

1 **Supplemental Materials and Methods**

2 **Bacterial strains.** *M. tuberculosis* Erdman (TMCC 107) was used for drug evaluations in mice.

3 Bacteria were prepared for infection as described previously [1, 2].

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5 **Drug compounds.** Isoniazid was purchased from Sigma. AN12855 was synthesized as described  
6 [3].

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8 ***In vivo* efficacy and resistance studies.** Female specific pathogen-free C3HeB/FeJ mice aged 8-  
9 10 weeks were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in a  
10 bio-safety level III animal facility and provided sterile bedding, water and mouse chow.

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12 **Ethics statement.** The animal protocols involving mice were approved by Colorado State  
13 University's Institutional Animal Care and Use Committee.

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15 **Infection of mice.** The *M. tuberculosis* Erdman strain (TMCC 107) was used for aerosol infections  
16 of mice, and the inocula were prepared as previously described [1]. C3HeB/FeJ mice were exposed  
17 to a low dose aerosol infection using a Glas-Col inhalation exposure system [2], resulting in an  
18 average of ~ 100 bacteria in the lungs of aerosolized mice 1 day following exposure.

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20 **Drug treatments of mice.** For drug treatments, mice were administered isoniazid at 25 mg/kg in  
21 sterile water or AN12855 at 100 mg/kg in 1% [w/v] methyl cellulose (Sigma) 0.1% [v:v]  
22 polysorbate-80 (Sigma) via oral gavage five times weekly for 2, 4, or 8 weeks starting 68 days  
23 (efficacy studies) or for 7 consecutive days (PK studies).

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25 **Agar MICs**

26 Agar MICs were determined using the agar proportion method using Middlebrook 7H11 with  
27 0.02% [v/v] glycerol (Sigma), 10% [v/v] OADC (oleic acid, albumin, dextrose, catalase; Difco),  
28 cycloheximide (0.03 mg/mL), carbenicillin (0.05 mg/mL) [7H11-OADC] and further  
29 supplemented with two-fold increasing concentrations of each antimicrobial agent. The inoculum  
30 was  $1 \times 10^4$  CFU per 0.1 mL spot. MICs were scored as the first consecutive plate for which no  
31 growth was visible after at least 21 days of incubation at 37°C.

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33 **Enumeration of CFU burdens and drug-resistant colonies from infected lung tissues.** Eight  
34 mice per group were used for studies involving C3HeB/FeJ mice owing to larger heterogeneity in  
35 treatment response. Infected mice were sacrificed at the start of treatment as pretreatment controls  
36 and to determine the *in vivo* frequency of resistance prior to the start of treatment. For endpoint  
37 analysis, mice were euthanized three days following the end of treatment, and the lungs were  
38 collected and frozen at -80°C. Whole lungs (C3HeB/FeJ mice) were disrupted with a tissue  
39 homogenizer (Bertin, Precellys), serially diluted in PBS, and plated on 7H11-OADC to determine  
40 lung CFU burdens. Samples were also replica plated onto a 7H11-OADC containing either  
41 isoniazid or AN12855 at 5× to 10× their respective agar MIC or a final concentration of 0.625  
42 mg/L INH or 1.25 mg/L AN12855 to isolate *M. tuberculosis* Erdman resistant mutants. Colonies  
43 were counted after at least 21 days of incubation at 37°C. The viable bacterial counts of whole  
44 organs were calculated and converted to logarithms. The data were expressed as mean log<sub>10</sub> CFU  
45 ± the standard error of the mean (SEM) for each group. In cases where no CFU were recovered,  
46 values were listed at the lower limit of detection. Frequency of resistance was calculated as the

47 number of resistant colonies over the total number of CFU plated. Companion studies evaluated  
48 the *in vitro* frequency of resistance using the same agar medium by plating serial dilutions of *M.*  
49 *tuberculosis* Erdman cultured to  $2 \times 10^9$  CFU/mL in 7H9-ADC with 0.05% [v/v] Tween 80 (Sigma).

