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CLINICAL THERAPEUTICS

Assessment of Bactericidal Drug Activity and Treatment Outcome in a Mouse Tuberculosis Model Using a Clinical Beijing Strain

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ABSTRACT *Mycobacterium tuberculosis* Beijing strains are associated with lower treatment success rates in tuberculosis (TB) patients. In contrast, laboratory strains such as H37Rv are often used in preclinical tuberculosis models. Therefore, we explored the impact of using a clinical Beijing strain on treatment outcome in our mouse tuberculosis model. Additionally, the predictive value of bactericidal activity on treatment outcome was assessed. BALB/c mice were infected with a Beijing strain and treated with one of 10 different combinations of conventional anti-TB drugs. Bactericidal activity was assessed by determining reductions in mycobacterial load after 7, 14, and 28 days and after 2, 3, and 6 months of treatment. Treatment outcome was evaluated after a 6-month treatment course and was based on lung culture status 3 months posttreatment. None of the anti-TB drug regimens tested could achieve 100% treatment success. Treatment outcome depended critically on rifampin. Four non-rifampin-containing regimens showed 0% treatment success compared to success rates between 81 and 95% for six rifampin-containing regimens. Bactericidal activity was predictive only for treatment outcome after 3 months of treatment. Our data advocate the use of multiple mycobacterial strains, including a Beijing strain, to increase the translational value of mouse TB models evaluating treatment outcome. Additionally, our findings support the notion that bactericidal activity in the first 2 months of treatment, as measured in clinical phase IIa/b trials, has limited predictive value for tuberculosis treatment outcome, thus emphasizing the need for better parameters to guide future phase IIII trials.

KEYWORDS mice, *Mycobacterium tuberculosis*, rifampin, bactericidal activity, treatment outcome, relapse

With 1.8 million deaths in 2015, tuberculosis (TB) surpassed HIV as a leading cause of death among infectious diseases (1). One factor contributing to this ongoing burden of TB is the rapid emergence of *Mycobacterium tuberculosis* strains of the Beijing genotype (2, 3). These strains specifically contribute to the spread of drug-resistant TB and are clinically associated with increased rates of treatment failure (2–7).

To overcome this new challenge in TB treatment, novel treatment strategies with increased efficacy are urgently needed. However, clinical trials evaluating TB treatment outcome are expensive, involve large numbers of patients, and may take many years from study design to possible approval for clinical use (8). Moreover, phase IIa and IIb trials, which rely on early bactericidal activity (EBA) and surrogate endpoints, such as 2-month sputum culture status, cannot predict treatment outcome in phase III trials in TB to a satisfying degree (8–10).

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TABLE 1 Schematic overview of the experiments

Drug regimen[a]	No. of mice used for determination of mycobacterial load by time point[b]							
	D0	D7	D14	D28	M2	M3	M6	M6 + 3
R	3	3	3	3				
H	3	3	3	3				
Z	3	3	3	3				
S	3	3	3	3				
E	3	3	3	3				
RE (6RE)	3	3	3	3	3	3	3	20
RZ (2RZ/4R)	3	3	3	3	3	3	3	15
RH (6RH)	3	3	3	3	3	3	3	15
RHZ (2RHZ/4RH)	3	3	3	3	3	3	3	21
RHZE (2RHZE/4RH)	3	3	3	3	3	3	3	21
RHZS (2RHZS/4RH)	3	3	3	3	3	3	3	21
HS (2HS/4H)	3	3	3	3	3	3	3	9
HZ (2HZ/4H)	3	3	3	3	3	3	3	20
HE (6HE)	3	3	3	3	3	3	3	18
ZES (2ZES/4E)	3	3	3	3	3	3	3	20

[a]Data in parentheses report treatment details. For instance, 2RZ/4R indicates 2 months of RZ treatment followed by 4 months of R treatment. R, rifampin; H, isoniazid; Z, pyrazinamide; S, streptomycin; E, ethambutol.

[b]Drugs were administered in their human pharmacokinetic equivalent doses, and mice were infected at day −14. D0, day 0 (start of treatment); M2, 2 months (i.e., 8 weeks) after start of treatment; M3, 3 months (i.e., 12 weeks); M6 + 3, 3 months after stop of a 6-month treatment course.

