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EXPERIMENTAL THERAPEUTICS

Moxifloxacin Replacement in Contemporary Tuberculosis Drug Regimens Is Ineffective against Persistent *Mycobacterium tuberculosis* in the Cornell Mouse Model

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ABSTRACT Tuberculosis (TB), which is caused by *Mycobacterium tuberculosis*, remains a leading killer worldwide, and disease control is hampered by the ineffective control of persistent infections. Substitution of moxifloxacin for isoniazid or ethambutol in standard anti-TB regimens reduces the treatment duration and relapse rates in animal studies, and 4-month regimens were not noninferior in clinical trials. Resuscitation-promoting factor (RPF)-dependent bacilli have recently been implicated in *M. tuberculosis* persistence. We aimed to investigate the therapeutic effects of the substitution of moxifloxacin for a drug used in the standard drug regimen in eradicating CFU count-positive and RPF-dependent persistent *M. tuberculosis* using the Cornell murine model. *M. tuberculosis*-infected mice were treated with regimens in which either isoniazid or ethambutol was replaced by moxifloxacin in the standard regimen. The efficacy of the regimens for bacterial CFU count elimination and removal of persistent tubercle bacilli, evaluated using culture filtrate (CF) derived from *M. tuberculosis* strain H37Rv, was compared to that of the standard regimen. We also measured disease relapse rates. The regimen in which moxifloxacin replaced isoniazid achieved total organ CFU count clearance at 11 weeks posttreatment, which was faster than that by the standard regimen (14 weeks), and showed a 34% lower relapse rate. The regimen in which moxifloxacin replaced ethambutol was similar to standard regimens in these regards. Importantly, neither the regimen in which moxifloxacin replaced isoniazid or ethambutol nor the standard regimen could remove CF-dependent persistent bacilli. The finding of CF-dependent persistent *M. tuberculosis* in TB treatment requires confirmation in human studies and has implications for future drug design, testing, and clinical applications.

KEYWORDS *Mycobacterium tuberculosis*, moxifloxacin, resuscitation-promoting factors, Cornell mouse model

Tuberculosis (TB), which is caused by *Mycobacterium tuberculosis*, remains a leading cause of mortality worldwide (1). Current combination antimicrobial regimens require a prolonged 6-month treatment period. This long regimen leads to poor patient compliance, which gives rise to the emergence of drug resistance and high relapse rates (2). Substitution of moxifloxacin (an 8-methoxy fluoroquinolone) for drugs in the contemporary anti-TB regimen has shown promise for improving treatment efficacy (3, 4). *In vivo*, replacement of isoniazid with moxifloxacin led to a shortened treatment duration (5, 6), reduced relapse rates (6, 7), and favorable outcomes (8) in BALB/c and granuloma-forming C3HeB/FeJ mice. In the recent REMoxTB trial, shorter (4-month) moxifloxacin replacement regimens (in which moxifloxacin was substituted for either isoniazid or ethambutol) in human clinical trials failed to achieve noninferiority compared to standard regimens (3, 4), mainly due to higher relapse rates (3, 4, 9). Persistent

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TABLE 1 Organ CFU counts before and after treatment with experimental regimens

Time of infection or treatment[a]	Mean no. of log CFU per lung ± SD				Mean no. of log CFU per spleen ± SD			
	Control	RHZE regimen	RHZM regimen	RMZE regimen	Control	RHZE regimen	RHZM regimen	RMZE regimen
Day 0	4.80 ± 0.14				5.29 ± 0.03			
Day 21	7.54 ± 0.03				6.99 ± 0.06			
2 wk		6.09 ± 0.03	5.92 ± 0.14	5.70 ± 0.12		5.43 ± 0.08	5.54 ± 0.12	4.77 ± 0.10
4 wk		4.80 ± 0.07	4.52 ± 0.28	3.61 ± 0.16		4.05 ± 0.02	4.49 ± 0.08	3.31 ± 0.21
6 wk		4.08 ± 0.11	4.00 ± 0.09	2.53 ± 0.29		3.49 ± 0.16	3.81 ± 0.06	2.27 ± 0.20
8 wk		3.00 ± 0	3.25 ± 0.48	2.00 ± 0		2.44 ± 0.22	3.24 ± 0.13	1.57 ± 0.20
11 wk[b]		2.00 ± 0	2.00 ± 0	0		1.19 ± 0.29	2.00 ± 0	0
14 wk[b]		0	0	0		0	0	0

