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

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

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26 ABSTRACT

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

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

52 INTRODUCTION

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

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

83 **MATERIALS AND METHODS**

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

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

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

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

107 **(ii) Chemotherapy.** As shown in Table 1, mice were randomly allocated into eight groups.
108 Control group consisted of infected and untreated mice; 4 of these were sacrificed at 2 hours
109 after infection (D0) and 4 were killed at the beginning of treatment, D14 and D21 days after
110 infection. The treatment groups were as follows: single drug treatment group, each contained
111 16 mice receiving RMP, INH or PZA, respectively, for 8 weeks. Combination groups, each
112 contained 76 mice were administrated with RMP-INH, RMP-PZA, INH-PZA or RMP-INH-
113 PZA, respectively, for 14 weeks. Single drug therapy started 14 days after infection, when a
114 large bacterial load in the organs (the mean CFU counts reached 10^7 per lung or spleen) had
115 been achieved with visible symptoms of disease. Combination therapy started at 21 days after
116 infection. All groups were treated by daily oral administration (0.2 ml) for 5 days per week at
117 the dosages of RMP 10 mg/kg, INH 25 mg/kg or PZA 150 mg/kg. The drug suspensions
118 were prepared freshly for the daily dosage. In the combination containing RMP, RMP was
119 administered 1 hour before the other drugs to avoid drug to drug interactions (4).
120 Immediately after termination of 14 weeks of chemotherapy, the remaining mice were
121 administered 0.5 mg/mouse of hydrocortisone acetate by daily oral administration for 8

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

124 **(iii) Assessment of infection and treatment efficacy.** As seen in Table 1, to examine *M.*
125 *tuberculosis* infection and baseline CFU counts before initiation of chemotherapy, 4 untreated
126 control mice were sacrificed at 2 hours, day 14 and day 21 after infection, respectively. For
127 assessment of treatment efficacy, 4 mice were sacrificed at the 2, 4, 6 and 8 weeks post
128 treatment for single drug treatment to monitor CFU counts. For combination therapy, a
129 sample of 8 mice was sacrificed at 2, 4, 6 and 8 weeks and 10 mice were used at 11 and 14
130 weeks of treatment (Table 1). Lungs and spleens from mice were removed rapidly after
131 sacrifice and a sterile autopsy was performed. The organs were transferred into 2 ml tubes
132 each containing 1 ml sterile distilled water and 2 mm diameter glass beads. Lungs and
133 spleens of mice were homogenised using a reciprocal shaker (Thermo Hybaid Ltd) for 40
134 seconds at 6.5 speed. CFU counts from each lung and spleen were performed using serial
135 dilutions of the homogenates. At 14th week treatment, the entire organ homogenates (the total
136 volume of each organ homogenate was approximately 1.5 ml including the organ and 1 ml of
137 water) from the 10 mice were aliquoted equally into three tubes which were used 1. CFU
138 counting by addition of the homogenate to 2 ml of sterile distilled water following by plating
139 out the entire organ homogenate suspension on 6 selective 7H11 agar plates, 2. culturing in
140 5 ml of selective Kirchner liquid medium by the addition of polymyxin B 200 U/ml,
141 carbenicillin 100 mg/L, trimethoprim 20 mg/L and amphotericin B 10 mg/L (Selectatab,
142 Mast Diagnostica GmbH) for 4 weeks with subsequent sub-culturing of the entire culture
143 onto Löwenstein-Jensen slopes for a further 4 weeks and 3. resuscitation of persistent bacteria.
144 Culture negative organs were defined as no colonies grown on 7H11 agar plates and no
145 growth in selective Kirchner liquid medium following inoculation on Löwenstein-Jensen
146 slopes.

