



26 **Abstract**

27 Pyrazinamide (PZA) is one of only two sterilizing drugs in the first-line anti-tuberculosis regimen.  
28 Its activity is strongly pH-dependent; the minimum inhibitory concentration changes by several  
29 orders of magnitude over a range of pH values that may be encountered in various in vivo  
30 compartments. We recently reported selective inactivity of PZA in a subset of C3HeB/FeJ mice  
31 with large caseous lung lesions. In the present study we evaluated whether such inactivity was  
32 explained by poor penetration of PZA into such lesions or selection of drug-resistant mutants.  
33 Despite demonstrating similar dose-proportional PZA exposures in plasma, epithelial lining fluid  
34 and lung lesions, no dose response was observed in a subset of C3HeB/FeJ mice with the highest  
35 CFU burden. Although PZA-resistant mutants eventually replaced the susceptible bacilli in  
36 BALB/c mice and in C3HeB/FeJ mice with low total CFU burdens, they never exceeded 1% of the  
37 total population in non-responding C3HeB/FeJ mice. The selective inactivity of PZA in large  
38 caseous lesions of C3HeB/FeJ mice is best explained by the neutral pH of liquefying caseum.

39

40

41 **Introduction**

42 Pyrazinamide (PZA) is one of only two drugs proven to be capable of shortening the duration of  
43 treatment for tuberculosis (TB) to less than 12 months (1, 2). Although it has been a part of first-  
44 line treatment regimens for 40 years, its mechanism of action remains incompletely understood  
45 (3, 4). PZA is a pro-drug that is converted to the active moiety pyrazinoic acid (POA) by a

46 bacterial amidase encoded by *pncA* (3). The MIC of both PZA and POA against *M. tuberculosis* is  
47 profoundly pH-dependent, changing by several orders of magnitude over the range of pH values  
48 that may be encountered *in vivo*. For example, the PZA MIC is 1000 µg/ml at a pH of 6.8, 50  
49 µg/ml at a pH of 5.5, and theoretically as low as 5µg/ml at a pH of 4.5, which *M. tuberculosis*  
50 may encounter in the phagolysosome of activated macrophages (5-7). Thus, just as its activity *in*  
51 *vivo* is assumed to vary according to drug exposures at the site of infection, it also should vary  
52 significantly according to the pH at the site of infection.

53 The treatment-shortening, or sterilizing, effect of PZA in human TB is not readily evident in its  
54 early bactericidal activity (EBA), as measured by the average daily fall in sputum CFU count over  
55 the first 14 days of treatment. The EBA of PZA monotherapy ranges from 0.04 to 0.1  
56 log<sub>10</sub>CFU/ml/day (8, 9). When administered in combination with isoniazid and rifampin, its  
57 contribution to the EBA of the regimen may be undetectable (8). Instead, the contribution of  
58 PZA is more evident during the later phase of sputum sterilization (8, 10). It is remarkable then  
59 that PZA exerts its treatment-shortening effect in the modern short-course regimen only during  
60 the first 2 months of treatment. Extending the duration of treatment has no additional benefit,  
61 in both humans and murine models (3, 11, 12).

62 Taken together, these characteristics suggest that PZA exerts its unique sterilizing effect against  
63 a subpopulation of tubercle bacilli residing in one or more specific compartments where the pH  
64 is sufficiently low to make the bacilli more susceptible to PZA than to other drugs. Where such  
65 subpopulations reside remains incompletely understood. It has been proposed that PZA acts  
66 against bacilli in inflammatory lung lesions where the pH is presumed to be acidic (e.g., 5.5-6.0)  
67 initially and then to increase as the lesions resolve with effective treatment (13). Alternatively,

68 the acidic milieu may persist but the bacillary sub-population that resides in that milieu making  
69 it more susceptible to PZA than to another first-line drug (e.g., rifampin), may simply be  
70 eradicated after 2 months of first-line therapy.

71 Active TB in humans is characterized by a variety of lesion types in which *M. tuberculosis*  
72 encounters different microenvironments. Although no single non-clinical model of TB  
73 recapitulates all aspects of human TB, existing models may be used in a complementary fashion  
74 to better understand the impact of lesion type and resultant microenvironmental conditions on  
75 the action of PZA in human disease (14, 15). Based on pharmacodynamics studies in an *in vitro*  
76 hollow fiber model of TB, Gumbo et al proposed that PZA exerts its sterilizing effect against  
77 extracellular bacilli because PZA accumulates to concentrations high enough to produce its  
78 observed EBA at pH of 5.8 only in alveolar epithelial lining fluid (ELF) (16), and not inside  
79 alveolar macrophages (17). However, there is no direct evidence that the ELF or the caseous  
80 material inhabited by extracellular *M. tuberculosis in vivo* is indeed this acidic. Moreover, PZA  
81 clearly exerts substantial bactericidal and sterilizing activity against established *M. tuberculosis*  
82 infection in BALB/c mice, where the infecting bacilli are virtually all intracellular. In fact,  
83 bactericidal activity is evident in BALB/c mice at doses producing plasma exposures  
84 approximately half those produced by standard human doses (e.g., 75 mg/kg in mice), both  
85 alone and in combination with rifampin and isoniazid (18). The pronounced effect of PZA in the  
86 intracellular compartment is presumably due to the more acidic milieu in the phagolysosomes  
87 of activated macrophages, where the pH can be as low as 4.5 (6, 7), making PZA capable of  
88 significant sterilizing effects. The key role of macrophage activation in optimizing PZA effect is

89 further supported by the poor activity of PZA in mice prior to the onset of the adaptive immune  
90 response and in athymic nude mice (19, 20).

91 C3HeB/FeJ mice have recently garnered significant attention as a murine TB model because,  
92 unlike BALB/c and other commonly used mouse strains, they develop caseous lung lesions in  
93 response to infection with *M. tuberculosis* (21-23). As expected, bacilli in the caseous core of  
94 these lesions are extracellular, while the cellular cuff of caseous granulomas and other non-  
95 necrotic cellular granulomas harbor intracellular bacilli. Moreover, due to differences in the rate  
96 and extent of development of caseous lesions, significant heterogeneity in the presence, size  
97 and degree of liquefaction of caseous lesions is often observed between mice and between  
98 lesions within the same mouse at the initiation of treatment. We recently described a surprising  
99 phenomenon of dichotomous activity of PZA in C3HeB/FeJ mice (24), whereby PZA had little or  
100 no detectable bactericidal activity in a subset of mice with large caseous lesions despite  
101 demonstrating the expected bactericidal effect in those with less extensive disease and in a  
102 parallel cohort of BALB/c mice. Based on this apparent lesion-dependent activity, we  
103 hypothesized that this dichotomous effect of PZA was due to its limited activity against  
104 extracellular bacilli in caseum, which comprise the majority population in C3HeB/FeJ mice with  
105 large caseous lesions, but bactericidal effects on the smaller numbers of bacilli in cellular lesions  
106 of BALB/c and C3HeB/FeJ mice and in the cellular cuff of necrotic granulomas of C3HeB/FeJ  
107 mice. However, in C3HeB/FeJ mice with large caseous lesions, the effect against intracellular  
108 bacilli is largely obscured by the limited effect on the majority bacillary population in caseum.