[a]Day 0 and day 21 represent 2 h and 21 days postinfection, respectively. Times in weeks represent times posttreatment.
[b]CFU counts were derived from one-third of the tissue homogenate, and the limit of detection was 3 CFU/organ.

bacteria that are tolerant to drug therapy may be implicated in the higher disease relapse rates (9).

M. tuberculosis persistence is the single most important hurdle hampering effective TB disease control (10). *M. tuberculosis* has the ability to survive in a dormant, nonmultiplying, and persistent state (11–14). These persistent bacteria do not grow on solid or liquid media and are undetectable using conventional diagnostic methods; however, they can be resuscitated using resuscitation-promoting factors (RPF), which are present in the *M. tuberculosis* culture supernatant (15). Recently, we found that, using a culture filtrate (CF) containing RPF (15), we could induce persistent bacteria to recommence multiplication, rendering them detectable once more in mice (16–18). Moreover, these tubercle bacilli resuscitated with CF could be completely eliminated using high-dose rifampin regimens, shortening the treatment duration with no disease relapses (16, 18).

In this study, we used the Cornell mouse model (19, 20) to investigate the therapeutic impact of moxifloxacin replacement of a drug in the standard anti-TB regimen against both CFU count-positive and CF-dependent bacteria. We compared the regimens in which either isoniazid or ethambutol was replaced by moxifloxacin to the standard regimen by measurement of the elimination rates of CFU counts, the presence of CF-dependent *M. tuberculosis* in mouse organs, and disease relapse rates.

RESULTS

Treatment with moxifloxacin-containing regimens in the Cornell mouse model. In the Cornell mouse model, after 3 weeks of infection, the mean CFU counts in the organs reached log 7.54 in lungs and 6.99 in spleens (Table 1).

When we investigated the substitution of moxifloxacin for either isoniazid or ethambutol in the current drug regimen on the rate of bacterial CFU count elimination, we found that the early bactericidal activities were similar among the three drug treatment regimens, which were 99% kill at 2.2 weeks for the regimen in which moxifloxacin replaced isoniazid (which contained rifampin, moxifloxacin, pyrazinamide, and ethambutol [the RMZE regimen]), 2.7 weeks for the standard regimen (which contained rifampin, isoniazid, pyrazinamide, and ethambutol [the RHZE regimen]), and 3 weeks for the regimen in which moxifloxacin replaced ethambutol (which contained rifampin, isoniazid, pyrazinamide, and moxifloxacin [the RHZM regimen]). Treatment with the RMZE regimen increased the rate of bacterial elimination, showing undetectable CFU counts at 11 weeks, whereas undetectable CFU counts were seen at 14 weeks for the standard regimen and the regimen in which moxifloxacin replaced ethambutol (Table 1).

These observations coincided with the bactericidal activities assessed using the monoexponential bacterial elimination rate constants (Fig. 1 and Table 2), where the exponential rate constants (logarithmic base 10) for net bacterial elimination during treatment ($k_{net\_with\_drug}$) for the standard regimen, the regimen in which moxifloxacin replaced ethambutol, and the regimen in which moxifloxacin replaced isoniazid were −0.46, −0.50, and −0.65, respectively, in lungs and −0.46, −0.46, and −0.60, respec-