Phase III trials can also be guided by preclinical testing of anti-TB drugs, which is often performed in mouse TB models (11–14). However, recent disappointing results of phase III clinical trials on moxifloxacin for anti-TB treatment, which were partly based on promising results from mouse experiments, have also raised skepticism regarding the predictive value of preclinical TB models and emphasize the need for their improvement (8). This has led to the formation of multiple international consortia, such as PreDiCT-TB and CPTR, aimed at improving the translational value of preclinical TB models (11).

Approaches that are currently being evaluated include the development of specific *in vitro* models that allow drug activity assessment against *Mycobacterium tuberculosis* in different metabolic states (13), increased appreciation of the pharmacokinetic aspects of treatment (8), and the use of mouse models that develop cavitating lesions, thus better representing human pathology (11).

Most mouse TB models that evaluate treatment outcomes use *Mycobacterium tuberculosis* laboratory strains such as H37Rv and Erdman, which were originally derived from clinical isolates in 1905 and 1945, respectively, but are no longer found in patients (11, 13).

Given the significant clinical impact of Beijing strain infections on treatment outcome, the use of Beijing strains in preclinical mouse TB models should increase their translational value. Therefore, the primary aim of this study was to assess treatment outcome, as measured in clinical phase III trials, in mice infected with a Beijing genotype strain (15–17).

Additionally, we evaluated the predictive value of bactericidal activity-based parameters, as measured in clinical phase IIa/b trials, on treatment outcome at multiple time points throughout the full 6-month treatment course.

RESULTS

Mortality and bactericidal activity after single-drug exposure. Mice infected with the Beijing strain were treated with isoniazid (H), rifampin (R), ethambutol (E), pyrazinamide (Z), or streptomycin (S) in three different doses (Table 1). Figure 1 shows mortality and bactericidal activity after 7, 14, and 28 days of single-drug exposure. Earlier observations in our model have shown that untreated Beijing-infected mice uniformly become moribund after 3 to 4 weeks of infection (15). Treatment with