109 In the present study we set out to determine whether this selective inactivity of PZA in large  
110 caseous lesions could be explained by reduced drug penetration, selection of PZA-resistant

111 mutants, or insufficiently acidic conditions for PZA activity at achievable PZA concentrations.  
112 The results indicate that the near neutral pH of liquefying caseum prevents PZA from exerting  
113 any significant bactericidal activity against the numerous extracellular bacilli in larger caseous  
114 lesions and support the concept that pronounced sterilizing effects of PZA are exerted against  
115 intracellular bacilli.

116 Portions of the results of this study have been presented previously at the International  
117 Workshop on the Clinical Pharmacology of Tuberculosis Drugs (Abstract No. 12, Washington,  
118 DC, September 2014), and the Interscience Conference on Antimicrobial Agents and  
119 Chemotherapy (Abstract No. A-20, Washington, DC, September 2014).

120

## 121 **Materials and methods**

### 122 *Mycobacterial strains*

123 *M. tuberculosis* H37Rv was used as a frozen stock prepared from a log-phase culture in  
124 Middlebrook 7H9 broth after mouse passage and was diluted in 7H9 broth supplemented with  
125 10% OADC (Oleic acid Albumin Dextrose Catalase) before infection.

### 126 *Drugs and Chemotherapy*

127 PZA was obtained from Acros Organics (Thermo Fisher Scientific, New Jersey) and formulated  
128 for oral administration in distilled water. The daily doses were (in mg/kg) 10, 30, 100, 150, 300  
129 and 900. The most highly concentrated dosing solutions were warmed before administration to  
130 keep PZA in solution.

131 Doses were administered once daily, 5 days/week, by oral gavage, except for the 900mg/kg  
132 dose, which was administered as 450mg/kg twice daily (BID) due to solubility issues.

133 *Mouse aerosol infection*

134 All animal procedures were approved by the Animal Care and Use Committee of Johns Hopkins  
135 University. Female BALB/c mice (Charles River, Wilmington, MA) and C3HeB/FeJ mice (Jackson,  
136 Bar Harbor, ME) were used. Age of the mice varied between 1.5 and 10 months.

137 Mice were aerosol infected using the Inhalation Exposure System (Glas-Col, Terre Haute, IN)  
138 with dilutions of a titered frozen stock of *M. tuberculosis* H37Rv to implant into the lung  
139 approximately 100-250 colony-forming units (CFU) for BALB/c and 50-100 CFU for C3HeB/FeJ  
140 mice. One day after infection, 4 mice from each aerosol run were humanely killed to determine  
141 the number of bacteria implanted.

142 *Guinea pig aerosol infection*

143 Female Hartley guinea pigs (Charles River, Wilmington, MA) 6-7 weeks old were aerosol infected  
144 with *M. tuberculosis* CDC1551 using the Madison Chamber as previously described (25).

145

146 *Pharmacokinetic studies*

147 Several pharmacokinetic (PK) studies were performed in C3HeB/FeJ mice to enable comparisons  
148 with results in BALB/c mice. The single dose plasma and lung concentration-time profiles were

149 determined in both 6-7 week-old and 8-10 month-old mice. Steady state plasma and lung  
150 concentration-time profiles were determined in infected 5-10 month-old mice.

151 PZA concentrations were measured in samples of plasma, epithelial lining fluid (ELF) and lung  
152 lesions obtained, depending of the study, at 0.08, 0.25, 0.45, 1.5, 3, 5, 7, 12 and 17 hours after  
153 PZA dosing. Three or four mice from each dose group were sampled at each time point. Plasma  
154 was obtained either by tail vein bleed or by cardiac puncture performed under anesthesia by  
155 isoflurane inhalation. ELF was obtained after centrifugation of bronchoalveolar lavage fluid  
156 (BALF) at 400 x g for 5min. BALF was obtained, after anesthesia by intra peritoneal injection of  
157 Ketamine (200mg/kg) + Xylazine (10mg/kg), by injection and aspiration of 300µl of phosphate  
158 buffered saline (PBS) via a 20G IV catheter (ProtectIV® Plus, Smith Medical ASD, Southington,  
159 CT). The procedure was performed under visual control using an optic fiber (UV/VIS Fiber  
160 0.22NA, 400µm; Edmund Optics, Barrington, NJ) and a fiberscope light source. Lung lesions  
161 were obtained by resecting single or coalescing tubercular lesions, minimizing the amount of  
162 normal-appearing lung resected to obtain at least 20mg of tissue. Lungs were rinsed in cold PBS  
163 and lesions were resected on dry ice to prevent PZA diffusion or degradation.

164 Samples were frozen at -80°C before being shipped to the Dartois laboratory, Rutgers New  
165 Jersey Medical School, for quantification.

#### 166 *Quantification of PZA in samples*

167 PZA standards were obtained from Acros Organics (Thermo Fisher Scientific, New Jersey).  
168 Analytes of interest were extracted by diluting 50µL of mouse serum with 50µL of  
169 acetonitrole:water (1:1), and 450µL of methanol:acetonitrile (1:1) containing 0.5µg/ml of



170 pyrazinamide-<sup>15</sup>N,d3 or pyrazinecarboxylic acid-d3 (Toronto Research Chemicals, Inc) as internal  
171 standards. The mixture was vortexed and centrifuged, and 200µL of the supernatant was  
172 recovered for analysis. LC/MS-MS analysis was performed with an Agilent 1260 system coupled  
173 to an AB Sciex 4000 Q-trap Mass Spectrometer (positive mode electrospray ionization), and an  
174 Agilent column SB-C8, 4.6 x 75mm, 3.5µm, with the column temperature fixed at 24 °C. Mobile  
175 phase A was 0.1% formic acid in 100% H<sub>2</sub>O and mobile phase B was 0.1% formic acid in 100%  
176 acetonitrile. Injection volumes were routinely 2µL. The Mass Selective Detector was set to MRM  
177 (multiple reaction monitoring) mode using positive polarity ionization, monitoring for the ions  
178 of interest (m/z 124.0/81.1 for PZA) and the internal standard (m/z 296/215). The lower limit of  
179 quantification was 0.2µg /ml.