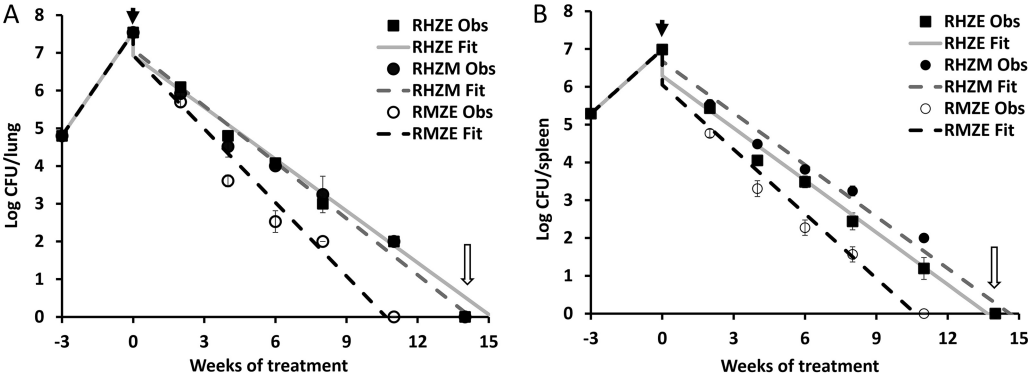

FIG 1 Profiles of *M. tuberculosis* H37Rv after treatment with the RHZE, RMZE, and RHZM regimens in the Cornell mouse model. (A) Elimination of CFU counts in lungs. (B) Elimination of CFU counts in spleens. Solid arrows, start of treatment at 3 weeks of postinfection; empty arrows, start of steroid treatment after the termination of 14 weeks of therapy; Obs, observed.

tively, in spleens (Table 2). The higher that the absolute value of this elimination rate constant is (i.e., the steeper that the slope of the elimination on the logarithmic scale with units of week^{-1} is), the quicker that the exponential elimination rate of CFU counts in the organs is. These values therefore indicate that, compared to the standard therapy, substitution of moxifloxacin for isoniazid gives a significant increase in bacterial elimination in both lungs and spleens, while substitution of moxifloxacin for ethambutol makes a statistically indistinguishable difference.

In the CFU count-free organs, no tubercle bacilli were recovered, which was determined by negative cultures of the organ homogenates in selective Kirchner broth for 4 weeks followed by growth on Löwenstein-Jensen medium.

Posttreatment level of CF-resuscitated MPN in the Cornell mouse model. In order to investigate the effect of the moxifloxacin-containing regimens on the posttreatment level of persistent bacilli detected through CF-induced resuscitation, lung and spleen homogenates obtained during the weeks of treatment when CFU counts of zero were achieved by each of the regimens were incubated with culture filtrates. As shown in Table 3, after 14 weeks of treatment with the RHZE and RHZM regimens, high levels of CF-resuscitated bacilli remained in both the lungs and spleens. For the treatment with the RMZE regimen, at 11 weeks posttreatment, although the CFU counts were zero, there averaged a most probable number (MPN) of CF-resuscitated bacilli per lung and spleen of 2.96 and 3.01 logs, respectively. At 14 weeks of treatment, there was still a MPN of 2 logs of bacilli present (Table 3). The numbers of CF-dependent bacteria among the three treatment groups were not significantly different at 14 weeks posttreatment ($P > 0.05$, $n = 8$).

TABLE 2 Elimination rate constants for different treatment groups

Treatment regimen	Elimination rate constant (wk^{-1})[a]			
	Lungs[b]		Spleens[c]	
	Alpha	% RSE	Alpha	% RSE
RHZE	−0.46	3.20	−0.46	4.76
RHZM	−0.50	8.57	−0.46	6.54
RMZE	−0.65	10.96	−0.57	6.40

[a]The elimination rate constant is equivalent to $k_{net\_with\_drug}$. RSE, relative standard error.
[b]$P = 0.008$ for the RMZE versus RHZE regimen, $P = 0.065$ for the RMZE versus RHZM regimen, and $P = 0.384$ for the RHZE versus RHZM regimen. A P value of <0.017 was significant at the 0.05 level after Bonferroni correction for 3 pairwise comparisons.
[c]$P = 0.018$ for the RMZE versus RHZE regimen, $P = 0.011$ for the RMZE versus RHZM regimen, and $P = 0.943$ for the RHZE versus RHZM regimen. A P value of <0.017 was significant at the 0.05 level after Bonferroni correction for 3 pairwise comparisons.