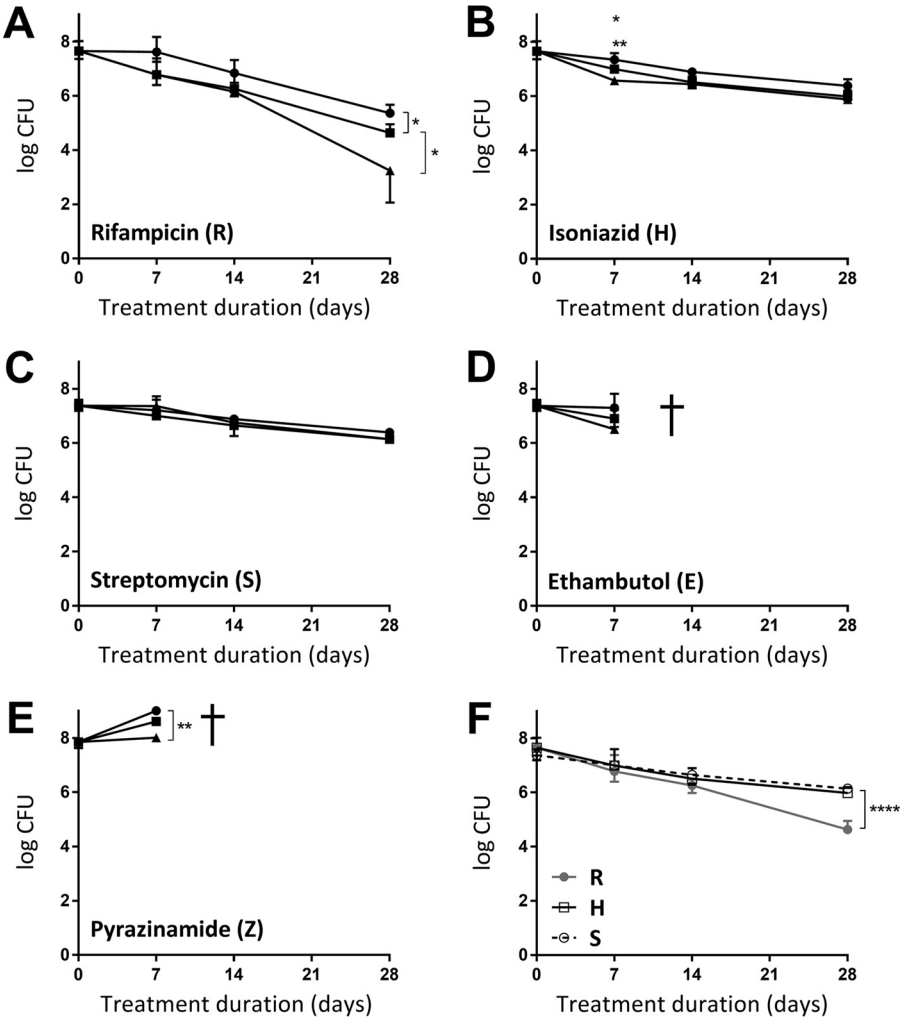

FIG 1 Bactericidal activity after single-drug exposure. Mycobacterial loads in the lungs after single-drug exposure over a 28-day treatment course, using 0.5× (dots), 1× (squares), and 2× (triangles) the human pharmacoequivalent dose (HED) of the selected drugs. (A) Rifampin showed significant dose responses after 28 days of treatment between 0.5× compared to 1× HED and 2× HED (*). (B) Isoniazid showed significant dose responses after 7 days of treatment between 0.5× and 2× HED (**). (C) Streptomycin showed limited bactericidal activity but prevented mortality. Ethambutol (D) and pyrazinamide (E) showed no dose responses and could not prevent mortality. (F) Comparison of 1× HED treatment with rifampin, isoniazid, and streptomycin shows significantly stronger bactericidal activity of rifampin compared to the other two drugs after 28 days (****). Data are shown as medians and ranges, with $n = 3$ mice per time point, with the exception of panels C and D, where 2 mice were used after 7 days due to early mortality. $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.0001$ (****) after Bonferroni correction for multiple comparisons.

rifampin, isoniazid, or streptomycin was able to prevent mortality, whereas mice treated with pyrazinamide or ethambutol showed mortality similar to that of untreated mice.

Rifampin effectively reduced mycobacterial loads in the lungs and showed a significant dose-dependent bactericidal effect after 28 days (Fig. 1A). Isoniazid also showed bactericidal activity, but significant dose-dependent effects were observed only at day 7 (Fig. 1B). Streptomycin reduced mycobacterial loads but did not show dose-dependent effects (Fig. 1C). Ethambutol showed bactericidal activity after 7 days that was comparable to that of rifampin or isoniazid but failed to prevent mortality (Fig. 1D). Pyrazinamide did not display bactericidal activity at any of the dosages tested and did not prevent mortality (Fig. 1E).

Comparison of the bactericidal activity of rifampin, isoniazid, and streptomycin over the 28-day exposure window showed no significant differences between the different drugs after 7 and 14 days of treatment (Fig. 1F). However, after 28 days rifampin showed markedly stronger bactericidal activity than the other two drugs.

TABLE 2 Treatment outcome against a Beijing strain

Drug regimen[a]	Treatment success[b] [% (no. culture negative/total no.)]
RZ (2RZ/4R)	95 (20/21)
RHZS (2RHZS/4RH)	95 (20/21)
RHZE (2RHZE/4RH)	90 (19/21)
RH (6RH)	87 (13/15)
RE (6RE)	85 (17/20)
RHZ (2RHZ/4RH)	81 (17/21)
HS (2HS/4H)	0 (0/9)
HZ (2HZ/4H)	0 (0/20)
HE (6HE)	0 (0/18)
ZES (2ZES/4E)	0 (0/20)

[a]Data in parentheses report treatment details. For instance, 2RZ/4R indicates 2 months of RZ treatment followed by 4 months of treatment with R only, 2RHZS/4RH indicates 2 months of RHZS treatment followed by 4 months of RH treatment, etc.