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

152 **Resuscitation of *M. tuberculosis* in mouse lungs and spleens.** For resuscitation of *M.*
153 *tuberculosis* grown in mouse organs, culture filtrates containing RPFs were used as described
154 previously (6, 7). *M. tuberculosis* H37Rv was grown in 7H9 medium for 15 to 20 days until
155 an optical density of 1 to 1.5 was reached. The cultures were harvested by centrifugation at
156 3000 g for 15 minutes and sterilised by filtration with 0.2 µm filter (Sartorius) twice. The
157 sterilised culture filtrates were made selective by addition of polymyxin B 200000 U/L,
158 carbenicillin 100 mg/L, trimethoprim 20 mg/L and amphotericin B 10 mg/L (Selectatab, Mast
159 Diagnostica GmbH) and immediately used for broth counting of the most probable number
160 (MPN) of the bacilli (7). Broth counting of lungs and spleens after 14 weeks of combination
161 therapy was performed as serial 10-fold dilutions in triplicate in which 0.5 ml of tissue
162 homogenates were added to 4.5 ml of the culture filtrates. At 10-day intervals over a 2-month
163 period of incubation at 37°C, the broth cultures were examined for visible turbidity changes.
164 Growth of *M. tuberculosis* in turbid tubes was confirmed by colonial morphology on 7H11
165 agar plates. The MPN of viable bacilli was then estimated from the patterns of positive and
166 negative tubes (7). The absence of microorganisms other than mycobacteria from turbid tubes
167 was confirmed by plating on blood agar medium (Oxoid) and Sabouraud dextrose agar
168 (Oxoid). In order to assess the sterility of culture filtrates free of *M. tuberculosis*, tubes
169 containing culture filtrates were incubated at 37°C for 2 months to ensure the absence of *M.*
170 *tuberculosis* in the culture filtrates.

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

191 **RESULTS**

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

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

200 **Treatment with RMP, INH and PZA singly and in two drug or three drug combination**
201 **in a modified Cornell mouse model.** We investigated the effect of RMP, INH and PZA
202 singly and in double and triple combinations on the rate of bacterial eradication and relapse in
203 a modified Cornell mouse model. The single dose of the drugs was tested in the animals for
204 8 weeks and terminated before resistant strain emergence (15). As shown in Table 2, Table 3
205 and Fig 2, RMP at 10 mg/kg, INH at 25 mg/kg or PZA at 150 mg/kg exhibited modest rates
206 of bacterial eradication in both lungs and spleens showing 99% kill (2-log reduction) at
207 around 8 weeks. The exponential rate constants (logarithmic base 10) for net bacterial
208 elimination during treatment ($k_{\text{net_with_drug}}$) for RMP, INH and PZA were -0.21, -0.27 and -
209 0.26 for lungs and -0.31, -0.29 and -0.26 for spleens (Table 4), respectively. Notably, the
210 drop in CFU counts in both lungs and spleens during the first 2 weeks of treatment with the
211 singly dosed drugs was minimal, though over the complete time course of therapy a clear
212 monoexponential decline in CFU counts was observed. No RMP or INH resistant strains
213 were isolated from 4 to 8 weeks of treatment. In addition, there was no significant difference
214 in activities amongst each of the single drug treatments (Table S1 and S2 in the supplemental
215 material). Interestingly, treatment with RMP combined with INH (Fig. 2A and 2E) or PZA
216 (Fig. 2B and 2F) accelerated the rate of bacterial eradication showing 99% kill (Table 2 and
217 Table 3) at 4 weeks of treatment for RMP-INH and at about 3 weeks for RMP-PZA with the
218 estimation of $k_{\text{net_with_drug}}$ at -0.53 and -0.51 for lungs and -5.2 and -0.43 for spleens (Table 4),
219 respectively. All the combined therapies were significantly more effective than the single
220 therapy (Table S1 and S2 in the supplemental material). As seen in Table 2, Table 3, Fig. 2C

221 and Fig. 2G, 99% kill with the RMP-INH-PZA combination was achieved at about 3 weeks
222 for both lungs and spleens showing a similar elimination rate constant (-0.51 for lung and -
223 0.48 for spleen) to RMP-INH or RMP-PZA (Table 4). There was no significant difference in
224 efficacies amongst these RMP containing regimens against *M. tuberculosis* in this mouse
225 model (Table S1 and S2 in the supplemental material). All the RMP containing combinations
226 achieved undetectable *M. tuberculosis* CFU counts (Table 2 and Table 3) and negative broth
227 growth in selective Kirchner liquid medium in murine lungs and spleens at 14 weeks of
228 treatment. However, when INH was combined with PZA (Fig. 2D and 2H), there was no
229 noticeably increased initial kill compared to each of the single drugs until 4 weeks of
230 treatment followed by a reduction of CFU count showing a 99% kill at 5.6 weeks post
231 treatment (Table 2) for lungs and 4 weeks for spleens (Table 3). This was reflected in the
232 estimates for $k_{\text{net_with_drug}}$ for the INH and PZA combination, which was -0.42 and -0.44 for
233 lungs and spleens, respectively (Table 4). Although the INH and PZA combinations failed to
234 achieve undetectable *M. tuberculosis* CFU counts in murine lungs after 14 weeks of treatment
235 (Fig. 2D and 2H), the difference in efficacies between the single drug treatment and the
236 combination was significant (Table S1 and S2 in the supplemental material).