180 The urea method was used to correct for dilution of ELF by PBS in BALF samples, as previously  
181 described (16). Thus the concentration of PZA in ELF ( $Z_{\text{ELF}}$ ) was derived from the following  
182 relationship:  $Z_{\text{ELF}} = Z_{\text{BAL}} \times (V_{\text{BAL}} / (V_{\text{BAL}} \times (U_{\text{BAL}} / U_{\text{PLA}})))$  where  $Z_{\text{BAL}}$  is the concentration of PZA  
183 measured in BALF,  $V_{\text{BAL}}$  is the volume of BALF,  $U_{\text{BAL}}$  is the concentration of urea in BALF, and  $U_{\text{PLA}}$   
184 is the concentration of urea in plasma. Five µl of plasma and 20µl of ELF were used with the  
185 QuantiChrom® urea assay kit (Gentaur, San Jose, CA), following manufacturer's instructions.

186 PK parameters (area under the concentration-time curve ( $AUC_{0-t}$ ,  $AUC_{0-\infty}$ ),  $C_{\text{max}}$ , half-life) were  
187 calculated from mean concentration data using Microsoft Excel (Office 2010, Microsoft Corp,  
188 Redmond, WA). AUC was calculated using the linear trapezoidal rule. Half-life and elimination  
189 rate constant were calculated by linear regression using semi-logarithmic concentration versus

190 time data. Concentration values below the lower limit of quantification were excluded from the  
191 pharmacokinetic evaluation.

#### 192 *Pharmacodynamics study*

193 To determine dose-ranging efficacy of PZA, 60 BALB/c and 60 C3HeB/FeJ 6-week-old mice  
194 received 10, 30, 100, 300 and 450 BID mg/kg of PZA for up to 8 weeks, beginning 6 weeks after  
195 infection. Treatment efficacy was assessed on the basis of lung CFU counts determined after 3  
196 and 8 weeks of treatment. Serial dilutions of whole lung homogenates were plated on selective  
197 Middlebrook 7H11 agar (Becton Dickinson, Franklin Lake, NJ). Plates were incubated for 6 to 8  
198 weeks at 37°C before determining final CFU counts. At the 8 week time point, quantitative  
199 cultures were performed with 0.5ml of lung homogenates on the same 7H11 agar  
200 supplemented with 900 mg/L of PZA (which is 3-6 times the MIC against the parent H37Rv strain  
201 on this media).

#### 202 *Whole genome sequencing of PZA-resistant mutants*

203 Genomic DNA extraction procedures were adapted from a previously described cetyltrimethyl  
204 ammonium bromide (CTAB)-lysozyme method (26). From 1 to 5 colonies per mouse were picked  
205 from PZA-containing plates and streaked on 7H11 agar to amplify the clone. Colonies were  
206 scraped and suspended by bead-beating (2mm sterile beads) in 5ml of PBS. Three ml of  
207 supernatant was centrifuged for 2 min (3340 x g) before the pellet was heat killed (30min at  
208 80°C) in 200µL of Tris-EDTA buffer (TE buffer, Gaithersburg, MD). After 5 min of centrifugation  
209 (3340 x g), the pellet was incubated at 37°C overnight in 100µL of lysozyme. The extract was  
210 incubated at 65°C twice for 10 min, once after addition of 20 µl of 5% sodium dodecylsulfate

211 (Bio-Rad, Hercules, CA) and 20  $\mu$ l of 1mg/ml Proteinase K (Thermo Scientific, Waltham, MA), and  
212 once after addition of 20  $\mu$ l of 3M NaCl and 10  $\mu$ l CTAB/NaCl solution. After adding  
213 chloroform:isoamylalcohol (24:1, v:v) the solution was centrifuged for 8 min at 15680 x g. The  
214 top layer was transferred in another tube containing 340 $\mu$ l of ice cold isopropanol before  
215 precipitating nucleic acids at -20°C for 30 minutes. After another centrifugation at 15680 x g for  
216 15min, the pellet was washed with ice cold 70% ethanol and then dissolved in 20 $\mu$ l of pure  
217 water after a second centrifugation.

218 Samples were sequenced on an Illumina GAIIx next-generation sequencer. DNA samples were  
219 prepared for sequencing using the standard genomic DNA sample preparation protocol  
220 (Illumina Inc., San Diego, CA). Paired-end data was collected with a read length of 54+54 bp.  
221 Base-calling was performed using RTA 1.9.35, and genome assembly was carried out using a  
222 comparative assembly technique as described in (27), using the genome of H37Rv as a reference  
223 sequence. The mean depth of coverage over all samples was 29.5x.

#### 224 *Assessment of pH of the lesions*

225 The pH of liquefied caseum from selected lesions of more than 3mm diameter was measured  
226 with a 16G needle tip micro-pH comb electrode (Thermo Scientific Orion, Chelmsford, MA) and  
227 a benchtop pH meter Mettler Toledo FE20 (Business Unit Analytical, Schwerzenbach,  
228 Switzerland). The probe was inserted directly into the tubercle to measure the pH of the  
229 liquefied material.

#### 230 *Data Analysis*

231 Lung CFU counts ( $x$ ) were log-transformed as  $\log_{10}(x + 1)$  before analysis. Group mean CFU  
232 counts after 2 months of treatment were compared using one-way analysis of variance with  
233 GraphPad Prism v.5 (GraphPad Software, San Diego, CA) and Bonferroni's posttest to adjust for  
234 multiple comparisons, as appropriate. A non-linear dose-response regression was used to  
235 calculate PZA dose-response using the same software.

236

237 **Results**238 *Pharmacokinetics of PZA*239 *Uninfected mice*

240 Total daily doses from 10 to 900mg/kg of PZA were administered. As stated in the methods  
241 section, PZA was administered orally once a day, except for 900 mg/kg which was administered  
242 as two 450mg/kg doses given 12 hours apart (BID). PZA concentrations were measured in  
243 plasma and ELF.

244 As shown in Figure 1 uninfected C3HeB/FeJ mice had at least dose proportional (possibly supra-  
245 proportional) exposures in plasma and also in ELF (data not shown). The PK parameter values  
246 for plasma are presented in Table 1 and were comparable to previous published results (28).  
247 The concentrations produced by the lowest doses of PZA were not quantifiable in ELF because  
248 of the high dilution factor. The median ratio of ELF/plasma concentrations ranged between 0.9-  
249 3 at 3h and tended to increase slightly over time to 1.2-3.7 independently of dose (except for  
250 300mg/kg, where the ratio decreased) (Table 2).

251 *Infected mice*

252 Infected C3HeB/FeJ mice received 150mg/kg once daily or 450mg/kg BID doses of PZA for 3  
253 days or 4 weeks before PZA concentrations were measured in plasma, ELF and tubercular  
254 lesions. No major difference was observed in plasma PZA concentrations between infected and  
255 uninfected mice, although AUC and  $C_{max}$  were numerically lower in infected mice receiving  
256 450mg/kg BID compared to uninfected mice (Table 1). Plasma PK parameters in infected

257 C3HeB/FeJ mice were comparable to past results in infected BALB/c mice (28) (Table 1). The  
258 dose proportionality was conserved both in plasma (Figure 2) and in ELF. In ELF, PZA  
259 concentrations seemed to be more variable between mice and between experiments than what  
260 was observed in plasma (data not shown). The ratio of PZA concentrations in ELF/plasma in  
261 infected mice was similar to that observed in uninfected mice (Table 2).

