Higher Dosing of Rifamycins Does Not Increase Activity against *Mycobacterium tuberculosis* in the Hollow-Fiber Infection Model

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ABSTRACT Improvements in the translational value of preclinical models can allow more-successful and more-focused research on shortening the duration of tuberculosis treatment. Although the hollow-fiber infection model (HFIM) is considered a valuable addition to the drug development pipeline, its exact role has not been fully determined yet. Since the strategy of increasing the dose of rifamycins is being evaluated for its treatment-shortening potential, additional *in vitro* modeling is important. Therefore, we assessed increased dosing of rifampin and rifapentine in our HFIM in order to gain more insight into the place of the HFIM in the drug development pipeline. Total and free-fraction concentrations corresponding to daily dosing of 2.7, 10, and 50 mg of rifampin/kg of body weight, as well as 600 mg and 1,500 mg rifapentine, were assessed in our HFIM using the *Mycobacterium tuberculosis* H37Rv strain. Drug activity and the emergence of drug resistance were assessed by CFU counting and subsequent mathematical modeling over 14 days, and pharmacokinetic exposures were checked. We found that increasing rifampin exposure above what is expected with the standard dose did not result in higher antimycobacterial activity. For rifapentine, only the highest concentration showed increased activity, but the clinical relevance of this observation is questionable. Moreover, for both drugs, the emergence of resistance was unrelated to exposure. In conclusion, in the simplest experimental setup, the results of the HFIM did not fully correspond to preexisting clinical data. The inclusion of additional parameters and readouts in this preclinical model could be of interest for proper assessment of the translational value of the HFIM.

KEYWORDS hollow-fiber infection model, rifampin, rifapentine, *Mycobacterium tuberculosis*, pharmacokinetics, pharmacodynamics

Tuberculosis (TB) treatment is long and complex, leading to poor adherence, which results in the emergence of drug resistance and low treatment success rates. To shorten the duration of TB treatment, it is important to focus on drug discovery, dose optimization, and the implementation of new drug combinations. Besides, it is necessary to improve the clinical predictability of preclinical models in order to make this process more effective. This became apparent in the REMoxTB trial, aimed at shortening the duration of treatment of drug-susceptible tuberculosis from 6 to 4 months by replacing ethambutol or isoniazid with moxifloxacin (1). Although previous *in vitro* and *in vivo* studies had shown promising results, this new combination lacked the expected treatment-shortening potential (2–5).

To further accelerate the improvement and development of preclinical models, several international consortia have been established (6, 7). These consortia focus on the (back)validation of preclinical models (both existing and new models) against

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TABLE 1 Observed drug concentrations^a in the hollow-fiber infection model at steady state

Drug and dose	$fC_{\max}$ (mg/liter)	$fAUC_{0-24}$ (mg/liter·h)	$C_{\max}$ (mg/liter)	AUC_{0-24} (mg/liter·h)
Rifampin (mg/kg)				
2.7	0.69 (0)	3.29 (0.13)		
10	1.82 (0.08)	10.94 (0.77)	11.30 (0.45)*	62.61 (2.44)*
50	11.30 (0.45)*	62.61 (2.44)*	54.48 (2.80)	312.30 (31.60)
Rifapentine (mg)				
600	0.50 (0.08)	7.71 (0.97)	15.27 (0.83)	240.00 (8.92)
1,500	1.00 (0.10)	14.64 (3.54)	33.18 (2.02)	496.20 (37.41)

^aAll values are means (standard deviations). Values labeled with asterisks in the same row are from the same experiment. $C_{\max}$, maximum concentration; AUC, area under the curve; $fC_{\max}$ and $fAUC$, for the free unbound fraction of the drugs.

performance in clinical trials (6). They aim to develop an optimal research path by combining different preclinical models, including pharmacokinetic/pharmacodynamic (PK/PD) modeling and a simulation framework to facilitate the prediction of optimal drug combinations for assessment in clinical studies (6, 7).

A well-known PK/PD model is the hollow-fiber infection model (HFIM). This method, for which the European Medicines Agency (EMA) issued a qualification opinion, was developed to simulate human PK dynamics *in vitro*, while PD sampling of a liquid *Mycobacterium tuberculosis* culture can be performed whenever required (8). This is an improvement over traditional *in vitro* methods, such as the time-kill kinetics (TKK) assay, which assesses only static TB drug concentrations (9). Furthermore, the HFIM allows better control of the PK profile and more frequent PK/PD sampling than can be performed in *in vivo* experiments for both practical and ethical reasons. Although the HFIM has been shown to be valuable in the assessment of PK/PD indices (10), its exact role in the preclinical drug development pipeline has not been fully determined yet.