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

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

248 **Determination of persisters after treatment with four drug regimens.** In order to
249 determine the effect of the four combination regimens on the post-treatment level of
250 persisters, we analysed lung and spleen homogenates at 14 weeks post-treatment using *M.*
251 *tuberculosis* culture filtrate resuscitation (6). As shown in Table 6, at 14 weeks post-treatment,
252 although CFU counts and growth in Kirchner liquid medium were negative for the drug
253 regimens INH-RMP, RMP-PZA and INH-RMP-PZA, there were significant amounts of
254 culture filtrate-dependent persisters present in lungs and spleens (1.89 log cells/lung and 2.09
255 log cells/spleen for RMP-PZA, 2 log cells/lung and 2.18 log cells/spleen for INH-RMP and
256 1.94 log cells/lung and 2.12 log cells/spleen for INH-RMP-PZA). After INH-PZA treatment,
257 there were 4 log culture filtrate-resuscitated bacilli in both lungs and spleens. If we exclude
258 CFU count positive bacilli, there were still 4-log culture filtrate-dependent persisters in the
259 organs of INH-PZA treated mice.

260 **DISCUSSION**

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

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

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

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

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

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

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

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

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

370 elimination rate constants estimated from the profiles of bacterial elimination over time; the
371 $k_{net_with_drug}$ was -0.51 for RMP-PZA and -0.51 for RMP-INH-PZA (significance of
372 difference $p>0.002$). We also observed that the INH-PZA combination was not antagonistic
373 against *M. tuberculosis* compared to the activities of each single drug. The differences in drug
374 interaction of the current regimens seen from different studies may be attributable to different
375 experimental conditions such as *M. tuberculosis* strains, mouse species, routes of infection
376 and length of treatment used by different research groups (26). Importantly, our
377 demonstration of RMP containing regimens being superior to a RMP-free regimen against *M.*
378 *tuberculosis* in the modified Cornell mouse model indicated the essential role RMP plays in
379 the current regimen to treat tuberculosis disease. However, the relationship between
380 elimination rate, MPN counts and relapse rates requires further evaluation across a broader
381 range of (possibly non-RMP containing) regimens.

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470

471 Figure legend

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

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

487



Differential equation:

$$\begin{aligned}d \text{ Bacteria} / dT &= k_{\text{growth}} * \text{Bacteria} - k_{\text{death}} * \text{Bacteria} \\ &= (k_{\text{growth}} - k_{\text{death}}) * \text{Bacteria} \\ &= k_{\text{net}} * \text{Bacteria}\end{aligned}$$

Analytical function of time:

$$\text{Bacteria}(t) = \text{Bacteria}_{\text{initial}} \times 10^{(k_{\text{net}} \cdot t)}$$