[b]All rifampin-containing regimens are shaded gray. Results are given as the percentage of mice with culture-negative lungs 3 months after stop of a 6-month treatment course.

Treatment outcome and bactericidal activity after treatment with different anti-TB drug regimens. Treatment outcome after a 6-month treatment course for 10 different anti-TB drug regimens is shown in Table 2. Interestingly, none of the regimens achieved 100% treatment success (cure).

Another finding was that treatment success depended critically on rifampin. The six rifampin-containing regimens showed treatment success rates between 81 and 95%, compared to 0% treatment success of four non-rifampin-containing regimens (Table 2). Among the different rifampin-containing regimens themselves, no significant differences in treatment success could be detected.

We next determined whether the degree of bactericidal activity after any given treatment duration could predict the impact of rifampin on treatment outcome, as observed in Table 2.

To this aim, we ranked the bactericidal activity of the different rifampin-containing regimens and non-rifampin-containing regimens after 7, 14, and 28 days and after 2, 3, and 6 months. The results are shown in Table 3. Of note, although each treatment group was infected at a different time point, no significant differences in mycobacterial load were detected at day 0 before treatment was initiated.

TABLE 3 Bacterial loads over a 6-month treatment course[a]

Treatment initiation (D0)		Intensive phase (all drugs administered)								Continuation phase[b] (no Z/S/E)			
		D7		D14		D28		M2		M3		M6	
Regimen	Load	Regimen	Load	Regimen	Load	Regimen	Load	Regimen	Load	Regimen	Load	Regimen	Load
RHZE	7.3	RH	6.7	RHZ	6.1	RHZS	4.3*	RHZS[c]	0.9****	RE	1.4****	RE	0
RZ	7.7	HS	6.8	HS	6.1	RE	4.3*	RHZE	2.3****	RH	1.4****	RH	0
HS	7.7	RHZE	6.9	RHZS	6.1	RZ	4.5	RZ	3.0**	RHZS	1.6****	RHZS	0
RH	7.7	RHZ	6.9	ZES	6.2	RH	4.7	RE	3.1*	RZ	1.8****	RHZ	0
HZ	7.7	RHZS	7.0	RH	6.2	RHZ	4.9	RHZ	3.8	RHZE	2.1****	RHZE	0
HE	7.9	HE	7.2	RHZE	6.3	RHZE	5.3	RH	3.9	RHZ	2.3****	HS[d]	0.4
RHZ	8.0	HZ	7.2	RZ	6.4	ZES	5.3	ZES	4.3	HS	3.9	RZ[d]	1.1
RE	8.0	ZES	7.3	HZ	6.6	HS	5.5	HS	4.3	ZES	4.0	HE	3.0
RHZS	8.0	RE	7.3	HE	6.7	HE	5.8	HZ	4.9	HZ	4.0	HZ	3.4
ZES	8.0	RZ	7.6	RE	6.7	HZ	6.2	HE	5.2	HE	4.9	ZES	4.9

[a]The different anti-TB drug regimens were ranked based on the mean log value of CFU of mycobacteria in the lungs (Load) of $n = 3$ mice per time point. Rifampin-containing regimens are shaded gray. Mice were infected at day −14, and treatment was started at day 0 (D0). D7, 7 days after start of treatment; M2, 8 weeks after the start of treatment; M3, 12 weeks after the start of treatment; M6, 24 weeks after the start of treatment. Culture-negative values were included for all (statistical) analyses. Asterisks indicate significance (after Bonferroni correction for multiple comparisons): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Significance for each rifampin-containing regimen was calculated against all non-rifampin-containing regimens at that specific time point, and the highest P value is displayed. Zero log indicates no CFU detected.

[b]After 2 months, Z, S, and/or E treatment was stopped for all regimens, with the exception of the ZES, RE, and HE regimens in which E was continued.

[c]Two out of three mice of the RHZS group were culture negative at M2.

[d]Two out of three mice of the HS and RZ groups were culture negative at M6.

