Assessment of the Additional Value of Verapamil to a Moxifloxacin and Linezolid Combination Regimen in a Murine Tuberculosis Model

Pieterman, Elise D.; te Brake, Lindsey H. M.; de Knegt, Gerjo J.; van der Meijden, Aart; Alffenaar, Jan-Willem C.; Bax, Hannelore I.; Aarnoutse, Rob E.; de Steenwinkel, Jurriaan E. M.

Published in:
Antimicrobial Agents and Chemotherapy

DOI:
10.1128/AAC.01354-18

Publication date:
2018

AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

EXPERIMENTAL THERAPEUTICS

Assessment of the Additional Value of Verapamil to a Moxifloxacin and Linezolid Combination Regimen in a Murine Tuberculosis Model

Elise D. Pieterman,[a] Lindsey H. M. te Brake,[b,c] Gerjo J. de Knegt,[a] Aart van der Meijden,[a] Jan-Willem C. Alffenaar,[d] Hannelore I. Bax,[a,e] Rob E. Aarnoutse,[b] Jurriaan E. M. de Steenwinkel[a]

[a]Department of Medical Microbiology and Infectious Diseases, Erasmus Medical Centre, Rotterdam, the Netherlands

[b]Department of Pharmacy, Radboud Institute for Health Sciences, Radboud University Medical Centre, Nijmegen, the Netherlands

[c]Department of Pharmacology and Toxicology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Centre, Nijmegen, the Netherlands

[d]University of Groningen, University Medical Centre Groningen, Department of Clinical Pharmacy and Pharmacology, Groningen, the Netherlands

[e]Department of Internal Medicine, Section of Infectious Diseases, Erasmus University Medical Centre, Rotterdam, the Netherlands

ABSTRACT The favorable treatment outcome rate for multidrug-resistant tuberculosis (MDR-TB) is only 54%, and therefore new drug regimens are urgently needed. In this study, we evaluated the activity of the combination of moxifloxacin and linezolid as a possible new MDR-TB regimen in a murine TB model and the value of the addition of the efflux pump inhibitor verapamil to this backbone. BALB/c mice were infected with drug-sensitive *Mycobacterium tuberculosis* and were treated with human-equivalent doses of moxifloxacin (200 mg/kg of body weight) and linezolid (100 mg/kg) with or without verapamil (12.5 mg/kg) for 12 weeks. Pharmacokinetic parameters were collected during treatment at the steady state. After 12 weeks of treatment, a statistically significant decline in mycobacterial load in the lungs was observed with the moxifloxacin-linezolid regimen with and without verapamil (5.9 and 5.0 log CFU, respectively), but sterilization was not achieved yet. The spleens of all mice were culture negative after 12 weeks of treatment with both treatment modalities, and the addition of verapamil caused a significant reduction in relapse (14/14 positive spleens without versus 9/15 with verapamil, $P = 0.017$). In conclusion, treatment with a combination regimen of moxifloxacin and linezolid showed a strong decline in mycobacterial load in the mice. The addition of verapamil to this backbone had a modest additional effect in terms of reducing mycobacterial load in the lung as well as reducing the spleen relapse rate. These results warrant further studies on the role of efflux pump inhibition in improving the efficacy of MDR-TB backbone regimens.

KEYWORDS *Mycobacterium tuberculosis*, efflux pump inhibitor, linezolid, moxifloxacin, verapamil

Tuberculosis (TB) is the leading cause of death from infectious disease worldwide. An estimated 1.7 million people died due to this disease in 2016, and an estimated 10.4 million people fell ill in the same year (1). Drug resistance remains a problem for the treatment of TB; an estimated 4% of the new TB cases and 19% of previously treated TB cases had rifampin-resistant or multidrug-resistant TB (MDR-TB) (1). Currently, patients with MDR-TB need to undergo up to 2 years of treatment, which is often associated with toxic side effects, while only 54% of patients are being cured (1). Novel

Received 25 June 2018 **Accepted** 26 June 2018

Accepted manuscript posted online 9 July 2018

Citation Pieterman ED, te Brake LHM, de Knegt GJ, van der Meijden A, Alffenaar J-WC, Bax HI, Aarnoutse RE, de Steenwinkel JEM. 2018. Assessment of the additional value of verapamil to a moxifloxacin and linezolid combination regimen in a murine tuberculosis model. Antimicrob Agents Chemother 62:e01354-18. https://doi.org/10.1128/AAC.01354-18.

Address correspondence to Elise D. Pieterman, e.pieterman@erasmusmc.nl.

treatment regimens that are more effective, short, and safe need to be developed to treat this devastating disease.

In order to increase the treatment efficacy in the short term, we focused in this study on drugs which are already registered for use. Fluoroquinolones, in particular, moxifloxacin (MXF) and levofloxacin, are considered to be among the most important groups of drugs in the treatment of MDR-TB (2, 3). In murine TB models moxifloxacin was found to be the most bactericidal of all quinolones. It also showed good activity against rifampin-tolerant TB and MDR-TB in different mouse TB models (4, 5). Furthermore, a randomized controlled trial showed promising success rates in patients with MDR-TB treated with a regimen that included moxifloxacin (6). Therefore, moxifloxacin is now recommended by the World Health Organization (WHO) in their most recent guideline outlining a shorter MDR-TB regimen (8 to 12 months instead of at least 18 months of treatment) for patients who were not previously treated with second-line drugs (7).

In the current WHO treatment guideline, linezolid (LZD) (an oxazolidinone) is classified as a core second-line agent (group C) (7). *In vitro* and *in vivo* studies showed good activity of linezolid against multiple MDR-TB strains (4, 8), and a systematic review and meta-analysis of patients treated with linezolid-containing MDR-TB and extensively drug-resistant TB (XDR-TB) regimens showed promising results (9). Furthermore, a meta-analysis of MDR-TB patients treated with (at that time) group 5 drugs showed that the use of linezolid increased the probability of a successful treatment outcome in this group by 57% (10). Ongoing studies include an evaluation of linezolid combined with bedaquiline and pretomanid in a short (6-month) trial of treatment for XDR-TB (Nix-TB) (11) and a phase 3 study evaluating various doses and treatment durations of linezolid, bedaquiline, and pretomanid in MDR-TB and XDR-TB patients (ZeNix trial; ClinicalTrials registration no. NCT03086486).