TABLE 3 MPN of *M. tuberculosis* H37Rv in CFU count-negative mouse lungs and spleens after treatment with different drug regimens[c]

Treatment regimen	MPN/lung[a]				MPN/spleen[b]			
	11 wk	95% confidence limits	14 wk	95% confidence limits	11 wk	95% confidence limits	14 wk	95% confidence limits
RHZE	—		2.50 ± 0.19	2.35–2.67	—		2.56 ± 0.16	2.44–2.70
RHZM	—		2.55 ± 0.14	2.44–2.67	—		2.60 ± 0.09	2.52–2.69
RMZE	2.96 ± 0.15	2.86–3.08	2.30 ± 0.23	2.15–2.50	3.01 ± 0.14	2.92–3.11	2.35 ± 0.16	2.27–2.45

[a]Determined from the MPN of bacilli in the diluted lung homogenates ($n = 8$) with the culture filtrates.
[b]Determined from the MPN of baclli in the diluted spleen homogenates ($n = 8$) with the culture filtrates.
[c]For a CFU count of 0, organs showed no growth in Kirchner liquid medium, followed by inoculation on Löwenstein-Jensen slopes. Broth counts were derived from one-third of the tissue homogenate and calculated to represent the MPN of bacilli in the entire organ. The limit of detection was 30 MPN/organ. —, the colony count was positive, and MPN counts were not performed for the organs. The limit of detection was 3 CFU/organ.

Relapse rates after treatment with the moxifloxacin-containing regimens in the Cornell mouse model. After 8 weeks of high-dosage steroid immunosuppression, the disease relapse rates in mice treated with one of the three drug regimens were determined according to the percentage of mice that developed *M. tuberculosis*-positive cultures (CFU counts) of lung or spleen tissues, or both. As shown in Table 4, treatment with the standard RHZE regimen gave rise to positive organs in 19 out of 21 mice (90% relapse rate), and that with the RHZM regimen led to a 95% relapse rate after 14 weeks of treatment. In contrast, treatment with the RMZE regimen resulted in a 59% relapse rate ($P = 0.03$ versus the RHZE regimen and $P = 0.009$ versus the RHZM regimen; a P value of <0.017 was significant at the 0.05 level after Bonferroni correction for 3 pairwise comparisons).

We also measured, using culture filtrates, CF-dependent bacilli in the organs which were CFU count negative after 8 weeks of steroid treatment. As shown in Table 4, the negative organs in each treatment group contained high numbers of CF-dependent cells. The average values were MPN of 3.3, 3.51, and 3.05 logs per organ in the groups receiving the RHZE, RHZM, and RMZE regimens, respectively.

DISCUSSION

This is the first study to use the reliable Cornell mouse model to characterize the therapeutic efficacy of moxifloxacin replacement *in vivo* against CF-dependent *M. tuberculosis* persistent cells. Compared to the standard anti-TB regimen, we found that moxifloxacin replacement of isoniazid (i) failed to remove CF-dependent bacilli, despite producing (ii) higher organ CFU count elimination rates and (iii) lower disease relapse rates. In contrast, moxifloxacin replacement of ethambutol failed to demonstrate any therapeutic benefits compared to the standard regimen. These results of CF resuscitation need to be confirmed in human studies but may provide a novel mechanistic explanation for the results of moxifloxacin replacement regimens in clinical trials. The findings in this study also have important future implications for novel anti-TB drug design, diagnostic testing, and clinical applications.