For the improvement of treatment, optimization of the dosing of old as well as new TB drugs is attracting more interest (11, 12). Although rifampin has been the cornerstone of TB treatment for decades, its optimum dose is still unknown (11, 12). Since its introduction in 1971, rifampin has been given to patients at 10 mg/kg of body weight, a dose presumed to be based mainly on fear of adverse events and price issues rather than on proper dose-finding studies (13). As early as 1980, a study evaluating different doses of rifampin (5, 10, and 20 mg/kg) in a small population showed that increasing the rifampin dose resulted in faster declines in CFU counts during the first 2 days of treatment (14). Later *in vitro* and *in vivo* studies confirmed that increasing the rifampin dose resulted in more activity and efficacy (15, 16). These observations led to the start of several clinical trials assessing increased rifampin dosing, all of which were suggestive of a positive dose-response relationship (11, 12, 17).

Rifapentine, approved by the FDA in 1998, has a longer half-life and a lower *M. tuberculosis* MIC than rifampin (18) and was intended to be used primarily for intermittent therapy to lower the patient's pill load (19). Later studies explored daily dosing and increasing the standard 600-mg dose in order to shorten the duration of TB treatment, with promising results in both cases (20–22).

In this work, we assessed the activities of standard and high doses of rifampin and rifapentine in our HFIM. We developed a PD model describing the observed change in CFU counts over time to test the effects of different dosages. We compared the results to those of other *in vitro* and *in vivo* studies as well as clinical trials, all in order to gain more insight into the place of this HFIM in the preclinical drug development pipeline.

RESULTS

The observed maximum concentrations in serum ($C_{\max}$) and total exposure (area under the time-concentration curve from 0 to 24 h [AUC_{0-24}]) of rifampin and rifapentine at all doses tested are shown in Table 1. The values measured agreed well with the

FIG 1 Activities of rifampin and rifapentine at different simulated daily doses and the emergence of resistance in H37Rv over 14 days towards initial log-phase growth in the hollow-fiber infection model. Values shown are means ($n=2$); error bars indicate ranges. The intended maximum concentrations of the drugs are given in the keys. (A) Rifampin activity; (B) emergence of resistance during rifampin exposure; (C) rifapentine activity; (D) emergence of resistance during rifapentine exposure. Activity is expressed as the decline of the total population, and the emergence of resistance is expressed as the growth of resistant colonies.

(preset) targets for all dose levels. The presence of oleic acid-albumin-dextrose-catalase (OADC) did not change the inhibition zones of rifapentine in our bioassay, suggesting that protein binding does not affect rifamycin activity in the HFIM (data not shown).

The CFU count of the control (unexposed) cultures did not increase over time, while during the experiment, turbidity within the cartridges increased, indicating bacterial growth, and during the last 2 days (days 13 and 14), even aggregation was observed. Mycobacterial killing and regrowth over 14 days of exposure to rifampin or rifapentine are shown in Fig. 1A and C, respectively. Exposure to either rifamycin did not result in culture negativity at any of the concentrations tested. For rifampin, the lowest concentration resulted in less bactericidal activity, as reflected by a difference of >2 log CFU/ml in the CFU count at day 8. With regard to rifapentine, the highest concentration resulted in more bactericidal activity than the three lower concentrations at all time points. The emergence of drug resistance was observed from day 6 onward for rifampin and from day 8 onward for rifapentine, resulting in regrowth (Fig. 1B and D). The emergence of resistance in the control cultures remained below 1.3 log CFU/ml.