Although the effectiveness of moxifloxacin and linezolid appears to be promising, the emergence of resistance to both drugs is a problem. There is accumulating evidence supporting a role for mycobacterial efflux pumps in the extrusion of TB drugs and emergence of drug resistance to both fluoroquinolones and oxazolidinones (12, 13). Thus, inactivation of these mycobacterial efflux pumps by efflux pump inhibitors (EPIs) could be a valuable strategy to increase intrabacterial drug concentrations and reduce the emergence of drug resistance (14).

Verapamil was the first discovered inhibitor of P-glycoprotein-mediated drug efflux (15) and was shown to be able to increase the accumulation of P-glycoprotein substrate drugs in macrophages (16). Another study showed that macrophage-induced tolerance of moxifloxacin could be reversed upon exposure to verapamil (12). Furthermore, verapamil not only affects macrophage efflux pumps but also has been identified as an inhibitor of *Mycobacterium tuberculosis* efflux systems (17–20). Besides, verapamil is widely used for the treatment of cardiovascular diseases, in contrast to several other EPIs which are not registered yet or have been withdrawn from the market due to severe side effects (21–23).

In the present study, we evaluated the value of the addition of verapamil to a backbone of moxifloxacin and linezolid. We assessed the efficacy of this regimen in BALB/c mice infected with a *M. tuberculosis* strain of the Beijing genotype and evaluated the pharmacokinetic profiles of drugs in this regimen.

RESULTS

Mycobacterial load (efficacy) assessment. Both moxifloxacin plus linezolid (group 1) and moxifloxacin plus linezolid plus verapamil (group 2) combination treatments were well tolerated, although mice showed mild distress during the first 3 weeks of treatment in both groups.

The mycobacterial load in lungs and spleen during 12 weeks of treatment and after a 12-week posttreatment period is presented in Fig. 1. The median total amount of mycobacteria in the lungs at start of treatment was 8.18 log CFU (interquartile range [IQR], 7.96 to 8.19). During the 12 weeks of treatment, the mycobacterial load in the

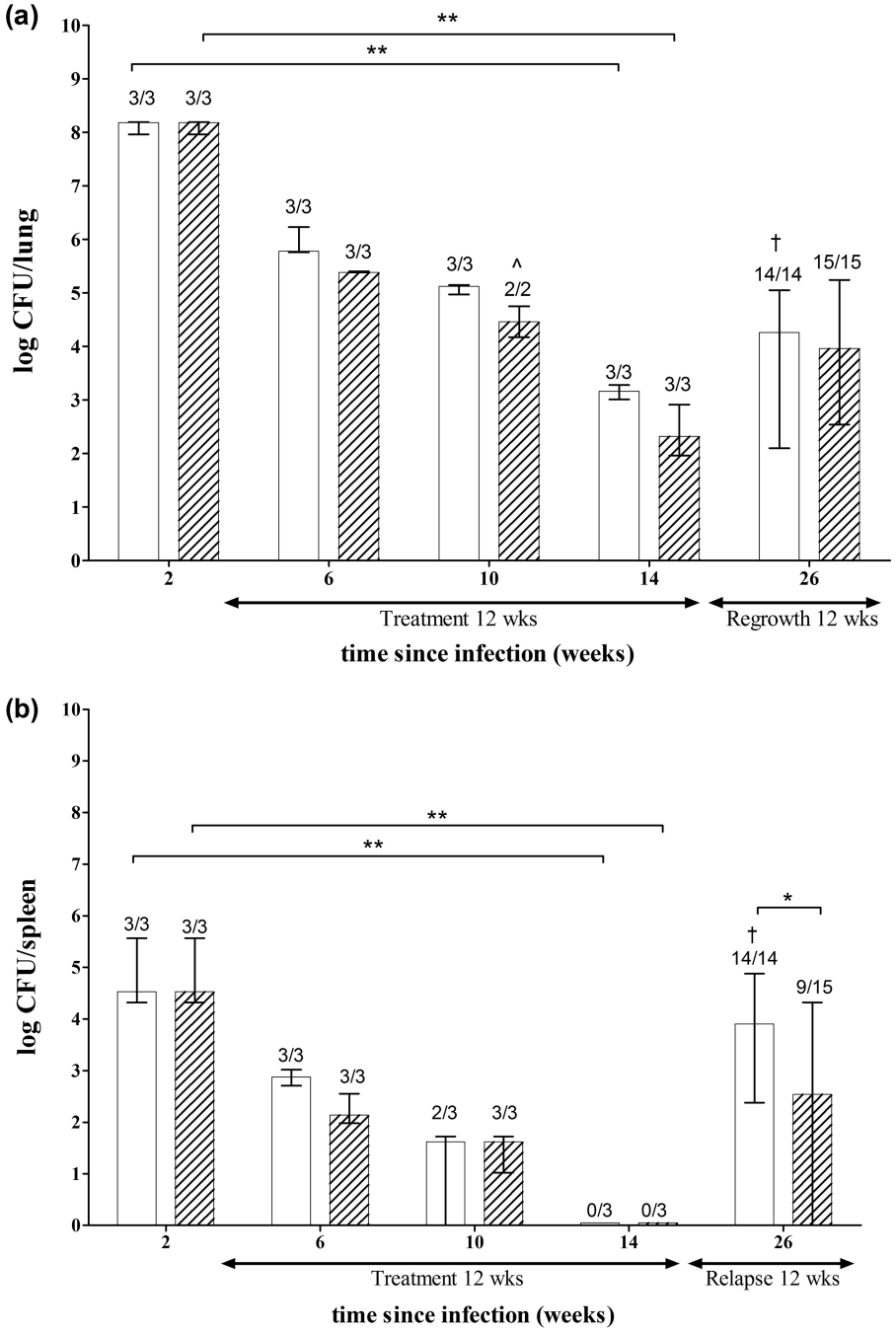

FIG 1 Mycobacterial load in lung (a) and spleen (b) expressed as median ± range (error bars) of the CFU per organ, at weeks 2, 6, 10, and 14 and at 12 weeks posttreatment (week 26). Gray bars represent mice treated with moxifloxacin and linezolid; striped bars represent mice treated with moxifloxacin, linezolid, and verapamil. Numbers above bars represent the numbers of culture-positive mice among the total numbers of mice at that time point. Significance is noted as follows: **, $P < 0.0001$; *, $P < 0.05$. †, 1 mouse became moribund before the planned dissection; ^, 1 measurement is missing due to a contaminated plate. LZD, linezolid; MXF, moxifloxacin; VPM, verapamil.