The treatment regimens in which moxifloxacin replaced the drugs used in the standard regimen are ineffective against CF-dependent tubercle bacilli. The

TABLE 4 Relapse rates of mice after treatment with different drug regimens[b]

Treatment regimen	No. of mice with positive culture results for:					Relapse rate pn/*N* (%)[a]	MPN of CFU count-negative organs
	Spleen only	Lung only	Both organs	Neither organs	Total		
RHZE	3	0	16	2	21	19/21 (90)	3.30 ± 0.13
RHZM	5	2	13	1	21	20/21 (95)	3.51 ± 0.11
RMZE	4	5	4	9	22	13/22 (59)	3.05 ± 0.18

[a]Relapse rates include all lungs or spleens or both organs positive for bacilli. *N*, total number of mice; pn, number of mice with CFU count-positive organs.
[b]$P = 0.03$ for the RMZE versus RHZE regimen, $P = 0.009$ for the RMZE versus RHZM regimen, and $P = 1$ for the RHZE versus RHZM regimen. A P value of <0.017 was significant at the 0.05 level after Bonferroni correction for 3 pairwise comparisons.

greater therapeutic efficacy and lower relapse rates achieved in this study with the regimen in which moxifloxacin replaced isoniazid than with the standard regimen are consistent with previous reports (5–8). De Groote et al. demonstrated that replacement of isoniazid by moxifloxacin in the standard regimen gave rise to a 63% disease relapse rate (6). In other studies, Nuermberger and colleagues showed that the same drug regimen produced a lower disease relapse rate of 33.3% (7). Late studies using two pathologically distinct murine tuberculosis models demonstrated very similar low disease relapse rates (8). Our study confirmed this interesting observation, showing a 56% relapse rate, indicating the consistency of the drug regimen in different mouse models.

The underlying mechanisms that moxifloxacin replacing isoniazid was more efficacious than the standard or moxifloxacin replacing ethambutol regimens were unknown. It has been shown previously that when mice were treated with rifampin-isoniazid-pyrazinamide, rifampin-isoniazid, or rifampin-pyrazinamide for 6 months, the rifampin-pyrazinamide-treated group demonstrated significantly lower relapse rates than the other two groups treated with regimens containing isoniazid (21), suggesting that isoniazid antagonized the actions of rifampin and pyrazinamide (21). It is possible that the replacement of isoniazid with moxifloxacin eliminated the antagonistic drug interaction, leading to the rapid organ CFU count clearance in mice.

The use of CFU counts as an endpoint reflects the clinical observations in patients to a large degree, which is related to clinical endpoints, such as sputum culture conversion in patients (3, 22, 23). The improved efficacy of the regimen in which moxifloxacin replaced isoniazid compared to that of the standard regimen reflected the clinical outcome in patients to some extent; for example, the regimen in which moxifloxacin was substituted for isoniazid showed effective bactericidal activity and was able to kill CFU count-positive bacilli faster than the standard regimen, leading to a higher sputum culture conversion rate in humans (3, 22, 23).

Despite the improved performance, the regimen in which moxifloxacin replaced isoniazid was ineffective against CF-dependent bacterial cells, which has not been demonstrated previously. At 11 weeks and the end of antibiotic therapy, despite the elimination of CFU counts indicating positivity for bacilli, a considerable MPN of CF-dependent bacilli remained in all the mice, and the number was similar to that in mice treated with the standard drug regimen or the regimen in which moxifloxacin replaced ethambutol. This indicates that although the regimen in which moxifloxacin replaced isoniazid was more bactericidal than the standard regimen, the drug regimen failed to show improved sterilizing activity against persistent bacteria. After 8 weeks of immunosuppression with steroids, 90% of mice treated with the standard regimen and 95% of mice treated with the regimen in which moxifloxacin replaced ethambutol that had CF-dependent bacterial cells became CFU count positive again. This high disease relapse rate can be feasibly explained only by the reactivation of CF-dependent bacterial cells since the mice had a CFU count of 0 before immunosuppression. In addition to a lower relapse rate, mice treated with the regimen in which moxifloxacin replaced isoniazid contained MPN counts in their negative organs similar to those in the other two groups. This indicates that certain drug regimens, such as the regimen in which moxifloxacin replaced isoniazid, may induce heterogeneously more diverse bacterial populations; therefore, not all CF-responding bacteria regained their ability to form colonies on agar plates at the time that we determined relapse, showing a lower disease relapse rate. Further studies on the induction of the heterogeneity of bacterial populations using different anti-TB drug regimens are warranted.

