RMP-INH	RMP-PZA	INH-PZA	RMP-INH-PZA
D0[a]	5.32 ± 0.04							
D14[b]	7.06 ± 0.01							
D21[c]	7.22 ± 0.21							
W2[d]		6.66 ± 0.06	6.85 ± 0.15	6.45 ± 0.51	5.59 ± 0.14	5.07 ± 0.12	6.14 ± 0.17	5.57 ± 0.15
W4		5.49 ± 0.10	5.58 ± 0.30	5.89 ± 0.10	4.52 ± 0.14	3.99 ± 0.22	5.29 ± 0.25	4.15 ± 0.10
W6		4.90 ± 0.24	5.19 ± 0.19	5.46 ± 0.24	3.52 ± 0.20	2.71 ± 0.45	5.01 ± 0.08	3.15 ± 0.29
W8		4.80 ± 0.24	4.99 ± 0.16	5.06 ± 0.08	3.01 ± 0.11	1.95 ± 0.19	4.57 ± 0.06	1.99 ± 0.07
W11					0.78 ± 0.50	0.64 ± 0.69	2.53 ± 0.43	0.73 ± 0.49
W14[e]					0	0	1.52 ± 0.50	0

a. 2 hours post-infection. b. 14 days post-infection. c. 21 days post-infection. d. week 2 post-treatment. e. CFU counts were derived from one third of tissue homogenate and limit detection was 3 CFU/spleen.

TABLE 4. Estimates of exponential rate constants during pre-treatment (knet_no_drug) and treatment (knet_with_drug) in mouse lungs and spleens

Treatment[a]	knet_no_drug in Lungs ($week^{-1}$)		knet_with_drug in lungs ($week^{-1}$)		knet_no_drug in spleens ($week^{-1}$)		knet_with_drug in spleens ($week^{-1}$)	
	est.[b]	%RSE[c]	est.[b]	%RSE[c]	est.[b]	%RSE[c]	est.[b]	%RSE[c]
RMP	1.03	1.99	-0.21	8.22	1.08	3.15	-0.31	6.09
INH	1.03	1.99	-0.27	10.37	1.08	3.15	-0.29	6.35
PZA	1.03	1.99	-0.26	9.05	1.08	3.15	-0.26	5.92
RMP-INH	0.85	5.05	-0.53	2.61	0.58	0.91	-0.52	2.15
RMP-PZA	0.85	5.05	-0.51	1.65	0.58	0.91	-0.43	4.95
INH-PZA	0.85	5.05	-0.42	3.00	0.58	0.91	-0.44	4.38
RMP-INH-PZA	0.85	5.05	-0.51	2.91	0.58	0.91	-0.48	3.23

[a] single drug treatments for 8 weeks. Double and triple drug treatments for 14 weeks. [b] estimate. [c] percentage relative standard error.

TABLE 5. Relapse of mice after double or triple drug treatment

Positive culture from	RMP-INH	RMP-PZA	RMP-INH-PZA
Spleen only	8	6	15
Lung only	5	4	1
Both organs	4	7	5
Neither organs	3	5	3
Total No. of mice with positive cultures	17	17	21
Total No. of mice	20	22	24
Relapse (%)	85	77.3	87.5

P values of relative relapse rates determined by Fisher's exact test: RMP-INH/RMP-PZA 0.7, RMP-INH/RMP-INH-PZA 1.0 and RMP-PZA/RMP-INH-PZA 0.45. With Bonferroni correction $P < 0.008$ would considered significant.

TABLE 6. Resuscitation of *M. tuberculosis* H37Rv in mouse lungs and spleens of a modified Cornell mouse model after treatment with different drug regimens

	Lung		Spleen	
Drug regimens[a]	Plate counts[b]	Broth counts RPF[c]	Plate counts[b]	Broth counts RPF[c]
RMP-PZA	0	1.89±0.12	0	2.09±0.29
INH-RMP	0	2.00±0.14	0	2.18±0.32
INH-RMP-PZA	0	1.94±0.14	0	2.12±0.26
INH-PZA	1.82±0.42	4.10 ±0.09	1.52±0.5	4.07±0.15

[a] 14 week treatment

[b] determined by CFU counts of the organ homogenies (n=10) on 7H11 agar plates, Mean Log CFU/organ ± standard deviations. CFU counts were derived from one third of tissue homogenate and calculated to represent the counts of entire organ. The limit of detection was 3 CFU/organs.

[c] determined by MPN of the diluted organ homogenies (n=10) with the culture filtrates, Mean of Log MPN/organ ± standard deviations. Broth counts were derived from one third of tissue homogenate and calculated to represent the MPN of entire organ. The limit of detection was 10 MPN/organ.