lungs declined, with 5.0 log CFU in group 1 ($P < 0.0001$) and with 5.9 log CFU in group 2 ($P < 0.0001$) compared to the start of treatment. No significant difference between group 1 and group 2 was observed at this time point. No sterilization of the lungs was achieved with either of the treatment modalities. As a result, no assessment of relapse in lung tissue was possible. In contrast, the spleens of all mice were culture negative after 12 weeks of treatment. The addition of verapamil was associated with a significant

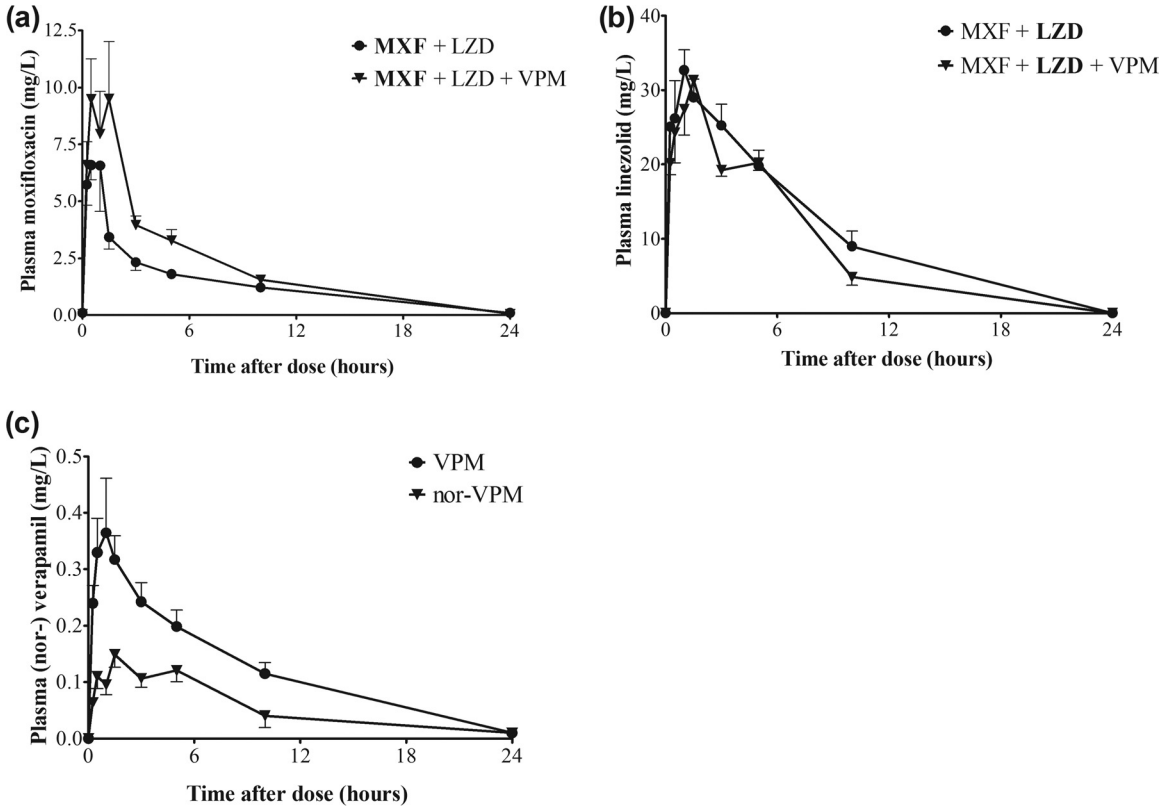

FIG 2 Moxifloxacin (a), linezolid (b), and (nor)verapamil (c) plasma concentration-time profiles at steady state following an oral dose of 200 mg/kg moxifloxacin, 100 mg/kg linezolid, and/or 12.5 mg/kg verapamil in *M. tuberculosis*-infected BALB/c mice. Plasma concentrations are plotted as means ± standard errors of the means (SEM) of results from three mice per study drug per time point. LZD, linezolid; MXF, moxifloxacin; VPM, verapamil; nor-VPM, norverapamil.

reduction in relapse after 3 months of treatment, as measured in the spleen (9/15 culture-positive samples in group 2 versus 14/14 in group 1, $P = 0.017$).

Pharmacokinetic evaluation. Plasma concentration-time profiles and pharmacokinetic parameters of the study drugs are shown in Fig. 2 and Table 1.

The total exposure to moxifloxacin was 53% higher in the mice that received verapamil; also, the peak concentration (C_{max}) was 44% higher with addition of verapamil (9.5 versus 6.6 mg/liter). In contrast, the area under the plasma concentration-time curve from 0 to 24 h (AUC_{0-24}) of linezolid was slightly (17%) lower

TABLE 1 Pharmacokinetic parameters at steady state of moxifloxacin and linezolid and (nor)verapamil in plasma following oral administration in *M. tuberculosis*-infected BALB/c mice[a]

Drugs	Dose (mg/kg)	$AUC_{0-\tau}$ (mg/liter · h)	C_{max} (mg/liter)	Cl/F (liters/h)	*V*/*F* (liters)	$t_{1/2}$ (h)
MXF (+ LZD)	200	30.0	6.6	0.15	0.92	4.3
MXF (+ LZD + VPM)	200	46.0	9.5	0.10	0.43	3.1
LZD (+ MXF)	100	215	32.7	0.010	0.028	1.9
LZD (+ MXF + VPM)	100	179	31.3	0.012	0.035	1.9
VPM (+ MXF + LZD)	12.5	2,670[b]	365[b]	0.00010	0.00067	4.5
nor-VPM (+ MXF + LZD)	NA	1,387[b]	149[b]	NA	NA	NA

[a]Pharmacokinetic parameters are based on 8 time points; concentrations at each time point are based on plasma samples of 3 mice. $AUC_{0-\tau}$, area under the plasma concentration-time curve within the dosing interval; Cl, clearance; C_{max}, maximum plasma concentration; *F*, bioavailability; LZD, linezolid; MXF, moxifloxacin; NA, not applicable; nor-VPM, norverapamil; $t_{1/2}$, half-life; *V*, volume of distribution; VPM, verapamil.

[b]For verapamil and norverapamil, $AUC_{0-\tau}$ and C_{max} are expressed in micrograms per liter per hour and micrograms per liter, respectively.

in mice that received verapamil, whereas the linezolid peak concentrations in the two groups were nearly identical (31.3 and 32.7 mg/liter). The total exposures of verapamil and its active metabolite norverapamil were 2,670 μg/liter · h and 1,387 μg/liter · h, respectively, with C_{max} levels of 365 μg/liter and 149 μg/liter, respectively.