Clinical trials and animal studies. (i) Different test beds but the same mechanism. None of the two phase III clinical trials in which moxifloxacin replaced a drug in the standard regimen demonstrated the noninferiority of those regimens to the standard regimen in terms of treatment duration and disease relapses (3, 4); the underlying mechanism is unclear. An important finding of the REMoxTB trial is that a proportion of patients showed sputum conversion quickly but continued to relapse after treatment with all three drug regimens (9). Our experiments suggest that the high

relapse rates may have been due to CF-dependent bacilli (9), which, until now, have remained undetectable using conventional culture methods, including those used in the clinical trials (3, 4, 9). Persistent bacteria are established causes of prolonged chemotherapy and disease relapse (10). It has been repeatedly shown that, in the Cornell mouse model, the high relapse rate after treatment with the standard drug regimen was due to the presence of CF-dependent persistent bacteria (16, 17). Recently, we showed that drug regimens containing high doses of rifampin (30 mg/kg of body weight or higher) could eliminate CF-dependent persistent bacilli, which led to a shortening of the treatment duration from 14 weeks to just 6 weeks, without disease relapses (16, 18). The lesson learned from the REMoxTB trial is that more rapid culture conversion in the short moxifloxacin-containing regimens may not allow for shortened regimens due to the presence of persistent bacteria (9). A previous study also showed that the early bactericidal activities of certain novel drug regimens were not necessarily predictive of any sterilizing effects (24). This may be attributed to the inability of the drug regimens to eliminate the persistent bacilli which were undetectable using traditional microbiological methods. Therefore, in addition to conventional microbiological methods, evaluation of anti-TB regimens by assessing their efficacies in eliminating RPF-dependent *M. tuberculosis* is important for providing a comprehensive profile of novel drug regimens before proceeding to human clinical trials. The combined data sets on CFU counts, growth in broth, and persister counts will strengthen any claims to be made about a regimen, which will ultimately increase the confidence of advancing it into trials with humans.

(ii) Different test beds and important considerations. The interpretation of the results obtained with the moxifloxacin replacement regimens in mice compared with interpretation of the results of clinical trials requires careful consideration. In the previous animal studies, for example, the burden of persistent bacteria was not detected or assessed (5, 7) largely due to the undetectable feature of the persisters (16, 17). Importantly, there are clear differences in the pathophysiology of TB between humans and mice. Patients with active TB have persisters residing in a milieu of different pathogenic sites, including necrotic/caseating lesions, central liquefactive lesions, open cavities, and closed fibrotic granulomas (25, 26). Indeed, a patient may develop a combination of these lesions over time (10). Consequently, these heterogeneous persistent bacteria coexist with fast-growing bacteria at the time of commencement of antibiotic treatment (10). In contrast, mice do not form granuloma structures after *M. tuberculosis* infection (25), and persistent bacteria are generally low in absolute number (similar to the CFU counts) at the beginning of treatment (16). In addition, an important study has shown that moxifloxacin does not diffuse into caseating lesions, which may also lead to reduced sterilizing activities against persisters in human TB (27).

The treatment of *M. tuberculosis* persisters is complex, and future clinical trials will require careful consideration of the results of mechanistic *in vivo* studies, which can elucidate further insights into potential therapeutic targets. The study reported here represents an important step in the right direction by showing that RPF-dependent persisters may be a novel and important clinical therapeutic target. In conclusion, the regimens in which moxifloxacin was substituted for the antibiotics used in contemporary drug regimens were ineffective against resuscitation-promoting factor-dependent persistent *M. tuberculosis*, despite having favorable therapeutic efficacy against actively multiplying bacteria *in vivo*.