After 7 and 14 days, no significant differences in mycobacterial load could be found between rifampin-containing regimens and non-rifampin-containing regimens. After 28 days, the rifampin-containing regimens started to show a trend toward stronger bactericidal activity compared to non-rifampin-containing regimens, which is in line with the single-drug exposure kinetics shown in Fig. 1F. At this time point, two out of six rifampin-containing regimens showed significantly lower mycobacterial loads in the lungs than all non-rifampin-containing regimens tested.

After 2 months of treatment, four out of six rifampin-containing regimens showed significantly lower mycobacterial loads than all of the non-rifampin-containing regimens. However, a clear distinction in bactericidal activity between all rifampin-containing regimens compared to all non-rifampin-containing regimens could be made only after 3 months of treatment (Table 3).

At the end of the 6-month treatment course, no mycobacteria could be cultured from the lungs of nearly all mice treated with rifampin-containing regimens. One exception was the group receiving rifampin and pyrazinamide (termed RZ), in which one out of three mice still had culture-positive lungs. Of the non-rifampin-containing regimens, all mice treated with isoniazid in the continuous phase (HE, HZ, and HS) showed reductions in mycobacterial load, but mycobacteria still could be cultured from the lungs. One exception was the HS group, in which two out of three mice had culture-negative lungs. All mice of the ZES group, which were treated with E in the continuous phase of treatment, showed an increase in mycobacterial loads compared to 3 months of treatment.

DISCUSSION

Three important findings of this study in our TB mouse model using a clinical Beijing genotype strain are that none of the anti-TB drug regimens could achieve clinical cure, that rifampin was essential for treatment success, and that bactericidal activity was an unreliable predictor for TB treatment outcome when assessed within the first 2 months of treatment.

Infections with Beijing strains are of increasing clinical significance due to their global spread and association with treatment failure in TB patients (3–7). The data obtained in our mouse TB model reflect these clinical findings. None of the regimens tested, including the standard-of-care regimen, 2RZH/4RH (2 months of RZH followed by 4 months of RH), achieved 100% treatment success. In contrast, at least four different studies using *Mycobacterium tuberculosis* H37Rv strains, including two previous studies in our own model, showed 100% treatment success of the 2RHZ/4RH regimen in the BALB/c aerosol mouse TB model (12, 16, 18, 19). However, no head-to-head comparison between Beijing and H37Rv was performed in this study, and it should be noted that one study in the H37Rv BALB/c aerosol model actually showed lower treatment success rates of 75% for the 2RHZ/4RH regimen (20). Another study showed similar treatment success rates for 2RZHE/4RH (21) compared to the present study using the H37Rv strain, while 95% treatment success was observed by de Groote et al. using the Erdman strain (22). With regard to treatment success for 2RHZ/4RH using a Beijing strain, our previous study showed 100% success (23), but only four mice were used, compared to 21 mice in the present study, which might account for the difference observed.

Potential differences in treatment outcome between Beijing and H37Rv strains could be explained by the observation that only Beijing strains constitutively express proteins belonging to the DosR dormancy regulon (24, 25). These proteins regulate the mycobacterial metabolic state in response to stressors induced by the host response. This might result in a more rapid or more profound conversion by Beijing genotype strains to a metabolic state in which the mycobacteria are less susceptible to anti-TB drugs. Other possibilities include the ability of Beijing strains to circumvent and manipulate host responses more effectively than H37Rv (26, 27), thus resulting in better localization in (intracellular) niches shielded from anti-TB drugs (28).

Rifampin was essential for treatment success after 6 months of treatment. Despite relatively large group sizes, the current method of treatment outcome evaluation did

not enable us to detect the added effects of other anti-TB drugs. Rifampin monoexposure resulted in previous studies in treatment success rates of 56 to 70% after 4 months of treatment in latent TB mouse models (29, 30). Considering the observed treatment success rates of rifampin-containing regimens of between 81 and 95% in our study, a minor contribution of the other anti-TB drugs could be assumed. However, since we did not test 6 months of rifampin monotherapy in our model, a synergistic effect of other anti-TB drugs on the efficacy of rifampin could not be evaluated. Nevertheless, these findings emphasize the major impact of rifampin resistance on anti-TB treatment outcome.