262 The concentrations of PZA in lesions largely mirrored concentrations in plasma (Figure 2). The  
263 measurements from a representative large necrotic granuloma sampled 7h after a dose of  
264 150mg/kg were similar for the capsule and the liquefied caseum (2.68 and 1.76 $\mu$ g/ml  
265 respectively). When comparing PZA concentrations in lesions of mice with large necrotic  
266 granulomas (>3mm) and mice without such lesions, at the same time points, means tended to  
267 be higher in larger lesions than in smaller lesions. For example, in samples obtained 90min after  
268 a 150mg/kg dose, 3 large lesions had a mean PZA concentration of 105.1 $\mu$ g/ml whereas 3  
269 smaller lesions averaged 80.1 $\mu$ g/ml, but the difference was not statistically significant ( $p=0.07$ ).  
270 In samples obtained 7h after the same dose, 3 large lesions had a mean PZA concentration of  
271 3.5 $\mu$ g/ml whereas 2 smaller lesions averaged 2 $\mu$ g/ml ( $p=0.055$ ). The median ratios of  
272 lesion/plasma concentration were 0.8 and 0.7 at 1.5h for 150mg/kg and 450mg/kg BID  
273 respectively, and remained stable between 0.8-1.2 over time.

#### 274 *Pharmacodynamics of PZA*

275 In the dose-ranging efficacy study, BALB/c and C3HeB/FeJ mice received daily doses ranging  
276 from 10 to 450 BID mg/kg of PZA for 3 to 8 weeks. Mean (SD) CFU counts at the start of  
277 treatment (D0) were 6.86 (0.17) for BALB/c mice and 7.09 (0.12) for C3HeB/FeJ mice. As

278 previously described (24), PZA was associated with a dichotomous dose-response relationship in  
279 C3HeB/FeJ mice but not in BALB/c mice. Dose-proportional bactericidal activity was observed in  
280 BALB/c mice and in most C3HeB/FeJ mice at each time point. At doses of 100 mg/kg and above,  
281 the bactericidal effect size increased between 3 and 8 weeks of treatment. After 8 weeks,  
282 increasing the dose from 30 to 300mg/kg increased the log-kill by 2 log<sub>10</sub>, and increasing the  
283 dose from 300 mg/kg to 450 mg/kg BID increased the log-kill by another 2 log<sub>10</sub> in both mouse  
284 strains (Figure 3). However, no dose response effect was observed in a subset of C3HeB/FeJ  
285 mice, even after 8 weeks of treatment (Figure 3). CFU counts after 8 weeks in these poor  
286 responders were one-half to one log lower compared to week 3, so some modest activity could  
287 not be excluded. Excluding these outliers, the goodness of fit of the logarithmic dose-response  
288 curve was  $r^2=0.92$  at week 3 and 0.87 at week 8; the maximum effect ( $E_{max}$ ) at week 3 was  
289  $3.27\log_{10}$  and the dose producing 50% of the  $E_{max}$  ( $EC_{50}$ ) was 128.4mg/kg (95% confidence  
290 interval [CI] = 58.32-281.2). Similar curve fits and parameters were observed in BALB/c mice,  
291 e.g., the goodness of fit was  $r^2=0.87$  and 0.98 at weeks 3 and 8 respectively;  $E_{max}$  was  $3.06\log_{10}$   
292 and  $EC_{50}$  was 139.6mg/kg (95%CI = 61.26-318.1) at week 3.

### 293 *Selection and characterization of PZA-resistant mutants*

294 After 8 weeks of treatment, 11 (37%) of 30 BALB/c mice had colonies on PZA-containing plates,  
295 compared to 18 (75%) of 24 C3HeB/FeJ mice ( $p < 0.01$ ). Resistance among BALB/c mice was only  
296 observed at PZA doses  $\geq 100\text{mg/kg}$ , whereas resistance among C3HeB/FeJ mice was observed at  
297 all dose levels. Despite the proclivity towards selection of resistant mutants in C3HeB/FeJ mice,  
298 replacement of the PZA-susceptible bacillary population with PZA-resistant mutants did not  
299 explain the poor activity of PZA in those with the highest CFU counts at the end of treatment.

300 Indeed, the greatest proportion of PZA-resistant CFU compared to total CFU was among those  
301 C3HeB/FeJ mice receiving the highest PZA doses in which the greatest bactericidal activity was  
302 observed (where up to 100% of the total population was resistant to PZA). On the contrary,  
303 among mice in which PZA did not exhibit bactericidal activity, PZA-resistant mutants remained  $\leq$   
304 1% of the total CFU (Figure 4). Nevertheless, both the absolute number and the proportion of  
305 bacteria resistant to PZA tended to increase with dose among the poorly responding C3HeB/FeJ  
306 mice, indicating that PZA was likely exerting a bactericidal effect against drug-susceptible bacilli  
307 and promoting selective amplification of resistant mutants within a minority sub-population  
308 within these mice, likely in the intracellular compartment. The mean CFU counts of PZA-  
309 resistant mutants were not statistically different between the 2 mouse strains ( $p=0.89$ ,  $0.11$ ,  
310  $0.17$  for 100, 300 mg/kg and 450 mg/kg BID doses respectively).

311 Although colonies from each mouse harboring resistant mutants were processed for whole  
312 genome sequencing, only 79% of colonies selected (20/27 for BALB/c and 53/65 for C3HeB/FeJ)  
313 were actually sequenced due to a variety of technical difficulties.

314 With one exception, all sequenced colonies isolated on PZA-containing media had mutations in  
315 *pncA*, confirming the utility of employing 3-6xMIC PZA concentrations in standard 7H11 agar (pH  
316 6.8) for isolating resistant mutants. In most cases, different colonies from the same mouse  
317 harbored the same *pncA* mutation. Out of 13 C3HeB/FeJ mice with several colonies sequenced,  
318 2 mice had 3 or more colonies with differing mutations in *pncA*, whereas 7 mice had 2 colonies  
319 with either of 2 different mutations, and 4 mice had colonies sharing only one mutation. Among  
320 5 BALB/c mice with several colonies sequenced, 2 mice had colonies with either of 2 different  
321 *pncA* mutations and 3 mice had colonies sharing only one mutation. Table 3 catalogs the



322 mutations identified, most of which have previously been reported as clinically relevant  
323 mutations likely to confer PZA resistance (29, 30). Notably, deletions spanning multiple open  
324 reading frames were quite frequent (8/29 for C3HeB/FeJ vs. 2/10 for BALB/c). Apart from  
325 deletions, seven different mutations in *pncA* were identified in BALB/c mice and 20 in  
326 C3HeB/FeJ mice. Each *pncA* mutation was observed in only one mouse. The only colony without  
327 a *pncA* mutation was selected in a C3HeB/FeJ mouse treated with 300mg/kg and harbored only  
328 an A3311T mutation in Rv3350c (PPE56). Three other colonies isolated from the same mouse  
329 had this mutation as well as identical 2 bp deletions in *pncA*. The fifth colony had no mutation in  
330 Rv3350c, only a different (G108R) mutation in *pncA*.

