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CLINICAL THERAPEUTICS

Advanced Quantification Methods To Improve the 18b Dormancy Model for Assessing the Activity of Tuberculosis Drugs *In Vitro*

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ABSTRACT One of the reasons for the lengthy tuberculosis (TB) treatment is the difficulty to treat the nonmultiplying mycobacterial subpopulation. In order to assess the ability of (new) TB drugs to target this subpopulation, we need to incorporate dormancy models in our preclinical drug development pipeline. In most available dormancy models, it takes a long time to create a dormant state, and it is difficult to identify and quantify this nonmultiplying condition. The *Mycobacterium tuberculosis* 18b strain might overcome some of these problems, because it is dependent on streptomycin for growth and becomes nonmultiplying after 10 days of streptomycin starvation but still can be cultured on streptomycin-supplemented culture plates. We developed our 18b dormancy time-kill kinetics model to assess the difference in the activity of isoniazid, rifampin, moxifloxacin, and bedaquiline against log-phase growth compared to the nonmultiplying *M. tuberculosis* subpopulation by CFU counting, including a novel area under the curve (AUC)-based approach as well as time-to-positivity (TTP) measurements. We observed that isoniazid and moxifloxacin were relatively more potent against replicating bacteria, while rifampin and high-dose bedaquiline were equally effective against both subpopulations. Moreover, the TTP data suggest that including a liquid culture-based method could be of additional value, as it identifies a specific mycobacterial subpopulation that is nonculturable on solid media. In conclusion, the results of our study underline that the time-kill kinetics 18b dormancy model in its current form is a useful tool to assess TB drug potency and thus has its place in the TB drug development pipeline.

KEYWORDS 18b, *Mycobacterium tuberculosis*, bedaquiline, dormancy, isoniazid, moxifloxacin, rifampin, time to positivity, time-kill curves, tuberculosis

To achieve the targets of the End TB Strategy, requiring profound reductions of tuberculosis (TB) incidence and death rates, there is an urgent need for therapy improvement (1). The current treatment for drug-susceptible TB consists of a combination of TB drugs for at least 6 months (1). This prolonged duration has an unfavorable effect on treatment compliance, and shorter regimens are therefore urgently required.

One of the reasons for this lengthy treatment is the difficulty in eradicating the nonmultiplying *Mycobacterium tuberculosis* subpopulation (2). In the nonmultiplying state, bacteria stop dividing, lower their metabolism, and thicken the cell wall in response to different stress conditions, with an unfavorable effect on the activity of TB drugs (3). Consequently, it is important to target the nonmultiplying bacilli when aiming at shortening TB treatment duration. In order to assess the ability of (new) TB

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drugs to target these bacteria, we need to incorporate dormancy models in our preclinical drug development pipeline.

In most available dormancy models, creating a dormant state is a lengthy process (4, 5). In addition, it can be difficult to identify and quantify the nonmultiplying mycobacterial subpopulation, since some of these mycobacteria are nonculturable on solid media (6, 7). Therefore, the 18b model could be an attractive alternative for studying TB drug potency against nonmultiplying *M. tuberculosis*. The 18b strain is a clinical *M. tuberculosis* isolate obtained from a patient in Japan in 1955 (8). It belongs to clade 3 of the ancient ancestral lineage of the Beijing family. This strain is dependent on streptomycin for growth due to an insertion of a single cytosine in the 530 loop of the 16S rRNA and a single amino acid insertion in the N-terminal domain of initiation factor 3 (9). The advantage of this strain is that in the absence of streptomycin, the mycobacteria stop multiplying in a short time frame of approximately 10 days. After streptomycin removal, the gene expression is altered, causing a shift in its metabolism from aerobic (high NADH-oxidase levels) to microaerophilic (induction of cytochrome *bd*-type menaquinol oxidase), allowing the cell to adapt to a nonmultiplying state (9, 10). The upregulation of "dormancy regulon" and "stationary-phase induced" genes in this state is overall consistent with other dormancy models, e.g., multiple stress, phosphate depletion, nutrient depletion, and hypoxia, as well as with the gene expression of intracellular *M. tuberculosis* (9). An additional advantage is that CFU enumeration can still be performed when 18b is plated on culture plates supplemented with streptomycin. Several studies have shown that liquid culture methods such as the most probable number (MPN) and time-to-positivity (TTP) methods allow the identification of a mycobacterial subpopulation that is nonculturable on solid media (11, 12). This indicates that combining solid and liquid culture methods is important for quantification of the total mycobacterial load present when assessing the activity of TB drugs.

In this study, we assessed the applicability of the 18b strain by comparing the activity of isoniazid, rifampin, moxifloxacin, and bedaquiline against both log-phase growth and nonmultiplying *M. tuberculosis* subpopulations. We performed time-kill kinetics assays, assessed CFU, and included a novel area under the curve (AUC)-based approach to compare drug activity against the nonmultiplying (streptomycin [STR]-starved) state relative to log-phase growth (STR-exposed). In addition, we assessed the TTP by liquid culturing for STR-exposed and STR-starved 18b in order to optimize the detection methods for mycobacteria in different metabolic states.