MATERIALS AND METHODS

Bacterium and growth conditions. *M. tuberculosis* strain H37Rv was passaged in mice and grown in 7H9 medium that contained 0.05% Tween 80 and that was supplemented with 10% albumin-dextrose-catalase complex (ADC; Becton, Dickinson, UK) at 37°C without disturbance for 15 days. The culture was stored at −70°C for subsequent animal infection. To determine the viable counts prior to infection, CFU counts were performed prior to freezing and once again after thawing. Counting of the number of CFU was carried out by plating serial 10-fold dilutions of the cultures on 7H11 agar medium supplemented with oleic-albumin-dextrose-catalase complex (OADC; Becton, Dickinson, UK). Colonies were counted after incubation of the plates at 37°C for 3 to 4 weeks. Viability was expressed as the number of log CFU

TABLE 5 Mouse tuberculosis experimental design

Treatment regimen[a]	No. of mice sacrificed at:									
	Total[b]	Day 0	Day 21	2 wk	4 wk	6 wk	8 wk	11 wk	14 wk	22 wk[c]
Control	8	4	4							
RHZE	54			4	4	4	8	8	8	22
RHZM	54			4	4	4	8	8	8	22
RMZE	54			4	4	4	8	8	8	22

[a]Mice were intravenously infected at day 0. Treatment commenced at 21 days. Dosages for each drug were as follows: 10 mg/kg for rifampin, 25 mg/kg for isoniazid, 150 mg/kg for pyrazinamide, 100 mg/kg for ethambutol, and 100 mg/kg for moxifloxacin.

[b]All mice, excluding the mice that died of natural causes during the course of treatment, were infected and treated.

[c]After 8 weeks of hydrocortisone treatment that began after 14 weeks of treatment.

per milliliter. The cultures were subsequently diluted in phosphate-buffered saline and used for inoculations in mice.

Cornell mouse model. The substitution of moxifloxacin for either the isoniazid or ethambutol used in the standard anti-TB drug regimen with rifampin and pyrazinamide was tested using the Cornell mouse model (19, 20). The model was conducted using the experimental design and procedure described previously (17).

Female BALB/c mice (6 to 8 weeks old; Harlan UK Ltd.) were infected intravenously via the tail vein with 1.2×10^5 CFU of mouse-passaged *M. tuberculosis* strain H37Rv per mouse as described previously (16, 17, 20). The animal husbandry guidelines and all animal experiments were performed according to the Animals Scientific Procedures Act, 1986 (an Act of the Parliament of the United Kingdom 1986 c. 14; Home Office Project license number 70/7077), with approval from the St. George's, University of London, ethics committee.

As shown in Table 5, the control group consisted of 8 infected and untreated mice. The treatment groups each contained 54 mice which were treated orally (0.2 ml) with the RHZE, RHZM, or RMZE regimen 5 days per week for 14 weeks. The dosages for the drugs were 10 mg/kg for rifampin, 25 mg/kg for isoniazid, 150 mg/kg for pyrazinamide, 100 mg/kg for ethambutol, and 100 mg/kg for moxifloxacin. Rifampin was administered 1 h before the other drugs to avoid drug-drug interactions.

For assessment of treatment efficacy, a sample of 4 mice each was sacrificed at 2, 4, and 6 weeks of treatment and a sample of 8 mice each was sacrificed at 8, 11, and 14 weeks of treatment (Table 5). Mouse lungs and spleens were transferred into 2-ml tubes each containing 1 ml sterile distilled water and 2-mm-diameter glass beads, followed by homogenization using a reciprocal shaker (Thermo Hybaid Ltd.) for 40 s at a speed of 6.5. The CFU counts from each lung and spleen were performed using serial dilutions of the homogenates, which were plated on 7H11 agar plates.

At 11 and 14 weeks of treatment, the entire organ homogenates from the 8 mice were aliquoted equally into three tubes, which were used for (i) determination of the CFU counts by plating out the organ homogenate suspension on 6 selective 7H11 agar plates, (ii) culturing in 5 ml of selective Kirchner liquid medium (28) with the addition of polymyxin B (200 U/ml), carbenicillin (100 mg/liter), trimethoprim (20 mg/liter), and amphotericin B (10 mg/liter) (Selectatab; Mast Diagnostica GmbH) for 4 weeks and with subsequent subculturing of the entire culture onto Löwenstein-Jensen slopes for a further 4 weeks, and (iii) resuscitation of persistent bacteria with culture filtrate. Kirchner liquid medium was used to isolate different species of mycobacteria from human specimens. Mitchison et al. (28) showed that liquid Kirchner medium, made selective by the addition of the antimicrobials, was more effective for the isolation of mycobacteria than the other media tested. Culture-negative organs were defined as organs for which no colonies grew on 7H11 agar plates and for which there was no growth in selective Kirchner liquid medium followed by inoculation on Löwenstein-Jensen slopes.