In TB, clinical phase IIa trials were found to be a poor predictor for treatment outcome (8, 9). These studies measure early bactericidal activity (EBA) in patient sputum samples between 2 and 7 days or between 2 and 14 days in the case of extended EBA (10). Our mouse TB model clearly supports this clinical finding, as no differences in anti-TB drug activity were observed between the rifampin-containing and the non-rifampin-containing regimens after 7 or 14 days of treatment despite their markedly different treatment outcomes after 6 months of treatment.

Our single-drug exposure experiments showed that rifampin started to show significantly stronger bactericidal activity compared to other anti-TB drugs only after a minimum of 28 days of treatment. Two clinical studies that continued EBA measurements up to 28 days indeed found a markedly stronger association between bactericidal activity and treatment outcome for anti-TB drug regimens containing pyrazinamide and rifampin (31, 32). These studies indicate that extending EBA to 28 days is a better predictor for treatment outcome. In our regimen experiments, we found that after 28 days the rifampin-containing regimens showed a trend toward lower mycobacterial loads in the lungs than the non-rifampin-containing regimens. However, a significant distinction in bactericidal activity between all rifampin-containing regimens and all non-rifampin-containing regimens still could not be made. Moreover, after 28 days the standard-of-care RHZE regimen showed mycobacterial loads in the lungs similar to those of the non-rifampin-containing ZES regimen while having markedly different treatment outcomes. Thus, based on our data we conclude that extending EBA for up to 28 days is more informative than 7 or 14 days but remains an unreliable parameter for predicting treatment outcome.

Clinical phase IIb trials measure bactericidal activity over a 2-month period, with sputum culture status as a surrogate endpoint for treatment outcome (9). These studies were initially thought to be indicative for phase III trial outcomes in TB (9), but the disappointing results of the recent phase III REMox trials show otherwise (8, 33). Our study shows that after 2 months of treatment, the rifampin-containing regimens RH and RHZ still do not show significant differences in lung mycobacterial loads compared to the non-rifampin-containing regimens. The inability at this time point to significantly distinguish between regimens with a markedly different treatment outcome after 6 months supports the limited predictive value of measuring bactericidal activity during longer treatment durations in TB.

Our mouse TB model did show a clear distinction in lung mycobacterial loads between rifampin-containing regimens and non-rifampin-containing regimens after 3 months of treatment. However, the value of such a late time point in clinical studies is highly questionable, especially when phase III trials strive to shorten total treatment duration to 4 months (33).

In conclusion, multiple approaches are currently being evaluated for their potential to further increase the translational value of preclinical TB models. Examples such as implementation of mouse strains that better mimic human disease and integration of advanced biostatistics to generate more informative models are likely to improve future anti-TB drug research. Our data complement these developments by advocating an increase in the translational value of preclinical models assessing treatment outcomes, including for *Mycobacterium tuberculosis* genotype strains that currently impact clinical treatment, such as Beijing strains. Also, our data in this mouse TB model support the notion that bactericidal activity in the first 2 months of treatment, as measured in

clinical phase IIa/b trials, has limited predictive value for treatment outcome, which emphasizes the need for better biomarkers to guide future phase III trials.

MATERIALS AND METHODS

Bacterial strain. For all experiments, the previously described Beijing VN 2002-1585 (BE-1585) *Mycobacterium tuberculosis* genotype strain (17) was used. This strain was isolated from a patient in Vietnam in 2002 and was verified as a typical Beijing strain based on single-nucleotide polymorphism analysis (34). Susceptibility assays performed according to CLSI guidelines (35) showed MICs for rifampin of 0.25 mg/liter, for isoniazid of 0.125 mg/liter, for ethambutol of 5 mg/liter, and for streptomycin of 2 mg/liter.

Mice. Specific-pathogen-free female BALB/c mice were obtained from Charles River (Les Oncins, France) and acclimatized at least 7 days prior to starting experiments. Mice received food and water *ad libitum*. On the day of infection, animals were 13 to 15 weeks old and weighed 20 to 25 g. Experimental protocols adhered to the rules specified in the Dutch Animal Experimentation Act and were in concordance with the EU animal directive 2010/63/EU. The Institutional Animal Care and Use Committee of the Erasmus MC approved the present protocols (117-12-08 and 117-12-13).