### 331 *pH assessment*

332 We recently reported that the pH of liquefied caseous material from lesions in C3HeB/FeJ mice  
333 was  $7.39 \pm 0.096$  (range 7.19 - 7.54) (24). To extend our evaluation to another non-clinical  
334 species, we measured pH in 12 different lesions in 4 untreated guinea pigs infected for 13  
335 weeks. An average pH of  $7.23 \pm 0.17$  (range 6.99 – 7.52) was found, only slightly lower than that  
336 of the adjacent normal-appearing lung ( $7.35 \pm 0.22$ ).

337

338 **Discussion**

339 In a previous study we observed that PZA had limited activity in a subset of C3HeB/FeJ mice  
340 with large caseous lesions and hypothesized that this was due to the neutral pH of the liquefied  
341 caseum in such lesions (24). In this study we bring additional evidence in support of this  
342 hypothesis by demonstrating that neither poor distribution of PZA into caseous lesions nor  
343 selection of PZA-resistant mutants explains the limited PZA activity and the observed lack of  
344 dose-response effect.

345 Indeed PZA exposures increased in a dose proportional fashion in plasma and ELF in both  
346 uninfected and infected mice and, at doses of 100 mg/kg or higher, met or exceeded plasma  
347 exposures observed in humans receiving PZA at doses recommended for TB treatment (16, 31).  
348 Moreover, PZA concentrations in the caseous lesions of infected C3HeB/FeJ mice also increased  
349 dose-proportionally and were, on average, 67% of the concurrent plasma concentration, a ratio  
350 similar to that recently observed in a rabbit TB model and consistent with evidence that PZA  
351 diffuses readily through caseum (32-34). To our knowledge, this is the first report of PZA  
352 concentrations in ELF in mice. Conte et al (16) described higher concentrations of PZA in ELF  
353 relative to plasma in uninfected human subjects (ratio ELF/plasma concentration of 13-24) than  
354 we observed in infected mice, except for the median ratio of 22.8 we observed in mice sampled  
355 12 hours after a 150mg/kg dose. This discrepancy may be due in part to the higher systemic  
356 clearance of PZA in mice allowing less accumulation in ELF compared to humans. However,  
357 determination of ELF concentrations of rapidly diffusing small molecules like PZA is also  
358 technically challenging because small differences in dwell time during bronchoalveolar lavage  
359 can introduce large differences in concentration due to rapid drug redistribution from tissue

360 into lavage fluid (35). If the drug in question distributes faster than urea, its ELF concentration  
361 could be over-estimated as the dwell time of the lavage fluid increases. Importantly, infection  
362 did not appear to increase the distribution of PZA into the ELF.

363 As indicated by the lack of a dose-response effect in C3HeB/FeJ mice with large caseous lesions,  
364 achieving higher PZA concentrations are useful only if the pH of the actual lung compartment  
365 inhabited by *M. tuberculosis* is sufficiently acidic for PZA to exert its effect. According to Zhang's  
366 model, intrabacillary accumulation of the active POA moiety increases inversely with the pH of  
367 the milieu (3). At neutral pH, the PZA MIC against *M. tuberculosis* H37Rv may exceed 1600  
368  $\mu\text{g/ml}$  (5), which is 3-4 times higher than the highest average steady state ELF concentrations  
369 observed in uninfected mice in this study or at 4 hours post-dose in uninfected human subjects  
370 administered 1 g daily for 5 days (16), as well as in infected mice receiving a PZA dose (150  
371 mg/kg) that most closely approximates plasma AUCs observed in patients receiving  
372 recommended PZA doses. Only the  $C_{\text{max}}$  in the ELF of infected mice receiving 450 mg/kg BID  
373 approached the MIC at neutral pH.

374 Against cultures of *M. tuberculosis* H37Rv adjusted to a pH of 5.8 in an *in vitro* hollow fiber  
375 model of TB, Gumbo et al established that human-like exposures producing a PZA AUC of  
376 approximately 1500  $\mu\text{g}\cdot\text{h/ml}$  (i.e., AUC/MIC of 120 x MIC of 12.5) produces the same 0.11  
377  $\log_{10}\text{CFU/ml/day}$  fall in CFU counts that was observed in 14-day clinical EBA trials (8, 17).

378 Although the typical plasma AUC produced by standard PZA doses is only 300-500  $\mu\text{g}\cdot\text{h/ml}$ , the  
379 degree to which PZA was shown to accumulate in ELF provided a parsimonious explanation for  
380 sufficient target attainment in lung cavities, leading the authors to conclude that, because PZA  
381 does not accumulate above plasma concentrations inside cells, PZA most likely exerts its

382 sterilizing activity against extracellular bacilli. However, the bactericidal and sterilizing activity of  
383 doses producing clinically relevant plasma PZA AUC values in commonly used mouse strains, in  
384 which virtually all bacilli are found intracellularly, is well demonstrated (11, 18, 36). In the  
385 present study, we found that after 15 doses of PZA administered to BALB/c mice, the EC<sub>50</sub> of  
386 approximately 150 mg/kg produced a 0.102 log<sub>10</sub>CFU/dose reduction. The mean plasma AUC  
387 produced by this dose is 388 µg.hr/ml. Thus, a dose producing similar plasma AUC in BALB/c  
388 mice and humans produces similar EBA at exposures that are approximately one-quarter of the  
389 exposure required for the same effect in the hollow fiber system at pH of 5.8. The lower  
390 exposures needed to observe the same kill in mice compared to the hollow fiber would be  
391 explained if the pH in infected murine macrophages is closer to 5.0, a level that is attained by  
392 activated macrophages (7, 37).