DISCUSSION

To our knowledge, this was the first study evaluating whether verapamil could increase the treatment efficacy of a combination regimen consisting of moxifloxacin and linezolid in a murine TB model. Previous studies examining the additional value of verapamil in the treatment of TB focused on combining verapamil with only one drug or with the standard drug regimen against drug-sensitive *M. tuberculosis* (24–26), whereas our study focused on improving a new two-drug MDR-TB backbone.

In our study, the combination of moxifloxacin and linezolid achieved a strong reduction in mycobacterial load in the lungs after 3 months of treatment and the mycobacterial load in the spleen was undetectable at that time point. Although multiple randomized controlled trials showed promising treatment outcomes of MDR-TB regimens containing linezolid or moxifloxacin, with success rates of approximately 80%, limited data are available on the combination of the two drugs in humans (6, 27). Most studies on MDR-TB regimens containing linezolid in humans were performed either in the presence of moxifloxacin resistance or with linezolid added to a wide variety of different TB drugs (28, 29). However, previous *in vitro* studies showed that combining the two drugs resulted in synergistic as well as indifferent or antagonistic effects, which has been postulated to be strain dependent (30, 31).

In the present study, the addition of verapamil did not significantly increase the treatment efficacy of the moxifloxacin-linezolid backbone in terms of treatment outcome at 3 months of treatment. However, we did observe a modest additional decline in mycobacterial load in the lungs at the end of treatment as well as a significantly reduced relapse rate in the spleen with the addition of verapamil. Several other studies showed promising effects of the use of verapamil in combination with different anti-TB drugs. For example, Gupta et al. showed that verapamil could increase the treatment efficacy of a standard TB drug regimen consisting of rifampin, isoniazid, and pyrazinamide in mice infected with drug-sensitive *M. tuberculosis* (24). Similarly, it was shown for the new TB drug bedaquiline that subinhibitory dosing of bedaquiline in a murine drug-sensitive TB model could become as effective as regular dosing after the addition of verapamil (25). Both murine TB studies showed a significant difference of $\pm$1 log CFU between regimens with and without verapamil at different time points (24, 25). This observation was comparable to our findings in the lung tissue. Also in line with the results of our study showing a reduced relapse rate in the spleen, Gupta et al. showed reduced relapse rates with the addition of verapamil to the standard TB drug regimen (24). However, since all mice in our study were still culture positive in the lungs, it is difficult to assess the clinical value of this observation. Therefore, it would be worthwhile to evaluate the efficacy of this regimen in a future study for an extended period of time, in order to assess whether sterilization could be achieved in all tissues. In that case, relapse reduction mediated by verapamil, as observed by Gupta et al. (24), might possibly be replicated and might support the idea of the value of the addition of verapamil to this backbone. Interestingly, our previous *in vitro* study did not show any additional effect of verapamil on the activity of the moxifloxacin-linezolid combination (32). It could be hypothesized that the effect observed in the present study was mediated through the effects of verapamil on the efflux pumps present in macrophages. Such an effect was shown by Adams et al. in a TB macrophage model where the addition of verapamil reduced tolerance to multiple TB drugs (12).

In order to evaluate the influence of verapamil on the pharmacokinetics of our backbone, we compared the pharmacokinetic parameters of moxifloxacin and linezolid with and without verapamil. We observed that the total exposure to moxifloxacin was approximately 53% higher upon the addition of verapamil. These results were comparable to the observations of Xu et al., who found a 46% increased AUC of bedaquiline

after addition of verapamil (26). The authors postulated that the increased effectivity of bedaquiline was likely due to the increased bioavailability mediated through an effect of verapamil on mammalian drug transporters rather than to inhibiting efflux pumps present in mycobacteria and/or macrophages (26). Since moxifloxacin is subject to P-glycoprotein-mediated efflux (33), the increased AUC could perhaps be ascribed to the inhibition of P-glycoprotein-mediated drug efflux at the site of the intestines of the mice (34) and might have been the cause of the modest decline in CFU in the lungs. Besides, it is unlikely that the increased effectivity of the backbone after verapamil addition was due to the antimycobacterial effect of verapamil only, since in our previous *in vitro* study verapamil showed (modest) activity only at concentrations of ≥64 mg/liter (32). Moreover, other *in vivo* studies also showed no effect of the verapamil for single drug exposures (20, 26).

The results of our pharmacokinetic analyses confirm that drug exposures in this murine study resemble those achieved in humans. Total exposures to moxifloxacin (AUC within the dosing interval [AUC_{0-t}], 30 mg/liter · h) were comparable with the range of exposures found in humans (AUC_{0-24}, 25 to 29 mg/liter · h), which were obtained with the recommended 400 mg of moxifloxacin orally (35, 36). Similarly, total exposures to linezolid in our study were comparable to AUC_{0-12} values found in pharmacokinetic studies in humans after an oral intake of 600 mg linezolid twice daily (AUC_{0-12}, 108 to 146 mg/liter · h) (37, 38). Verapamil exposure in our study mimicked the range observed in humans, namely, an AUC_{0-24} of 3,253 μg/liter · h after daily oral intake of 240 mg of slow-release verapamil (39) and an AUC_{0-8} of 1,999 μg/liter · h after thrice-daily oral intake of 120 mg immediate-release verapamil with a norverapamil total exposure of 2,312 μg/liter · h (40). Although total exposures were comparable to human exposures, the C_{max} levels of moxifloxacin and linezolid in this study were higher than those seen in humans (6.6 mg/liter versus 3.9 mg/liter and 32.7 mg/liter versus 20.4 mg/liter, respectively) (36, 37). Since the AUC/MIC ratio is considered to be the driver of efficacy for moxifloxacin and linezolid (41, 42) and the moxifloxacin C_{max}/MIC ratio showed a poor correlation in efficacy in BALB/c mice (42), we assumed that this has a limited effect on the translational value.

In conclusion, the present study in mice showed a strong and steady CFU decline with the combination of moxifloxacin and linezolid. The addition of verapamil had a modest additional effect in terms of reducing mycobacterial load in the lung after 3 months of treatment as well as in terms of reducing spleen relapse rates. These results warrant further studies on the role of efflux pump inhibition in improving the efficacy of MDR-TB backbone regimens.

MATERIALS AND METHODS

Animals. Female BALB/c mice that were specified to be free of pathogens were obtained from Charles River (Les Oncins, France). At the start of the experiments, 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 EU animal directive 2010/63/EU. The Institutional Animal Care and Use Committee of the Erasmus Medical Centre (MC) approved the present protocols (117-12-14).