Immediately after the termination of 14 weeks of chemotherapy, the remaining mice were administered 0.5 mg/mouse of hydrocortisone acetate by daily oral administration for 8 weeks to suppress host immunity, followed by counting of the number of CFU from the lungs and spleens to determine disease relapse.

Resuscitation of *M. tuberculosis* in mouse lungs and spleens. For resuscitation of *M. tuberculosis* bacilli growing in mouse organs, culture filtrates containing RPFs were used as described previously (15–17).

M. tuberculosis H37Rv was grown in 7H9 medium without disturbance at 37°C for 15 to 20 days until an optical density of 1 to 1.5 was reached. The culture supernatants were collected by centrifugation at $3{,}000 \times g$ for 15 min and sterilized by double filtration through 0.2-μm-pore-size filters (Sartorius). The sterilized culture filtrates were made selective by the addition of polymyxin B (200 U/ml), carbenicillin (100 mg/liter), trimethoprim (20 mg/liter), and amphotericin B (10 mg/liter) (Selectatab; Mast Diagnostica GmbH) and immediately used for broth dilution to count the most probable number (MPN) of the bacilli (29).

Broth counting of the bacteria from lung and spleen tissue homogenates with culture filtrates was performed by the use of serial 10-fold dilutions, in which 0.5 ml of tissue homogenates was added to 4.5 ml of the culture filtrates. At 10-day intervals over a 2-month period of incubation at 37°C, the broth cultures were examined for visible turbidity changes. The growth of *M. tuberculosis* in turbid tubes was confirmed by the detection of the colonial morphology on 7H11 agar plates. The MPN of viable bacilli

was then estimated from the patterns of positive and negative tubes according to the method of the U.S. Food and Drug Administration (29). The absence of microorganisms other than mycobacteria from turbid tubes was confirmed by plating of the contents of the tubes on blood agar medium (Oxoid) and Sabouraud dextrose agar (Oxoid). In order to assess the sterility of culture filtrates free of *M. tuberculosis*, tubes containing culture filtrates were incubated at 37°C for 2 months to ensure the absence of *M. tuberculosis* in the culture filtrates.

Statistical analysis. A simple model for monoexponential bacterial growth and elimination (17, 30) was fitted to the profiles of the number of CFU versus time obtained experimentally. As simultaneously occurring exponential replication and death rates cannot be differentiated with this type of data, a k_{net} exponential rate constant was estimated separately before treatment began ($k_{net\_no\_drug}$, where it would take a net positive value) and during treatment ($k_{net\_with\_drug}$, where it would take a net negative value). During therapy, k_{net} is a first-order elimination rate constant, which can be interpreted as the slope of the modeled line fit through the logarithmic transform of the data (with the units of these data being week^{-1}). Parameter estimation was carried out with nonlinear regression using the nonlinear least-squares optimization function lsqnonlin as part of the pracma package in the R statistical software language with an objective function weighted by 1/(predicted value)^2. The standard errors of the parameter estimates were calculated using the method described previously (31), with the Jacobian of the model parameter sensitivities being estimated using a numerical central difference method. For data analysis purposes, rather than using the average for the data at each time point, the data sets for multiple individual subject animals were treated as a naive pool (32). The significance of the differences between model parameter estimates under different therapies was examined with pairwise Z-tests incorporating a Bonferroni correction of 3, where P values of <0.017 were considered significant. The significance of the differences between the relapse rates was determined with pairwise Fisher's exact tests, also with a Bonferroni correction of 3, with P values of <0.017 being considered significant.

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