Infection. A suspension of *Mycobacterium tuberculosis* stored at −80°C was thawed at room temperature for 30 min and centrifuged for 10 min at 14,000 × *g*. The pellet of mycobacteria was resuspended and diluted in fresh phosphate-buffered saline (PBS). Mice were infected under general anesthesia using a mixture of medetomidine (Sedator; 0.5 mg/kg of body weight; Eurovet Animal Health, Bladel, the Netherlands), midazolam (5 mg/kg; Actavis, Baarn, the Netherlands), and fentanyl (0.05 mg/kg; Hameln Pharmaceuticals, Hameln, Germany) by intratracheal instillation of a 40-μl suspension containing 1.4×10^5 (0.3×10^5 to 2.0×10^5) CFU of BE-1585, using a repeating dispenser (Hamilton Company, Bonaduz, Switzerland) and a 1-ml syringe with a 22-gauge mouse gavage feeding needle (Fine Science Tools, Heidelberg, Germany), followed by proper inhalation. Mice were antagonized using a mixture of atipamezole (Antisedan; 2.5 mg/kg; Orion Corporation, Espoo, Finland), flumazenil (0.5 mg/kg; Pharmachemie, Haarlem, the Netherlands), and naloxone (1.2 mg/kg; Orpha-Devel Handels und Vertriebs, Purkersdorf, Germany). Anesthetic and antagonistic agents were administered intraperitoneally in total volumes of 175 μl and 250 μl, respectively. Mice from different treatment groups were infected at different time points but using the same standardized procedure with mycobacteria from the same batch.

Antibiotic treatment. All treatment schedules started 14 days after infection. In the experiments assessing bactericidal activity of single anti-TB drugs, mice received 0.5×, 1×, or 2× the human pharmacokinetic equivalent dose (HED) (36) of rifampin (R) (5, 10, or 20 mg/kg; Erasmus MC Hospital pharmacy), isoniazid (H) (12.5, 25, or 50 mg/kg; Erasmus MC Hospital Pharmacy), streptomycin (S) (100, 200, or 400 mg/kg; Sigma Chemical Co, St. Louis, MO), ethambutol (E) (50, 100, or 200 mg/kg; Sigma), or pyrazinamide (Z) (75, 150, or 300 mg/kg; Sigma) for 5 days a week, up to 28 days. In the experiments assessing bactericidal activity and treatment outcome of the different anti-TB drug regimens, mice received treatment for up to 6 months with different regimens of 1× the HED of each antibiotic 5 days a week. Drugs for each regimen were diluted and mixed in sterile H_2O and were administered together in a total volume of 200 μl via oral gavage, except for streptomycin, which was administered via subcutaneous injections with a volume of 200 μl. The different drug regimens are shown in Table 1.

Assessment of mycobacterial load in the lungs. In order to assess the mycobacterial load in the lungs, mice were sacrificed by CO_2 exposure. To prevent carryover of anti-TB drugs on subculture plates, treatment was stopped 72 h before sacrificing the mice. In addition, activated charcoal (0.4%) was added to the agar to inhibit the antibiotic residue from the tissue samples (37). The lungs were removed aseptically and homogenized according to the protocol for the gentleMACS Octo dissociator (Miltenyi Biotec BV, Leiden, the Netherlands) in 2 ml PBS. From each tissue homogenate 10-fold serial dilutions were made, and then 200 μl per dilution was cultured on drug-free 7H10 Middlebrook agar and incubated for 28 days at 37°C with 5% CO_2, followed by colony enumeration. The time points at which mycobacterial loads were evaluated are shown in the schematic overview of the experiments in Table 1. The lower limit of quantification was 5 CFU (log 0.7).

Data analysis and statistics. Analyses were performed and graphs were made using PRISM GraphPad 6 (GraphPad Software, La Jolla, CA). All data are expressed as median ± range. Student's *t* test was used to calculate significance shown in Fig. 1. Two-way analysis of variance followed by Bonferroni correction was used to calculate significance in Table 3. *P* values of less than 0.05 were considered statistically significant.

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