Bacterial strain. The *M. tuberculosis* Beijing VN 2002-1585 genotype strain (43), with a moxifloxacin MIC of 0.25 mg/liter and a linezolid MIC of 1 mg/liter, was used (43, 44). MICs were determined according to Clinical and Laboratory Standards Institute (CLSI) standards (45).

Experimental setup. For treatment efficacy and pharmacokinetic analyses, a total of 99 mice (51 mice for efficacy assessment and 48 mice for pharmacokinetic analysis) were infected with *M. tuberculosis* as described previously (46). In short, mice under anesthesia were infected by intratracheal instillation with a suspension (40 μl) containing 2.9×10^5 CFU (2.5×10^5 to 3.2×10^5) of *M. tuberculosis*, followed by inhalation to ensure the formation of a bilateral TB infection (46). Two weeks after infection, three mice were used as controls to determine infection efficacy and reproducibility over time. A total population of 48 mice was divided in two combination therapy groups; the first group received 200 mg moxifloxacin/kg of body weight (Bayer, Leverkusen, Germany) plus 100 mg/kg linezolid (Sigma-Aldrich, Zwijndrecht, the Netherlands), and the second group received the same backbone in combination with 12.5 mg/kg verapamil (Sigma-Aldrich, Zwijndrecht, the Netherlands). Mice were treated for 12 weeks. After 12 weeks of treatment, 30 mice ($n = 15$ per group) were kept for another 12 weeks without treatment to assess relapse in lung and spleen. Selected doses were chosen based on previous *in vivo* mouse studies (25, 47, 48). Dry powder moxifloxacin was dissolved in distilled water plus 0.05% agarose using a mortar and pestle. Dry powder linezolid was dissolved in distilled water and added to the moxifloxacin suspension. Verapamil was dissolved in distilled water and added to the moxifloxacin-plus-

linezolid suspension. Drugs were administered orally 5 times per week, using a feeding cannula, in a total volume of 0.2 ml per day.

Mycobacterial load (efficacy) assessment. A total of 51 of the infected mice receiving treatment were sacrificed at the start of therapy or after 4, 8, and 12 weeks of therapy ($n = 3$ per time point per regimen) and at 12 weeks posttreatment to assess relapse ($n = 15$ per regimen). Relapse was defined as the presence of ≥1 CFU on a culture plate, observed after complete sterilization at 12 weeks of treatment. To prevent carryover of TB drugs, therapy was stopped 72 h before the mice sacrificed and activated charcoal (0.4%) was added to the culture media. The lungs and spleen were removed aseptically and homogenized separately in M-tubes using a gentleMACS Octo Dissociator and the RNA program (Miltenyi Biotec BV, Leiden, the Netherlands) in 2 ml phosphate-buffered saline. Serial 10-fold dilutions were performed with each tissue homogenate, and samples of 200 μl were cultured on drug-free 7H10 Middlebrook agar containing activated charcoal and incubated for 28 days at 37°C with 5% CO_2 to perform colony counting.

Pharmacokinetic analyses. Pharmacokinetic analyses were performed at the steady state after 4 weeks of treatment in the other population of the infected mice ($n = 48$). These mice were sacrificed by CO_2 exposure, and blood samples were taken via cardiac puncture at 0.25, 0.5, 1, 1.5, 3, 5, 10, and 24 h after the dose. Three animals were euthanized for each of the eight sampling time points in each group ($n = 48$ total). Blood was collected in microcentrifuge tubes containing EDTA. Subsequently, blood was centrifuged at 10,000 × *g* for 5 min to obtain plasma, which was stored at −80°C upon analysis.

Moxifloxacin concentrations in plasma were analyzed with a liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay, validated for human plasma, at the University Medical Center Groningen, the Netherlands (49). Cross-validation of the assay for human plasma to murine plasma was performed by comparing responses of 5-fold measurements of quality control (QC) moxifloxacin samples (at concentrations of 0.5, 2.5, and 5.0 mg/liter) in human plasma with those determined in murine plasma after analysis of all samples with the LC-MS/MS assay for human plasma. The accuracy of measurement of murine samples with the assay for human samples was between 88% and 97%, and the within-run coefficient of variation (CV) amounted to 4.0% to 6.2%.

Linezolid concentrations in plasma were analyzed by a validated high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay at the University Medical Center Groningen, Groningen, the Netherlands (50). The assay accuracy was 96.3% to 108.5%, the within-run CV ranged from 2.5% to 7.1%, and the lower limit of quantitation (LLOQ) was 0.05 mg/liter. Cross-validation between human and murine plasma matrices was performed by comparing the responses seen with three QC linezolid concentrations (0.5, 15, and 30 mg/liter in 5-fold) in human plasma with those in murine plasma. The accuracy of murine plasma measurements relative to human plasma measurements was between 101% and 114%, with a within-run CV of 1.7% to 2.6%.

Verapamil and norverapamil concentrations were analyzed on a Thermo Fisher (San Jose, CA, USA) triple-quadrupole LC-MS/MS system with a Finnigan Surveyor LC pump and a Finnigan Surveyor autosampler. The mobile phase consisted of an aqueous buffer (containing ammonium acetate at 5 g/liter, acetic acid at 35 ml/liter, and trifluoroacetic anhydride at 2 ml/liter water), water, and acetonitrile and had a flow rate of 0.3 ml/min. Samples were prepared by the use of 100 μl serum or plasma and 750 μl precipitation reagent (mixture of methanol and acetonitrile [4:21 {vol/vol}]) containing cyanoimipramin as an internal standard, subjected to vortex mixing for 1 min, and subsequently centrifuged at 11,000 × *g* for 5 min. From the clear upper layer, 5 μl was injected onto the LC-MS/MS system. A Finnigan TSQ Quantum Discovery mass selective detector was used in electrospray positive ionization mode and performed selected reaction monitoring. The mass transitions for verapamil were 455.3 *m/z* to 165.1 *m/z* and for norverapamil were 441.2 *m/z* to 165.0 *m/z*; a scan width of 0.5 *m/z* was used. The calibration curves were linear within the concentration range of 18 to 1,800 μg/liter for verapamil and 19 to 1,900 μg/liter for norverapamil, with correlation coefficients (R^2) of 0.997 and 0.999, respectively. This method was precise and accurate; within-day precision ranged between 1.0% and 5.5% for verapamil and between 1.6% and 5.9% for norverapamil, and between-day precision ranged from 1.5% to 3.1% for verapamil and 0.0% to 1.2% for norverapamil. The calculated accuracy ranged from 1.8% to 4.5% for verapamil and 0.2% to 2.0% for norverapamil.