393 If extracellular bacilli are a target of PZA's sterilizing activity, then the pH of the ELF or caseum in  
394 which they are found should be sufficiently low for the expected PZA concentrations to be  
395 active. For example, a pH of 5.8 was studied in the hollow fiber model (17). The technique of  
396 sampling ELF via BAL makes it difficult to measure ELF pH. However, it is possible to measure  
397 the pH of airway lining fluid *in vivo*. The pH of the tracheal lining fluid of anesthetized mice was  
398 reported to be 7.1 (38). Similarly, a study using a bronchoscopically directed pH electrode  
399 reported the pH of subsegmental bronchi to be 6.6 in humans (6.48 in patients presenting with  
400 bacterial pneumonia) (39). Our finding of a more neutral pH in caseum from C3HeB/FeJ mouse  
401 and guinea pig tubercles extends previous work in rabbits showing an increase in pH of caseum  
402 (from 6.4 to 7.4) as lesions mature, caseate and liquefy (40). We are aware of only one report of  
403 the pH of caseum from human cavities which described the pH of resected cavity tissue

404 homogenates as ranging between 6.1 and 7.4, but noted that the pH was 6.8 or above in 15 out  
405 of 17 lesions (41). This report is well in line with the aforementioned results from animal  
406 models. Taken together, these data run counter to the prevailing notion that caseum is acidic  
407 and make the assumption that extracellular bacilli routinely encounter conditions of pH  $\leq$  6.5  
408 rather tenuous (17).

409 The remarkable dichotomous activity of PZA in C3HeB/FeJ mice in the present study confirms  
410 and extends our prior results (24). It also is consistent with the important role of pH in the  
411 action of PZA. Among those mice with less severe disease, the pharmacodynamics of PZA were  
412 similar to that observed in BALB/c mice, suggesting similar conditions wherever bacilli are found  
413 intracellularly in C3HeB/FeJ mice, such as in small cellular granulomas or in the cellular cuff of  
414 caseous granulomas. Based on the exposure-response relationship defined in the hollow fiber  
415 model (17), the PZA exposures attained inside mouse macrophages would not be expected to  
416 exert a bactericidal effect comparable to the EBA in humans unless the pH in the macrophage  
417 compartment was below 5.8 and closer to 5.0, a value attainable in activated macrophages (7).  
418 On the other hand, in those C3HeB/FeJ mice with large caseous lesions where the pH  
419 approaches 7.4 (24), little or no bactericidal activity of PZA was observed despite attaining  
420 plasma AUCs that are 3-5 times those produced in plasma at typical human doses but similar to  
421 the AUC associated with a 0.11 log<sub>10</sub> CFU reduction per day at pH of 5.8 in the hollow fiber  
422 experiments (17). Moreover, the estimated PZA AUC in these lesions of approximately 850  
423  $\mu\text{g}\cdot\text{h}/\text{ml}$  is the same as that associated with a 2 log kill over 28 days in the hollow fiber  
424 experiments. Taken together, these results are consistent with the idea that local pH has a  
425 profound effect on PZA activity.

426 The facts that PZA exerts its sterilizing contribution during the first 2 months of treatment in the  
427 current first-line regimen and that patients unable to take PZA can still be cured when rifampin-  
428 containing regimens are given for 9 months rather than the usual 6 months with PZA (42),  
429 suggests that the bacillary population eradicated by PZA is not susceptible only to PZA, just  
430 more susceptible to PZA than to rifampin, and that this population is of a limited size. When the  
431 pH is 6.5 and above, it is unlikely that PZA exposures routinely achieved in TB patients will  
432 produce the kind of bactericidal effect that will lend significant additive sterilizing activity to the  
433 first-line regimen. The high pH of the caseum lining the cavity wall likely explains the limited  
434 contribution of PZA to the activity of the first-line regimen over the first 14 days of treatment.  
435 There is ample evidence however, that PZA contributes to the sterilization of sputum cultures  
436 later in the initial phase in patients receiving the first-line regimen, including recent evidence  
437 that this contribution is exposure-dependent (10). While the poor results in mice with large  
438 caseous lesions seems inconsistent with the well-known treatment shortening potential of PZA  
439 in humans, the results may be quite consistent if one considers its sterilizing activity is only  
440 evident in the context of combination therapy. Our results suggest that while other drugs like  
441 rifampin and isoniazid eradicate the bacilli in caseous material, PZA eliminates a subpopulation  
442 that is likely residing inside macrophages, in phagolysosomes at low pH, where achievable PZA  
443 concentrations exceed the local MIC. The pH dependence of PZA activity, the near neutral pH of  
444 caseum across species, and the dramatic lesion-dependence of PZA activity in C3HeB/FeJ mice is  
445 compelling evidence that PZA exerts its most prominent sterilizing effects against intracellular  
446 bacilli. This model is not mutually exclusive with the model proposed by Gumbo et al in which  
447 PZA exerts activity against extracellular bacilli in sufficiently acidic environments. Rather, given

448 the remarkable heterogeneity observed in human TB, these models might be considered  
449 complementary. Further studies to evaluate the sterilizing activity of PZA in combination  
450 therapy and to isolate the lesions in C3HeB/FeJ mice in which PZA exerts a sterilizing effect are  
451 warranted.

452 We found that, like C3HeB/FeJ mice and rabbits, guinea pigs develop necrotic lesions with  
453 neutral caseum by 13 weeks post-infection. Despite this, PZA has been reported to have  
454 bactericidal activity in guinea pigs (36). This apparent discrepancy may be explained by the fact  
455 that examinations of lung histopathology revealed only poorly formed granulomas and limited  
456 necrosis at the time of treatment initiation (4 weeks post-infection) and active doses of PZA  
457 were associated with reduced numbers and size of granulomas (at 8 weeks post-infection) (36).  
458 Thus, it is likely that PZA exerted its bactericidal effect against bacilli under low pH conditions  
459 inside activated macrophages rather than inside caseous regions. Further studies of PZA activity  
460 against more established disease in guinea pigs are warranted to test this hypothesis.

461 PZA resistance was not the explanation for the dichotomous activity of PZA in C3HeB/FeJ mice,  
462 since resistant mutants never replaced the sensitive population in the mice with worst response  
463 to PZA. However, it is interesting to see that selective amplification of PZA-resistant mutants  
464 occurs more readily, including at lower doses, in C3HeB/FeJ mice compared to BALB/c mice  
465 (18/24 vs 11/30 respectively). Many C3HeB/FeJ mice receiving doses  $\geq 100$  mg/kg for 8 weeks  
466 harbored more than 0.1% PZA-resistant CFU, including mice in which the week 8 CFU count was  
467 likely not significantly lower than the D0 CFU count. This is evidence that, even when PZA was  
468 not exerting bactericidal effects on the largest population of bacilli in the large caseous lesions,  
469 it was exerting significant bactericidal activity against PZA-susceptible bacilli and selectively

470 amplifying PZA-resistant mutants in smaller granulomas and cellular lesions similar to those in  
471 BALB/c mice. The limited effect of PZA in liquefied caseum, where the highest bacterial counts  
472 are observed, may help to explain why PZA resistance typically emerges only after resistance to  
473 rifampin and isoniazid, and why PZA is not very effective at preventing the emergence of  
474 resistance to these and other companion agents that are bactericidal in that compartment (43).  
475 However, additional experiments comparing the selection of mutants resistant to PZA and to  
476 companion agents in large caseous lesions versus the rest of the lung are needed to confirm  
477 these results.