The model developed to describe total (T) and resistant (R) colony counts over time (t) included two separate compartments, one for wild-type (WT) and one for drug-resistant bacteria. The inocula (I) for the WT and R compartments were defined as follows:

$$I_{WT} = 10^{TV\log I_{WT} \times \exp(\eta_t)}$$

$$I_R = I_{WT} \times 10^{-6}$$

where $TV\log I_{WT}$ is the typical WT inoculum on the log scale and η_t is the random inter-experiment variability drawn from a normal distribution with a mean of 0 and estimated variance of ω_t^2 . The maximal carrying capacity of the system (N_{max}), limiting the

TABLE 2 Final parameter estimates, including uncertainty^a

Parameter (unit) ^b	Value	RSE (%)
Typical values		
$\log I_{WT}$ ($\log_{10}$ CFU)	6.63	1.7
DELAY with RIF (days) ^c	4.94	21
DELAY with RPT (days) ^c	4.37	21
kg_{base} (/h)	0.0307	11
k_{kill} of RIF (/h)	0.0555	6.0
k_{kill} of RPT (/h)	0.0761	6.5
Extra kill with RIF when C_{max} is >0.74 mg/liter ^d	0.631	16
Extra kill with RPT when C_{max} is >15.7 mg/liter ^d	0.264	32
Interexperiment variability (CV [%])		
$\log I_{WT}$	107	19
DELAY with RIF	43	22
DELAY with RP	20	73
k_{kill} of RIF	7.8	23
k_{kill} of RPT	0 FIX	
Residual variability (proportional error [%])	16.4	9

^aUncertainty is expressed as the relative standard error (RSE).

^b $\log I_{WT}$, inoculum for the wild type; DELAY, delay in the beginning of the growth of the resistant population; RIF, rifampin; RPT, rifapentine; kg_{base} , growth rate in the unlimited state; k_{kill} , kill rate; CV, coefficient of variation; 0 FIX, fixed to zero.

^cEstimated through the MTIME parameter in NONMEM.

^dParameterized with the equation k_{kill} high rifamycin = $k_{kill} \times (1 + \text{extra kill with high rifamycin})$.

growth rate of the bacteria, was set to the total inoculum ($I_{WT} + I_R$) given that no growth was observed in the control arms. The growth rate was defined as follows:

$$kg(t) = kg_{base} \frac{[N_{max} - WT(t) - R(t)]}{N_{max}}$$

where $kg(t)$ is the rate (k) of growth (g) at time t and kg_{base} is the growth rate in the unlimited state ($T \ll N_{max}$). The dynamics of the amounts of WT and R bacteria in the system was described by the following equations:

$$\frac{dWT(t)}{dt} = [kg(t) \times WT(t)] - [k_{kill} \times WT(t)]$$

$$\text{If } t \leq \text{DELAY, then } \frac{dR(t)}{dt} = 0$$

$$\text{If } t > \text{DELAY, then } \frac{dR(t)}{dt} = kg(t) \times R(t)$$

where k_{kill} is the kill rate and DELAY is the delay in the beginning of the growth of the resistant population. The total observed CFU (T) was simply CFU (WT) + CFU (R).

k_{kill} was found to differ between the lowest rifampin exposure (C_{max} , 0.74 mg/liter) and the other arms ($P < 0.005$). No additional effect was seen at higher rifampin exposures. k_{kill} did not differ statistically between the three lower levels of rifapentine, but some additional effect was seen for the highest exposure (C_{max} , 33 mg/liter) ($P < 0.005$). An exposure effect on the delay in the growth of the resistant population could not be reliably estimated for either drug.

The parameters in the final model were all estimated with good precision and are listed in Table 2. The model described the observed data well, as illustrated by the visual predictive check included in Fig. 2. The estimated growth rate of the unexposed mycobacterial population translated to a doubling time of 22.6 h, which is in line with what is expected for *M. tuberculosis* (23). The model structure relied on certain assumptions, e.g., that there is a small preexisting population of drug-resistant bacteria and

FIG 2 Visual predictive check of the final model. Blue rings represent the observed data; the red dashed line indicates the median of the observed data; the black line shows the median model prediction; and the shaded area is the 90% confidence interval of the predicted median.

that the start of growth in this population is delayed. Alternatively, one could, for example, assume no preexisting resistant population, so that the first resistant bacteria appear during the experiment and start growing from that moment. This structure could probably also describe the data and would likely give the same results in terms of evaluation of the drug effects. Both structures make sense theoretically, but with the information at hand, we have no way to determine which is better.