All measured concentrations represent total (i.e., protein-bound plus unbound) drug concentrations. Pharmacokinetic parameters were assessed using standard noncompartmental methods in Phoenix WinNonlin version 6.4 (Pharsight Corporation), as described previously (51).

Statistical analysis. CFU counts were log10 transformed before analysis. Group mean CFU counts at various time points after start of treatment were compared using one-way analysis of variance with a Bonferroni multiple-comparison test. Proportions of mice relapsing were compared using Fisher's exact test. In a previous study, we observed that a sample size of 3 or more mice is sufficient to achieve 100% power to detect a statistically significant difference in potency of 50% between different treatments (52). The statistical significance level adopted was a P value of <0.05. Analyses were performed using Prism 5 (GraphPad Software, San Diego, CA, USA).

ACKNOWLEDGMENTS

We thank Sanne van den Berg, Marian ten Kate, and Heleen van der Spek for their technical assistance.

This work was funded by the Dutch Organization for Health Research and Development (ZonMw), Priority Medicines Antimicrobial Resistance, project 205200002.

We have no conflicts of interest to declare.

REFERENCES

1. WHO. 2017. Global tuberculosis report 2017.1. WHO, Geneva, Switzerland.
2. Johnston JC, Shahidi NC, Sadatsafavi M, Fitzgerald JM. 2009. Treatment outcomes of multidrug-resistant tuberculosis: a systematic review and meta-analysis. PLoS One 4:e6914. https://doi.org/10.1371/journal.pone.0006914.
3. Caminero JA, Sotgiu G, Zumla A, Migliori GB. 2010. Best drug treatment for multidrug-resistant and extensively drug-resistant tuberculosis. Lancet Infect Dis 10:621–629. https://doi.org/10.1016/S1473-3099(10)70139-0.
4. Fattorini L, Tan D, Iona E, Mattei M, Giannoni F, Brunori L, Recchia S, Orefici G. 2003. Activities of moxifloxacin alone and in combination with other antimicrobial agents against multidrug-resistant Mycobacterium tuberculosis infection in BALB/c mice. Antimicrob Agents Chemother 47:360–362. https://doi.org/10.1128/AAC.47.1.360-362.2003.
5. Hu Y, Coates AR, Mitchison DA. 2003. Sterilizing activities of fluoroquinolones against rifampin-tolerant populations of Mycobacterium tuberculosis. Antimicrob Agents Chemother 47:653–657. https://doi.org/10.1128/AAC.47.2.653-657.2003.
6. Kang YA, Shim TS, Koh WJ, Lee SH, Lee CH, Choi JC, Lee JH, Jang SH, Yoo KH, Jung KH, Kim KU, Choi SB, Ryu YJ, Kim KC, Um S, Kwon YS, Kim YH, Choi WI, Jeon K, Hwang YI, Kim SJ, Lee HK, Heo E, Yim JJ. 2016. Choice between levofloxacin and moxifloxacin and multidrug-resistant tuberculosis treatment outcomes. Ann Am Thorac Soc 13:364–370. https://doi.org/10.1513/AnnalsATS.201510-690BC.
7. WHO. 2016. WHO treatment guidelines for drug-resistant tuberculosis 2016 update 2016.7. WHO, Geneva, Switzerland.
8. Yang C, Lei H, Wang D, Meng X, He J, Tong A, Zhu L, Jiang Y, Dong M. 2012. In vitro activity of linezolid against clinical isolates of Mycobacterium tuberculosis, including multidrug-resistant and extensively drug-resistant strains from Beijing, China. Jpn J Infect Dis 65:240–242. https://doi.org/10.7883/yoken.65.240.
9. Zhang X, Falagas ME, Vardakas KZ, Wang R, Qin R, Wang J, Liu Y. 2015. Systematic review and meta-analysis of the efficacy and safety of therapy with linezolid containing regimens in the treatment of multidrug-resistant and extensively drug-resistant tuberculosis. J Thorac Dis 7:603–615.
10. Chang KC, Yew WW, Tam CM, Leung CC. 2013. WHO group 5 drugs and difficult multidrug-resistant tuberculosis: a systematic review with cohort analysis and meta-analysis. Antimicrob Agents Chemother 57:4097–4104. https://doi.org/10.1128/AAC.00120-13.
11. Conradie FDA, Everitt D, Mendel C, van Niekerk C, Howell P, Comins K, Spigelman M. 2017. The NIX-TB trial of pretomanid, bedaquiline and linezolid to treat XDR-TB, abstr 80LB. Abstr Conf Retroviruses Opportunistic Infect, Seattle, WA, USA.
12. Adams KN, Szumowski JD, Ramakrishnan L. 2014. Verapamil, and its metabolite norverapamil, inhibit macrophage-induced, bacterial efflux pump-mediated tolerance to multiple anti-tubercular drugs. J Infect Dis 210:456–466. https://doi.org/10.1093/infdis/jiu095.
13. Escribano I, Rodriguez JC, Llorca B, Garcia-Pachon E, Ruiz M, Royo G. 2007. Importance of the efflux pump systems in the resistance of Mycobacterium tuberculosis to fluoroquinolones and linezolid. Chemotherapy 53:397–401. https://doi.org/10.1159/000109769.
14. te Brake LHM, de Knegt GJ, de Steenwinkel JE, van Dam TJP, Burger DM, Russel FGM, van Crevel R, Koenderink JB, Aarnoutse RE. 17 July 2017. The role of efflux pumps in tuberculosis treatment and their promise as a target in drug development: unraveling the black box. Annu Rev Pharmacol Toxicol https://doi.org/10.1146/annurev-pharmtox-010617-052438.
15. Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y. 1981. Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Res 41:1967–1972.