478 PZA resistance was explained by *pncA* mutations in all mice. Virtually all of these mutations have  
479 been described in PZA-resistant clinical isolates and most of them were found only in PZA-  
480 resistant isolates, adding to the evidence presented here that these mutations confer PZA  
481 resistance (30). Therefore, C3HeB/FeJ mice may be an excellent model to study factors  
482 associated with the selection of PZA-resistant mutants and the clinical significance of specific  
483 mutations. It is noteworthy that large multigenic deletions including *pncA* were observed in  
484 some PZA-resistant isolates from mice. Multigenic deletions are rare among reported clinical  
485 isolates (44, 45). However, they may be more difficult to detect by selective sequencing  
486 approaches commonly used with clinical isolates.

487

488 Our study has important limitations. Firstly, we did not plate large caseous lesions in C3HeB/FeJ  
489 mice separately from the rest of the lung, which prevented specific confirmation of where the  
490 selective killing of PZA-susceptible bacteria and amplification of PZA-resistant mutants was



491 occurring. Secondly, in extrapolating the pH of TB lesion compartments from mice and other  
492 animal models to humans, we are limited by the scant data available on the pH of human  
493 caseum and the intracellular compartments inhabited by phagocytosed bacteria *in vivo*.  
494 Although further confirmation of the pH of human caseum is needed, the available data  
495 presented above suggest a similar pH across species. Although there is evidence that  
496 macrophages activated with interferon-gamma *in vitro* deliver *M. tuberculosis* to an acidic  
497 compartment with a pH as low as 4.5-5.0 (7), confirmation of delivery of Mtb to acidified  
498 phagosomes in a living infected host was elusive until recently (46). Still, new tools are needed  
499 to more accurately quantify the pH of intracellular vacuoles and other lesion compartments  
500 inhabited by *M. tuberculosis in vivo* in a manner that can be correlated with PZA  
501 pharmacodynamics. A third limitation is that, due to the more rapid clearance of PZA in mice  
502 compared to humans, the concentration-time profiles produced in our mice reproduced human  
503 AUCs but did not necessarily mimic the time course of PZA concentrations in humans. As  
504 mentioned above, this may be one reason for less accumulation of PZA in ELF of mice compared  
505 to humans. It also may lead to discordance between the relationships of important PK/PD-based  
506 exposure indices and targets to efficacy and therefore demands caution when extrapolating  
507 between mice and humans or the hollow fiber model, as we have here. A final limitation of this  
508 study is the high level of dilution (median=40, IQR=21-64) of ELF with PBS, which made the  
509 lower limit of PZA quantification higher than that of plasma. The technical limit of quantification  
510 in each sample was 0.2µg/ml, making the lower limit of quantification in ELF 4-13µg/ml.  
511 Furthermore BAL was performed with 300µL of PBS rendering infeasible to duplicate samples or  
512 to repeat runs when results were not within 20% of controls (1 case out of 11).

513

514 **Conclusions**

515 In this study, we confirm that PZA has variable, lesion-dependent activity in C3HeB/FeJ mice in  
516 which poor PZA activity occurs in large caseous lesions due to the neutral pH of the caseum  
517 therein. Such lesion-dependent activity of PZA in C3HeB/FeJ mice, like that recently  
518 demonstrated for clofazimine and oxazolidinones (24, 47), promotes the C3HeB/FeJ mouse  
519 model as a valuable tool for studying the influence of lesion type and microenvironment on  
520 drug distribution and drug action, including the selection of drug-resistant mutants. Although  
521 additional studies to evaluate the contribution of PZA to the standard first-line regimen in  
522 C3HeB/FeJ mice are warranted, these results suggest that future investigations in C3HeB/FeJ  
523 mice may give a more holistic and nuanced appraisal of the potential contribution of PZA to  
524 novel regimens.

525

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534

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657 **Tables and Figures**

658 Table 1: Plasma PK parameters of PZA in C3HeB/FeJ mice

Infection status	PZA dose (mg/kg)	AUC <sub>0-24</sub> ( $\mu\text{g}\cdot\text{h}/\text{ml}$ )	C <sub>max</sub> ( $\mu\text{g}/\text{ml}$ )	T <sub>1/2</sub> (h)
Uninfected C3HeB/FeJ mice (single dose)	10	9.2	5.6	0.6
	30	59.2	23	1
	150	577.9	187.8	1.4
	300	1,081.6	263	1.9
	450 BID	5,326.8	665.3	3.5
Infected C3HeB/FeJ mice (steady state)	150	455.6	166	1.3
	450 BID	3,589.6	467	1.6
Infected BALB/c mice (single dose)	150 (28)	388	163	1.2
	450 BID*	3241.8	472.9	2.3

659 Legend: Parameters were calculated from mean concentration data. The data presented are  
660 from one of two representative experiments in each infection condition. \*= unpublished data

661

662

663 Table 2: Ratio of ELF/plasma PZA concentrations in C3HeB/FeJ mice.



	PZA dose (mg/kg)	Time			
		Median ELF/plasma ratio (number of mice)			
		1.5h	3h	7h	12h
Uninfected mice (single dose)	30		1.2 (2)	3.7 (1)	
	150		0.9 (7)	1 (4)	22.8 (4)
	300		2 (6)	1 (5)	
	450 BID		1.1 (3)	2.2 (3)	1.2 (3)
Infected mice (steady state)	150	1.1 (6)	3 (7)	3.1 (8)	NA
	450 BID	1.3 (6)	1.6 (8)	1.2 (7)	

664 Legend: NA=not applicable (due to non-quantifiable PZA concentration in ELF or in plasma). No

665 PZA was measurable at 0h and 17h time point or in the 10mg/kg arm.

666

667

668 Table 3: Results of mutations in *pncA* gene (Rv2043c) observed in PZA-resistant isolates

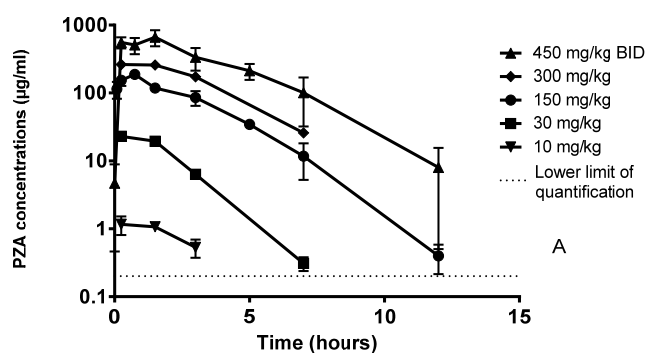
Type of mutation	BALB/c mouse strain	C3HeB/FeJ mouse strain	
Point mutations	K96M G132A C138Y T142P L159V H171R	M1I L19R A46E S67P H71Y C72F Y99stop G108R I133N	T135P H137D V139M A146V S164P L172P E173stop
Small Indels*	+A in E127	-G in P54, -CG in A26, -T in I133 -CGTCAGCGGTACTC in V73-P77	
Large-scale Deletions	Rv2023c-Rv2048c (27 kb) Rv2027c-Rv2047c (19.8 kb)	Rv2030c-Rv2048c (21 kb) Rv2034-Rv2045c (9 kb) Rv2039c-Rv2048c (13 kb) Rv2040c-Rv2045c (3.5 kb) Rv2041c-Rv2043c (1.1 kb)	