DISCUSSION

In the present study, higher rifampin exposure did not result in increased antibacterial activity, even at extremely high drug concentrations. In line with this, increased activity was observed only for the highest rifapentine concentration, which is probably not representative of what can be reached in TB patients, considering the high protein binding of rifapentine as well as the reported relation between high exposure and drug intolerance (24, 25). The reason for choosing these high rifamycin concentrations was to compensate for the expected protein binding in the HFIM. However, it was subsequently shown that protein binding did not play a significant role *in vitro*. This is an important finding and should be considered when one is interpreting the relation between rifampin exposure and activity in other *in vitro* studies. In our study, the

(concentration-independent) emergence of rifamycin resistance appeared to be the limiting factor for further CFU decreases after 6 days of monoexposure. This is not expected to impact clinical efficacy, because in patients, rifamycins are always combined with other antimicrobial agents. Previous HFIM studies also failed to show an exposure-response relationship for rifampin (26, 27), and to our knowledge, no other HFIM experiments with rifapentine have been described. Interestingly, and in line with the findings of the present study, simulated high rifampin dosages (ranging from 100 to 900 mg) were not enough to reach sterilization in those studies (26, 27).

These findings in the HFIM are in contrast to the results observed in our *in vitro* time-kill curves, where no CFU were detectable after 6 days of exposure to 8 mg/liter rifampin (28, 29), a concentration with a lower C_{max} and AUC_{0-24} than those of the highest dose given in the current study. The same holds true for rifapentine: in our previous time-kill experiments with the *M. tuberculosis* Beijing-1858 strain, rifapentine showed concentration- and time-dependent activity, with 4 mg/liter resulting in sterilizing activity (unpublished results). It could be hypothesized that this discrepancy is due to the presence of a mycobacterial subpopulation with lower metabolic activity in our HFIM than in time-kill experiments, given the significantly longer duration of HFIM experiments. Our previous time-kill studies of rifampin showed that rifampin activity was lower in *M. tuberculosis* populations with low metabolic activity, which was associated with the emergence of drug resistance (28, 29). In line with this, Hu et al. showed that 16-fold-higher concentrations of rifampin were required for the eradication of stationary-phase cultures than for the eradication of log-phase cultures (16). Therefore, the presence of such a population in our HFIM might explain the reduced bactericidal activity observed in our HFIM relative to that in our time-kill experiments.

Previous *in vivo* studies on rifampin and rifapentine did show that increasing the dose in *M. tuberculosis*-infected mice resulted in higher efficacy (15, 30). Our group showed that an 8-fold increase in the standard rifampin dose of 10 mg/kg allowed a reduction of treatment duration from 6 to 2 months in a murine TB model (15). In addition, Rosenthal et al. showed that when the rifampin dose was increased to 40 mg/kg, no relapse of infection was observed after 12 weeks of treatment in TB-infected mice (versus 100% of the mice treated with the standard dose) (30). The same study showed dose-dependent efficacy with doses up to 20 mg/kg rifapentine in combination with standard dosing of isoniazid and pyrazinamide (30). Generally, clinical studies were also suggestive of an exposure-response relationship for both rifamycins. Several studies with TB patients showed less early bactericidal activity (EBA) with low dosing of rifampin (5 mg/kg) than with the standard dose of 10 mg/kg (14, 31, 32). This finding is comparable to our HFIM results, confirming that lower rifampin exposure is indeed associated with lower success rates. However, two of these studies also showed increased EBA of rifampin when a 20-mg/kg dose was used (14, 32). Also, studies assessing doses as high as 35 mg/kg rifampin were suggestive of increased efficacy of high rifampin dosing when liquid cultures were used (11, 33). For rifapentine, an exposure-response relationship was shown by combining the results of the liquid cultures of two phase II trials assessing daily dosing ranging from 10 to 20 mg/kg and 450 to 1,500 mg (21). However, whether these clinical observations would indeed lead to improved cure rates has yet to be determined, since only surrogate markers, such as time to culture conversion, were used, and clinical follow-up was done for a maximum of 12 months (17, 22, 33). In that respect, the recently released results of Study 31/A5349, showing the noninferiority of two 4-month TB drug regimens including high-dose rifapentine relative to the standard of care, are worth mentioning (34). It could be speculated that the discrepancy between these studies and our results might be caused by various factors influencing rifamycin efficacy that are present in *in vivo* models as well as in TB patients but are absent in our HFIM. One could think of the presence of intracellular mycobacteria and variability in mycobacterial subpopulations, as well as different immunological factors, the severity of disease, and differences in drug penetration of tissue. The clinical predictability of the HFIM might therefore be

TABLE 3 Simulated target drug concentrations in the hollow-fiber infection model^a