16. Seral C, Michot JM, Chanteux H, Mingeot-Leclercq MP, Tulkens PM, Van Bambeke F. 2003. Influence of P-glycoprotein inhibitors on accumulation of macrolides in J774 murine macrophages. Antimicrob Agents Chemother 47:1047–1051. https://doi.org/10.1128/AAC.47.3.1047-1051.2003.
17. Coelho T, Machado D, Couto I, Maschmann R, Ramos D, von Groll A, Rossetti ML, Silva PA, Viveiros M. 2015. Enhancement of antibiotic activity by efflux inhibitors against multidrug resistant Mycobacterium tuberculosis clinical isolates from Brazil. Front Microbiol 6:330. https://doi.org/10.3389/fmicb.2015.00330.
18. Machado D, Couto I, Perdigao J, Rodrigues L, Portugal I, Baptista P, Veigas B, Amaral L, Viveiros M. 2012. Contribution of efflux to the emergence of isoniazid and multidrug resistance in Mycobacterium tuberculosis. PLoS One 7:e34538. https://doi.org/10.1371/journal.pone.0034538.
19. Rodrigues L, Villellas C, Bailo R, Viveiros M, Ainsa JA. 2013. Role of the Mmr efflux pump in drug resistance in Mycobacterium tuberculosis. Antimicrob Agents Chemother 57:751–757. https://doi.org/10.1128/AAC.01482-12.
20. Louw GE, Warren RM, Gey van Pittius NC, Leon R, Jimenez A, Hernandez-Pando R, McEvoy CR, Grobbelaar M, Murray M, van Helden PD, Victor TC. 2011. Rifampicin reduces susceptibility to ofloxacin in rifampicin-resistant Mycobacterium tuberculosis through efflux. Am J Respir Crit Care Med 184:269–276. https://doi.org/10.1164/rccm.201011-1924OC.
21. Harrigan EP, Miceli JJ, Anziano R, Watsky E, Reeves KR, Cutler NR, Sramek J, Shiovitz T, Middle M. 2004. A randomized evaluation of the effects of six antipsychotic agents on QTc, in the absence and presence of metabolic inhibition. J Clin Psychopharmacol 24:62–69. https://doi.org/10.1097/01.jcp.0000104913.75206.62.
22. López-Muñoz F, Bhatara VS, Alamo C, Cuenca E. 2004. Historical approach to reserpine discovery and its introduction in psychiatry. Actas Esp Psiquiatr 32:387–395. (In Spanish.) https://doi.org/10.1016/S0210-4806(04)73094-6.
23. Grossman TH, Shoen CM, Jones SM, Jones PL, Cynamon MH, Locher CP. 2015. The efflux pump inhibitor timcodar improves the potency of antimycobacterial agents. Antimicrob Agents Chemother 59:1534–1541. https://doi.org/10.1128/AAC.04271-14.
24. Gupta S, Tyagi S, Almeida DV, Maiga MC, Ammerman NC, Bishai WR. 2013. Acceleration of tuberculosis treatment by adjunctive therapy with verapamil as an efflux inhibitor. Am J Respir Crit Care Med 188:600–607. https://doi.org/10.1164/rccm.201304-0650OC.
25. Gupta S, Tyagi S, Bishai WR. 2015. Verapamil increases the bactericidal activity of bedaquiline against Mycobacterium tuberculosis in a mouse model. Antimicrob Agents Chemother 59:673–676. https://doi.org/10.1128/AAC.04019-14.
26. Xu J, Tasneen R, Peloquin CA, Almeida DV, Li SY, Barnes-Boyle K, Lu Y, Nuermberger E. 21 December 2017. Verapamil increases the bioavailability and efficacy of bedaquiline but not clofazimine in a murine model of tuberculosis. Antimicrob Agents Chemother https://doi.org/10.1128/AAC.01692-17.
27. Sotgiu G, Centis R, D'Ambrosio L, Alffenaar JW, Anger HA, Caminero JA, Castiglia P, De Lorenzo S, Ferrara G, Koh WJ, Schecter GF, Shim TS, Singla R, Skrahina A, Spanevello A, Udwadia ZF, Villar M, Zampogna E, Zellweger JP, Zumla A, Migliori GB. 2012. Efficacy, safety and tolerability of linezolid containing regimens in treating MDR-TB and XDR-TB: systematic review and meta-analysis. Eur Respir J 40:1430–1442. https://doi.org/10.1183/09031936.00022912.
28. Lee M, Lee J, Carroll MW, Choi H, Min S, Song T, Via LE, Goldfeder LC, Kang E, Jin B, Park H, Kwak H, Kim H, Jeon HS, Jeong I, Joh JS, Chen RY, Olivier KN, Shaw PA, Follmann D, Song SD, Lee JK, Lee D, Kim CT, Dartois V, Park SK, Cho SN, Barry CE, III. 2012. Linezolid for treatment of chronic extensively drug-resistant tuberculosis. N Engl J Med 367:1508–1518. https://doi.org/10.1056/NEJMoa1201964.
29. Pang Y, Lu J, Huo F, Ma Y, Zhao L, Li Y, Liang Q, Chu N, Gao M, Huang H. 2017. Prevalence and treatment outcome of extensively drug-resistant tuberculosis plus additional drug resistance from the National Clinical Center for Tuberculosis in China: a five-year review. J Infect 75:433–440. https://doi.org/10.1016/j.jinf.2017.08.005.
30. Zou L, Liu M, Wang Y, Lu J, Pang Y. 2015. Determination of in vitro synergy between linezolid and other antimicrobial agents against Mycobacterium tuberculosis isolates. Tuberculosis (Edinb) 95:839–842. https://doi.org/10.1016/j.tube.2015.07.003.
31. Deshpande D, Srivastava S, Nuermberger E, Pasipanodya JG, Swaminathan S, Gumbo T. 2016. Concentration-dependent synergy and antagonism of linezolid and moxifloxacin in the treatment of childhood tuberculosis: the dynamic duo. Clin Infect Dis 63:S88–S94. https://doi.org/10.1093/cid/ciw473.
32. de Knegt GJ, van der Meijden A, de Vogel CP, Aarnoutse RE, de Steenwinkel JE. 2017. Activity of moxifloxacin and linezolid against Mycobac-