		Rv2042c-Rv2043c (1 kb)
		Rv2043c-Rv2044c (0.2 kb)
5' UTR	A>G -11 bp upstream	
No mutation	-	one (A3311T mutation in Rv3350c)

669 Legend: \*frame-shifts

670

671 Figure 1



672

673 Legend: Dose-ranging PZA concentration-time profiles in uninfected C3HeB/FeJ mice in plasma.

674 Data are plotted as mean and SD.

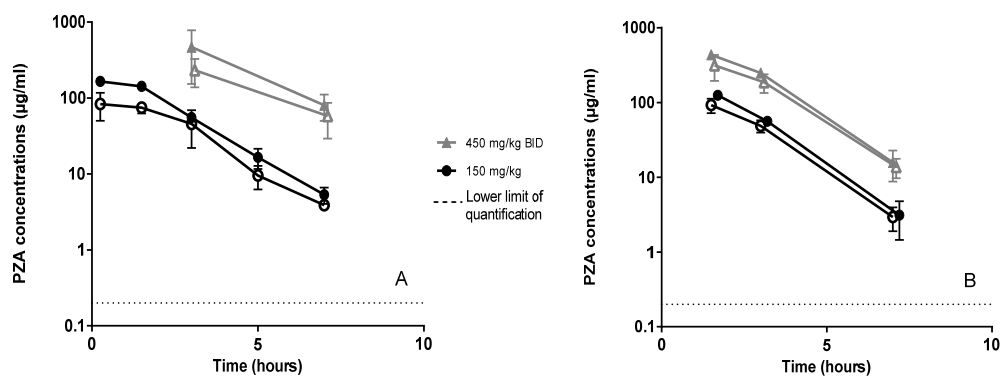
675

676

677

678

679 Figure 2



680

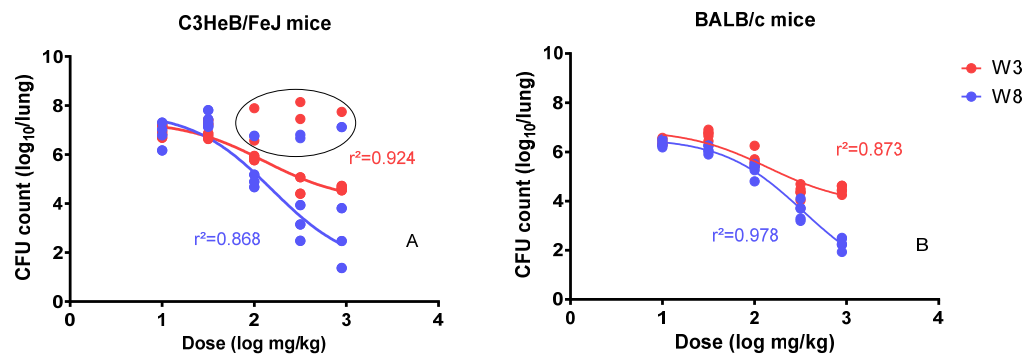
681 Legend: Dose-ranging PZA concentration-time profiles in infected C3HeB/FeJ mice in plasma

682 (solid shapes) and lesions (open shapes) for 150 and 450 BID mg/kg. Panel A and panel B

683 represents 2 different sets of experiment. Data are plotted as mean and SD.

684

685 Figure 3

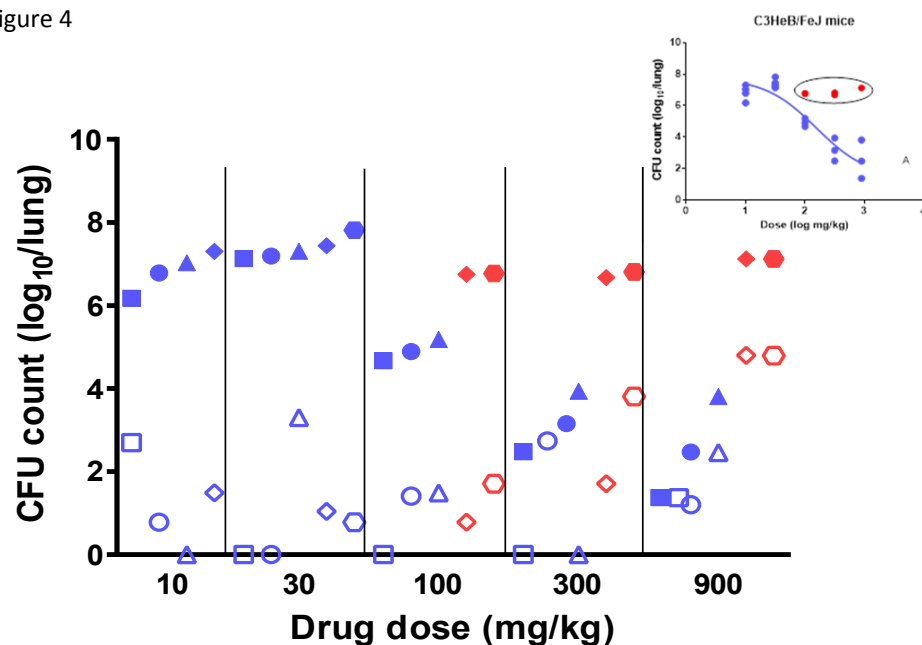


686

687 Legend: Dose-response profiles in C3HeB/FeJ mice (panel A) and BALB/c mice (panel B) after 3  
688 weeks (blue symbols and line) and 8 weeks (red symbols and line) of treatment (W3 and W8  
689 respectively). Open ellipse: C3HeB/FeJ mice excluded from curve fit (outliers).

690

691 Figure 4



692

693 Legend: Total CFU counts (solid symbols) and PZA-resistant CFU counts (open symbols) in  
694 C3HeB/FeJ mice treated with escalating PZA doses. Each symbol shape represents an individual  
695 mouse in its dose group. Shown in blue are the CFU counts from mice considered to fit the  
696 dose-response curve shown in the inset. Shown in red are the CFU counts from mice considered  
697 non-responsive to the dose increase. Solid symbols indicate the total CFU counts determined on

698 drug-free media for each individual mouse. The open symbols indicate the resistant CFU counts  
699 determined on PZA-containing plates for the same mouse.