Drug and dose	$fC_{\max}$ (mg/liter)	$fAUC_{0-24}$ (mg/liter·h)	$C_{\max}$ (mg/liter)	AUC_{0-24} (mg/liter·h)
Rifampin (mg/kg)				
2.7	0.74	4.19		
10	2.00	11.34	10.00*	60.28*
50	10.00*	60.28*	50.20	286.70
Rifapentine (mg)				
600	0.47	7.60	15.70	257.40
1,500	0.99	14.80	33.00	544.30

^aValues labeled with asterisks are from the same experiment.

increased by including more metabolic populations (35), the use of macrophages (36), and other intracellular approaches (37), as well as different mycobacterial strains (18). A remarkable observation is that the results of liquid cultures as used in clinical trials were suggestive of increased efficacy of both rifamycins, whereas the results of solid cultures were less conclusive (11, 21, 33). Given the fact that in the present study mycobacterial cultures were grown on solid media, this subpopulation might have been missed, providing an additional factor contributing to the lack of concentration-dependent activity of rifamycins in our HFIM. The use of additional readouts, such as time to positivity and/or most-probable-number assessment, could therefore be of interest for future HFIM studies (38).

The reliability of our results is supported by the PK results, which were in line with what was intended. On top of that, the semimechanistic model described the observed data well, enabling statistical analysis of the differences in the mycobacterial killing rate at different rifamycin concentrations. Of general concern for dose finding in the HFIM is the requirement that clinical PK information is available. As such, several important PK parameters, including drug half-life, absorption rate, $C_{\max}$, and AUC, have to be known beforehand. When these parameters are known, the HFIM can be a useful tool for dose scheduling by finding the PK/PD parameter with optimal effect (39, 40).

To conclude, the results of the experiments performed in a “standard” HFIM did not fully correspond to preexisting clinical data for high dosing of rifampin and rifapentine. The use of additional parameters and readouts in this model could be of interest for further determination of the place of the HFIM in the drug development pipeline.

MATERIALS AND METHODS

Strain. Experiments were performed using the H37Rv strain, with a rifampin MIC of 0.5 mg/liter and a rifapentine MIC of 0.125 mg/liter. MICs were determined according to CLSI standards (41). Stock cultures were stored at -80°C , thawed at the start of experiments, and incubated at 37°C for 5 days in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment (Becton, Dickinson and Company [BD], Sparks, MD, USA), 0.5% glycerol (Scharlau Chemie S.A, Sentmenat, Spain), and 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO, USA) under shaking conditions to facilitate log-phase growth prior to inoculation in our HFIM.

Antimicrobials. Rifampin (Sigma-Aldrich, St. Louis, MO, USA) and rifapentine (Sanofi Aventis, Frankfurt, Germany) were both dissolved in dimethyl sulfoxide (DMSO) and further diluted in broth to the desired concentration. Both free and total concentrations of the drugs were assessed in the HFIM. Rifampin and rifapentine protein binding levels in human plasma of 80% and 97%, respectively, were used to calculate the free drug concentrations (42). Target maximum concentrations ($C_{\max}$) were based on previous clinical studies reporting mean $C_{\max}$ values in patients (11, 21). The doses simulated in the HFIM were 2.7, 10, and 50 mg/kg rifampin and 600 mg and 1,500 mg rifapentine (Table 3). For rifapentine, no weight-based dosage was chosen, since weight does not influence its concentrations in plasma (21).

Hollow-fiber infection model. Twenty-milliliter samples of *M. tuberculosis* log-phase cultures (density, $\sim 2 \times 10^6$ CFU/ml) preconditioned with 7H9 broth at 37°C were inoculated into the peripheral compartment of the hollow-fiber cartridge (medium cellulosic cartridge; molecular weight cutoff, 5 kDa; catalog no. C3008; FiberCell Systems, New Market, MD, USA). These cartridges consist of a peripheral

TABLE 4 Pharmacokinetic parameters

Parameter (unit)	Value for:	
	Rifampin	Rifapentine
Vol (liters)	0.349	0.349
Half-life (h)	3	16
$T_{\max}$ (h)	2	4
Protein binding (%)	80	97

compartment inside which are semipermeable hollow fibers with pores that allow drugs and nutrients to transfer freely in and out of the peripheral compartment, while keeping the *M. tuberculosis* bacteria in the peripheral compartment. Twenty-four hours after the cartridges were inoculated, the mycobacteria in each cartridge were exposed to different dosing schemes of rifampin or rifapentine every 24 h (q24h) for 14 days (Table 3), while at the same time, one cartridge was left unexposed to drugs and served as a control. All experiments were performed in duplicate.