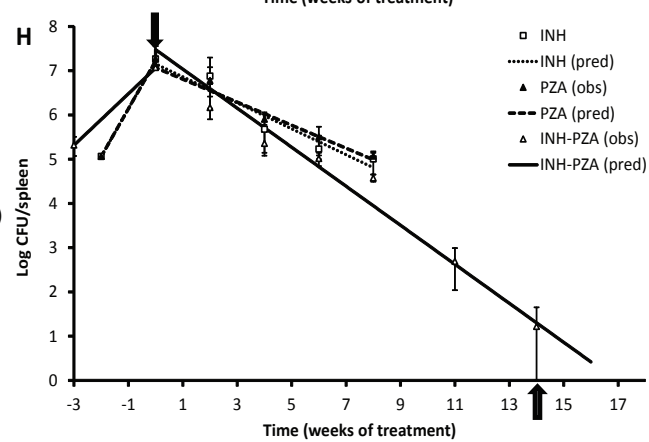
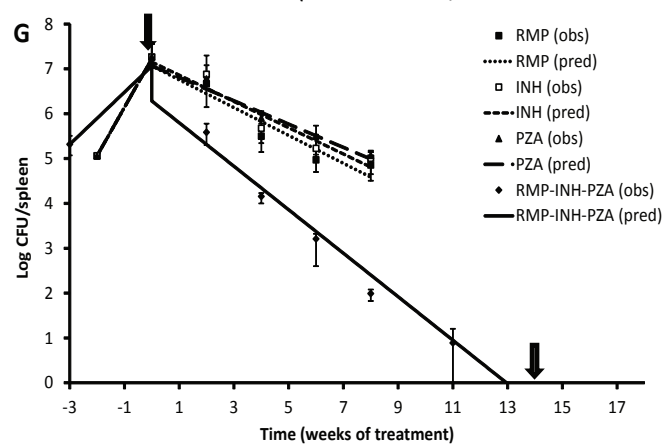
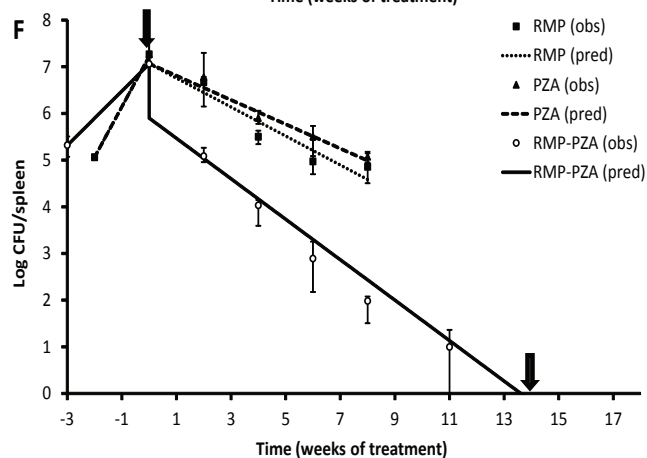
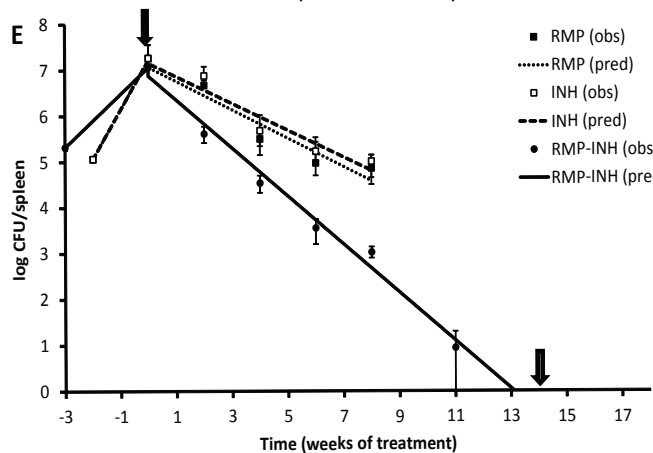
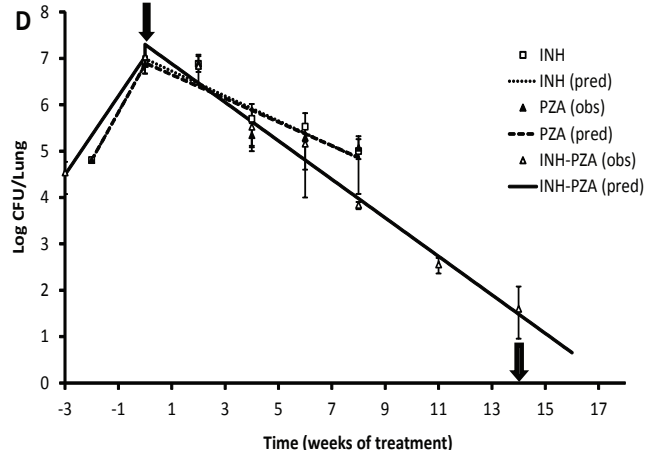
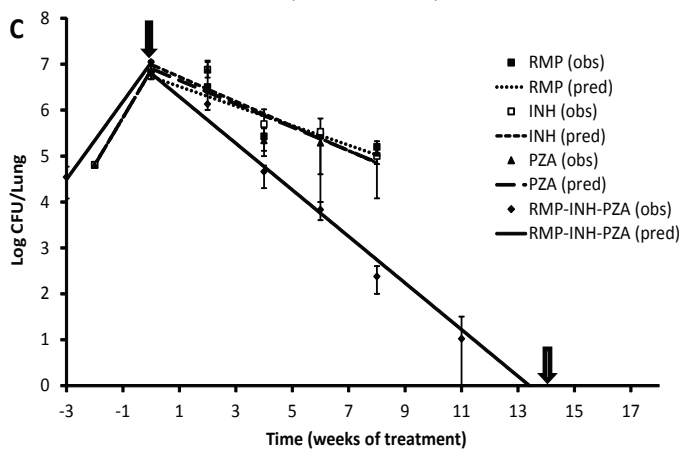
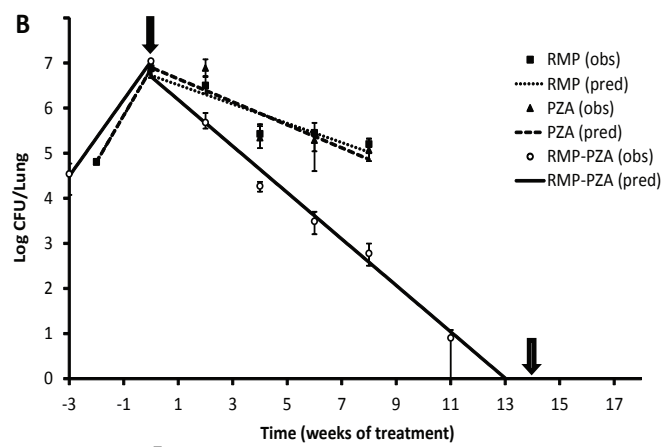
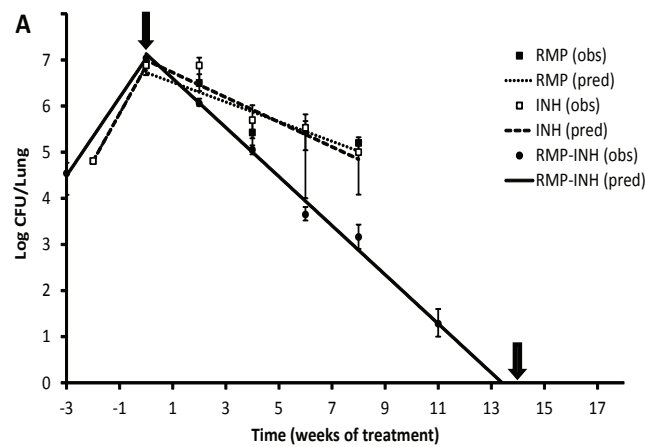