50  
51 **Isolation of DNA and sequencing of candidate genes.** DNA was extracted from strains of interest  
52 using a Fastprep-24 (MP Biomedicals). Briefly, 1 ml of culture was transferred to a 2 ml Fastprep  
53 Lysing Matrix B tube and agitated for 20 s at a speed of 4 m/s. Extracts were centrifuged for 5  
54 mins and then filtered through a 0.2  $\mu$ M filter. Extracted DNA was stored at 4°C. PCR of candidate  
55 genes were performed using the following primer pairs fabG1-inhA (TB-FabG1-MMF1,  
56 TCAATACACCCGCAGCCA; InhA\_F2, TTCATAGGTTTCGGTCTCC; InhA-R1,  
57 GTGATACCCACCGAAATGC) and katG (TB-KatG-MMF3, TCTATACCGGACTACGCC;  
58 TB-KatG-MMR4, TGGACCGTTTCGACAA).

59  
60 **Determination of compound MIC against drug resistant *M. tuberculosis*.** The IC<sub>90</sub> of  
61 compound was determined as previously described [4]. Briefly, bacterial growth was measured in  
62 the presence of test compounds. Compounds were prepared as 10-point two-fold serial dilutions  
63 in DMSO and diluted into 7H9 broth with 10% [v/v] OADC and 0.05% [w/v] tween 80 in 96-well  
64 plates with a final DMSO concentration of 2%. Each plate included assay controls for background  
65 (medium/DMSO only, no bacterial cells), zero growth (2  $\mu$ M rifampicin) and maximum growth  
66 (DMSO only), as well as a rifampicin dose response curve. Growth was measured after 5 days by  
67 OD<sub>590</sub>. Dose response curves were generated using the Levenberg-Marquardt algorithm and the  
68 concentrations that resulted in 90% inhibition of growth were determined (IC<sub>90</sub>).

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70 **Lesion PK and bioanalysis.** Drug levels in plasma, lung tissues and pulmonary lesions in drug  
71 treated C3HeB/FeJ mice were determined by bioanalytical analysis (PHARMout Laboratories,  
72 Inc.). Briefly, C3HeB/FeJ mice were infected by low dose aerosol and treatments started 10 weeks  
73 later. Mice received AN12855 (100 mg/kg) or INH (25 mg/kg) for seven consecutive days by oral  
74 gavage to achieve steady state levels in plasma and tissues. Plasma, whole lungs, fibrotic caseous  
75 lesions (Type I lesion), and excised caseum were harvested at defined intervals, weighed, and  
76 treated with methanol and acetonitrile at a ratio of 0.01 mL:0.44 mL per 0.05 mL of tissue  
77 homogenate to ensure sterility. Samples were kept frozen at -80°C until use. Antibiotic  
78 concentrations in extracted samples were analyzed by liquid chromatography-tandem mass  
79 spectrometry (LC-MS/MS) using an API5000 or 4000 instrument (AB Sciex). Quantification was  
80 achieved by comparing the analyte/internal standard peak areas with the internal standard. The  
81 limit of quantitation was 1 ng/mL.

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- 83 1. Lenaerts, A.J., et al., Preclinical testing of the nitroimidazopyran PA-824 for activity  
84 against *Mycobacterium tuberculosis* in a series of in vitro and in vivo models. *Antimicrob*  
85 *Agents Chemother*, 2005. **49**(6): p. 2294-301.
- 86 2. Kelly, B.P., et al., Low-dose aerosol infection model for testing drugs for efficacy against  
87 *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*, 1996. **40**(12): p. 2809-12.
- 88 3. Xia, Y., et al., Discovery of a cofactor-independent inhibitor of *Mycobacterium*  
89 *tuberculosis* InhA. *Life Science Alliance*, 2018. **1**(3).
- 90 4. Ollinger, J., et al., A dual read-out assay to evaluate the potency of compounds active  
91 against *Mycobacterium tuberculosis*. *PLoS One*, 2013. **8**(4): p. e60531.

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