Drugs were administered by digitally controlled syringe pumps (World Precision Instruments, Sarasota, FL, USA) through a dosing port just before the central compartment, simulating the absorption rate of the drugs in humans (Table 4) (42). By use of digitally controlled peristaltic pumps (Watson-Marlow, Falmouth, Cornwall, United Kingdom), fresh broth was pumped into the afferent port of the central compartment of the cartridge while drug-containing broth was isovolumetrically removed from the efferent port of the system at rates programmed to simulate the half-lives of the drugs in humans (Table 4) (42).

PD samples (1 ml) were collected from each cartridge just before drug administration at days 0, 1, 2, 3, 6, 8, 10, 13, and 14. To prevent drug carryover, samples were washed and serially diluted as described previously (43) before the addition of 200 μ l to solid-culture plates. All cultures were plated onto 7H10 agar plates with or without 4 mg/liter rifampin or 1 mg/liter rifapentine (4 times the clinical breakpoint) in order to assess total and resistant populations, respectively (44).

Pharmacokinetic assessment. The pharmacokinetic profiles of the TB drugs to which the mycobacteria were exposed in the HFIM were validated by sampling the afferent port of the central compartment leading to the cartridges of each HFIM twice per experiment (with 1 week between samplings) at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, and 24 h for rifampin and at 0, 1, 2, 3, 4, 5, 8, 12, and 24 h for rifapentine. Samples were frozen at -80°C until further analysis.

A bioassay was performed to assess the concentrations of rifampin and rifapentine in the HFIM (45). *Sarcina lutea* was grown on blood agar plates (BD, Vianen, the Netherlands) for 48 h at 35°C . Subsequently, a 0.5 McFarland standard suspension was made and plated onto Mueller-Hinton II plates (BD, Vianen, the Netherlands) in which 0.5-cm-diameter wells were punched. To create a standard curve, 100 μ l of a known concentration of thawed rifampin or rifapentine (8, 4, 2, 1, 0.5, 0.25, or 0.125 $\mu\text{g/ml}$) was added in duplicate to the wells of the agar plate. In addition, 100 μ l of each thawed HFIM PK sample was added to the other wells in the agar (samples were diluted when necessary). Plates were incubated for 24 h at 35°C . The next day, the inhibition zones of the standard concentrations were measured, and a standard curve was made. The inhibition zones of the PK samples were measured, and correlating concentrations were calculated based on the formula of the standard curve. With the same bioassay, the inhibition zones of rifapentine in the presence and absence of OADC were assessed with a standard curve of the seven known concentrations mentioned above in order to assess any protein binding in the HFIM.

Modeling and statistics. The CFU data were analyzed by developing a semimechanistic nonlinear mixed-effects model describing the change in total and resistant colony counts over the 14 days in the HFIM. Data from all experiments were analyzed jointly, and the structure of the model was inspired by previous work with TTK data (46). Since estimating both the number of resistant bacteria in the inoculum and the delay in the growth of resistant bacteria would lead to identifiability issues, the number of resistant bacteria in the inoculum was fixed to 10^{-6} times the number of wild-type bacteria in the inoculum. The growth rates for the wild-type and resistant bacteria were assumed to be the same. Random interexperiment variability was assumed to follow log-normal distributions. The effects of different rifamycin concentrations were tested on the kill rate of the wild-type bacteria and the delay in the start of growth of resistant bacteria. The initial evaluation simply tested a difference between the arms (with different target $C_{\max}$ levels), while full dynamic concentration-effect relationships could be evaluated. Statistical significance was assessed with likelihood ratio tests using a 5% confidence level.

Plotting of the raw CFU data, PK analyses, and AUC calculations were performed using Prism, version 5 (GraphPad Software, San Diego, CA, USA). Modeling, data management, graphical evaluation, and postprocessing of results were performed in R (Foundation for Statistical Computing, Vienna, Austria), partly using the Xpose package (Department of Pharmaceutical Biosciences, Uppsala University, Uppsala, Sweden). The model was developed in NONMEM, version 7.4 (Icon Development Solutions, Ellicott City, MD, USA), aided by PsN (Department of Pharmaceutical Biosciences, Uppsala University, Uppsala, Sweden) and Pirana (Pirana Software & Consulting, San Francisco, CA, USA) (47).

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