TABLE 1. Mouse tuberculosis experimental design

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 \pm SD							
	Control	RMP	INH	PZA	RMP-INH	RMP-PZA	INH-PZA	RMP-INH-PZA
D0 ^a	4.38 \pm 0.04							
D14 ^b	6.86 \pm 0.13							
D21 ^c	7.04 \pm 0.01							
W2 ^d		6.48 \pm 0.14	6.83 \pm 0.25	6.87 \pm 0.13	6.05 \pm 0.07	5.66 \pm 0.13	6.84 \pm 0.04	6.10 \pm 0.16
W4		5.40 \pm 0.15	5.57 \pm 0.37	5.32 \pm 0.15	5.05 \pm 0.07	4.26 \pm 0.08	5.46 \pm 0.24	4.63 \pm 0.17
W6		5.37 \pm 0.29	5.27 \pm 0.70	5.19 \pm 0.35	3.64 \pm 0.12	3.46 \pm 0.18	5.16 \pm 0.04	3.81 \pm 0.14
W8		5.18 \pm 0.13	4.89 \pm 0.40	5.05 \pm 0.15	3.12 \pm 0.21	2.73 \pm 0.22	3.83 \pm 0.07	2.32 \pm 0.24
W11					1.20 \pm 0.27	0.77 \pm 0.48	2.54 \pm 0.12	0.63 \pm 0.70
W14 ^e					0	0	1.82 \pm 0.42	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/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 \pm SD							
	Control	RMP	INH	PZA	RMP-INH	RMP-PZA	INH-PZA	RMP-INH-PZA
D0 ^a	5.32 \pm 0.04							
D14 ^b	7.06 \pm 0.01							
D21 ^c	7.22 \pm 0.21							
W2 ^d		6.66 \pm 0.06	6.85 \pm 0.15	6.45 \pm 0.51	5.59 \pm 0.14	5.07 \pm 0.12	6.14 \pm 0.17	5.57 \pm 0.15
W4		5.49 \pm 0.10	5.58 \pm 0.30	5.89 \pm 0.10	4.52 \pm 0.14	3.99 \pm 0.22	5.29 \pm 0.25	4.15 \pm 0.10
W6		4.90 \pm 0.24	5.19 \pm 0.19	5.46 \pm 0.24	3.52 \pm 0.20	2.71 \pm 0.45	5.01 \pm 0.08	3.15 \pm 0.29
W8		4.80 \pm 0.24	4.99 \pm 0.16	5.06 \pm 0.08	3.01 \pm 0.11	1.95 \pm 0.19	4.57 \pm 0.06	1.99 \pm 0.07
W11					0.78 \pm 0.50	0.64 \pm 0.69	2.53 \pm 0.43	0.73 \pm 0.49
W14 ^e					0	0	1.52 \pm 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 be 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

Drug regimens ^a	Lung		Spleen	
	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.