terium tuberculosis in combination with potentiator drugs verapamil, timcodar, colistin and SQ109. Int J Antimicrob Agents 49:302–307. https://doi.org/10.1016/j.ijantimicag.2016.11.027.
33. Brillault J, De Castro WV, Harnois T, Kitzis A, Olivier JC, Couet W. 2009. P-glycoprotein-mediated transport of moxifloxacin in a Calu-3 lung epithelial cell model. Antimicrob Agents Chemother 53:1457–1462. https://doi.org/10.1128/AAC.01253-08.
34. Rabbaa L, Dautrey S, Colas-Linhart N, Carbon C, Farinotti R. 1996. Intestinal elimination of ofloxacin enantiomers in the rat: evidence of a carrier-mediated process. Antimicrob Agents Chemother 40:2126–2130.
35. Pranger AD, van Altena R, Aarnoutse RE, van Soolingen D, Uges DR, Kosterink JG, van der Werf TS, Alffenaar JW. 2011. Evaluation of moxifloxacin for the treatment of tuberculosis: 3 years of experience. Eur Respir J 38:888–894. https://doi.org/10.1183/09031936.00176610.
36. Ruslami R, Ganiem AR, Dian S, Apriani L, Achmad TH, van der Ven AJ, Borm G, Aarnoutse RE, van Crevel R. 2013. Intensified regimen containing rifampicin and moxifloxacin for tuberculous meningitis: an open-label, randomised controlled phase 2 trial. Lancet Infect Dis 13:27–35. https://doi.org/10.1016/S1473-3099(12)70264-5.
37. Alffenaar JW, van Altena R, Harmelink IM, Filguera P, Molenaar E, Wessels AM, van Soolingen D, Kosterink JG, Uges DR, van der Werf TS. 2010. Comparison of the pharmacokinetics of two dosage regimens of linezolid in multidrug-resistant and extensively drug-resistant tuberculosis patients. Clin Pharmacokinet 49:559–565. https://doi.org/10.2165/11532080-000000000-00000.
38. Gee T, Ellis R, Marshall G, Andrews J, Ashby J, Wise R. 2001. Pharmacokinetics and tissue penetration of linezolid following multiple oral doses. Antimicrob Agents Chemother 45:1843–1846. https://doi.org/10.1128/AAC.45.6.1843-1846.2001.
39. Fuenmayor NT, Faggin BM, Cubeddu LX. 1992. Comparative efficacy, safety and pharmacokinetics of verapamil SR vs verapamil IR in hypertensive patients. Drugs 44(Suppl 1):1–11.
40. Shand DG, Hammill SC, Aanonsen L, Pritchett EL. 1981. Reduced verapamil clearance during long-term oral administration. Clin Pharmacol Ther 30:701–706. https://doi.org/10.1038/clpt.1981.223.
41. Srivastava S, Magombedze G, Koeuth T, Sherman C, Pasipanodya JG, Raj P, Wakeland E, Deshpande D, Gumbo T. 25 July 2017. Linezolid dose that maximizes sterilizing effect while minimizing toxicity and resistance emergence for tuberculosis. Antimicrob Agents Chemother https://doi.org/10.1128/AAC.00751-17.
42. Shandil RK, Jayaram R, Kaur P, Gaonkar S, Suresh BL, Mahesh BN, Jayashree R, Nandi V, Bharath S, Balasubramanian V. 2007. Moxifloxacin, ofloxacin, sparfloxacin, and ciprofloxacin against Mycobacterium tuberculosis: evaluation of in vitro and pharmacodynamic indices that best predict in vivo efficacy. Antimicrob Agents Chemother 51:576–582. https://doi.org/10.1128/AAC.00414-06.
43. de Steenwinkel JE, ten Kate MT, de Knegt GJ, Verbrugh HA, Aarnoutse RE, Boeree MJ, den Bakker MA, van Soolingen D, Bakker-Woudenberg IA. 2012. Consequences of noncompliance for therapy efficacy and emergence of resistance in murine tuberculosis caused by the Beijing genotype of Mycobacterium tuberculosis. Antimicrob Agents Chemother 56:4937–4944. https://doi.org/10.1128/AAC.00124-12.
44. de Steenwinkel JE, ten Kate MT, de Knegt GJ, Kremer K, Aarnoutse RE, Boeree MJ, Verbrugh HA, van Soolingen D, Bakker-Woudenberg IA. 2012. Drug susceptibility of Mycobacterium tuberculosis Beijing genotype and association with MDR TB. Emerg Infect Dis 18:660–663. https://doi.org/10.3201/eid1804.110912.
45. CLSI. 2012. Susceptibility testing of mycobacteria, nocardia, and other aerobic actinomycetes; approved standard—2nd ed, 31. CLSI, Wayne, PA.
46. De Steenwinkel JE, De Knegt GJ, Ten Kate MT, Van Belkum A, Verbrugh HA, Hernandez-Pando R, Van Soolingen D, Bakker-Woudenberg IA. 2009. Immunological parameters to define infection progression and therapy response in a well-defined tuberculosis model in mice. Int J Immunopathol Pharmacol 22:723–734. https://doi.org/10.1177/039463200902200318.
47. Gengenbacher M, Duque-Correa MA, Kaiser P, Schuerer S, Lazar D, Zedler U, Reece ST, Nayyar A, Cole ST, Makarov V, Barry Iii CE, Dartois V, Kaufmann SHE. 2017. NOS2-deficient mice with hypoxic necrotizing lung lesions predict outcomes of tuberculosis chemotherapy in humans. Sci Rep 7:8853. https://doi.org/10.1038/s41598-017-09177-2.
48. Tasneen R, Betoudji F, Tyagi S, Li SY, Williams K, Converse PJ, Dartois V, Yang T, Mendel CM, Mdluli KE, Nuermberger EL. 2016. Contribution of oxazolidinones to the efficacy of novel regimens containing bedaquiline and pretomanid in a mouse model of tuberculosis. Antimicrob Agents Chemother 60:270–277. https://doi.org/10.1128/AAC.01691-15.
49. Pranger AD, Alffenaar JW, Wessels AM, Greijdanus B, Uges DR. 2010. Determination of moxifloxacin in human plasma, plasma ultrafiltrate, and cerebrospinal fluid by a rapid and simple liquid chromatography-tandem mass spectrometry method. J Anal Toxicol 34:135–141. https://doi.org/10.1093/jat/34.3.135.
50. Harmelink IM, Alffenaar JW, Wessels AM, Greijdanus B, Uges DR. 2008. A rapid and simple liquid chromatography-tandem mass spectrometry method for the determination of linezolid in human serum. EJHP Sci 14:3–7.
51. Ruslami R, Nijland HM, Adhiarta IG, Kariadi SH, Alisjahbana B, Aarnoutse RE, van Crevel R. 2010. Pharmacokinetics of antituberculosis drugs in pulmonary tuberculosis patients with type 2 diabetes. Antimicrob Agents Chemother 54:1068–1074. https://doi.org/10.1128/AAC.00447-09.
52. Mourik BC, Svensson RJ, de Knegt GJ, Bax HI, Verbon A, Simonsson USH, de Steenwinkel JEM. 2018. Improving treatment outcome assessment in a mouse tuberculosis model. Sci Rep 8:5714. https://doi.org/10.1038/s41598-018-24067-x.