RESULTS

Time-kill kinetics. STR-exposed and STR-starved 18b cultures were exposed for 6 days to 4 concentrations of isoniazid, rifampin, moxifloxacin, and bedaquiline (1/25, 1/5, 1, and 5 times the peak free serum concentration [fC_{max}]). The TB drugs were added at day 6 postinoculation to the STR-exposed cultures and at day 14 postinoculation to the STR-starved mycobacteria. The concentration- and/or time-dependent activity of these four TB drugs against STR-exposed and STR-starved 18b are shown in Fig. 1. Isoniazid showed time-dependent activity against both populations, with similar CFU counts for all concentrations tested after 6 days of exposure. An increased CFU count of STR-exposed 18b was observed at day 6 after isoniazid exposure, while the CFU count of STR-starved 18b dropped further. Rifampin displayed time- and concentration-dependent activity against both STR-exposed and -starved 18b, with 10 μg/ml nearly eliminating STR-starved 18b. Also, moxifloxacin and bedaquiline showed time- and concentration-dependent activity. Interestingly, moxifloxacin concentrations of 0.4 μg/ml and 2 μg/ml were more potent against STR-exposed 18b than 10 μg/ml, while in the STR-starved experiment, 10 μg/ml was only slightly less active compared to 2 μg/ml.

To affirm persistence of the streptomycin-dependent phenotype, mycobacterial growth of the samples that were not exposed to drugs (controls) was assessed at day 0 and day 6 on Middlebrook plates without streptomycin (indicating streptomycin independency) and compared to the CFU counts on streptomycin containing plates. In

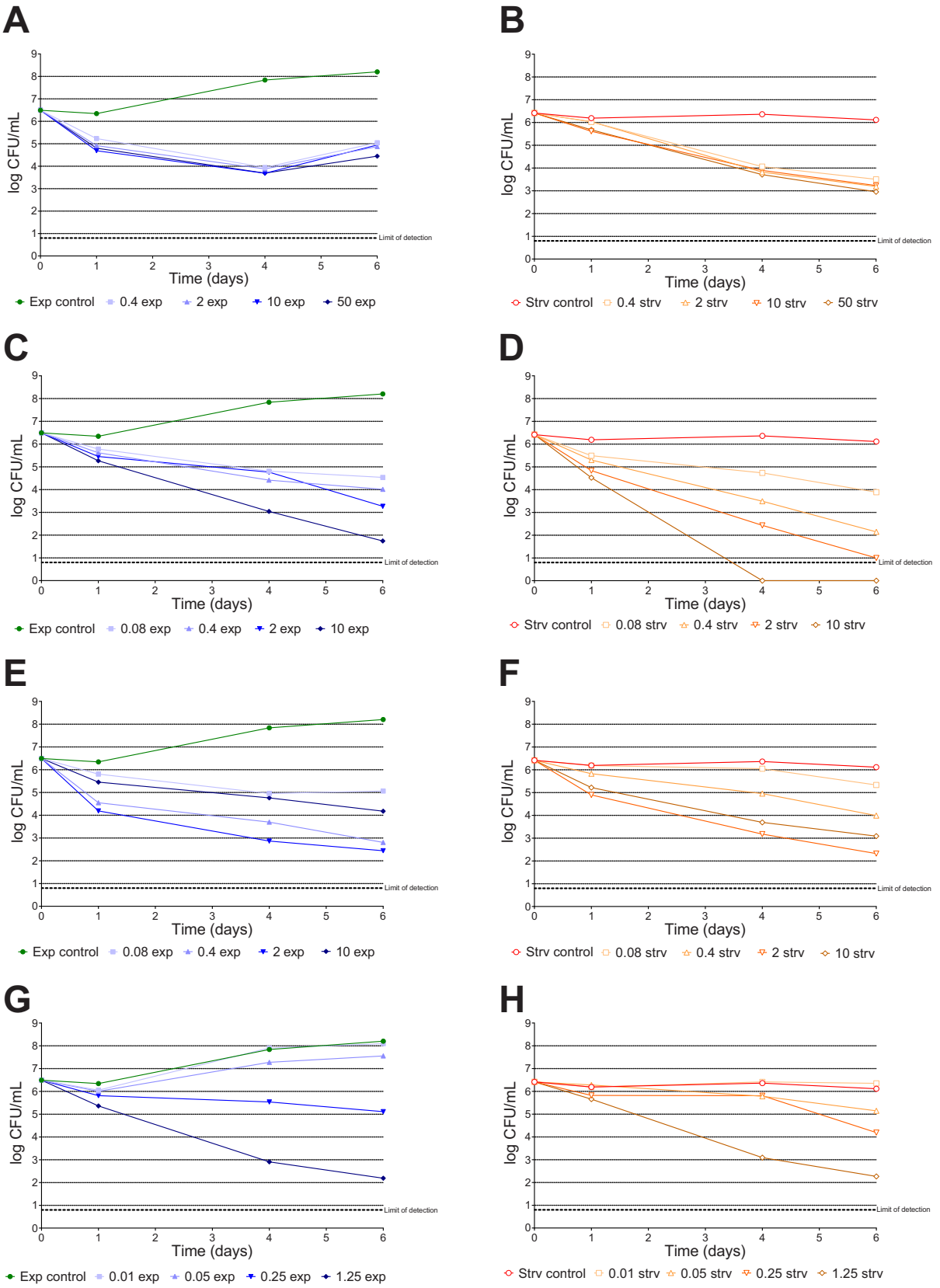

FIG 1 Bactericidal activity of isoniazid (0.4, 2, 10, and 50 μg/ml) (A and B), rifampin (0.08, 0.4, 2, and 10 μg/ml) (C and D), moxifloxacin (0.08, 0.4, 2, and 10 μg/ml) (E and F) and bedaquiline (0.01, 0.05, 0.25, and 1.25 μg/ml) (G and H) against strain 18b in STR-exposed (A, C, E, and G) and STR-starved states (B, D, F, and H). TB drugs were added at day 6 after inoculation in experiments with STR-exposed 18b and at day 14 after inoculation with STR-starved 18b. Data are presented as one representative of two experiments.

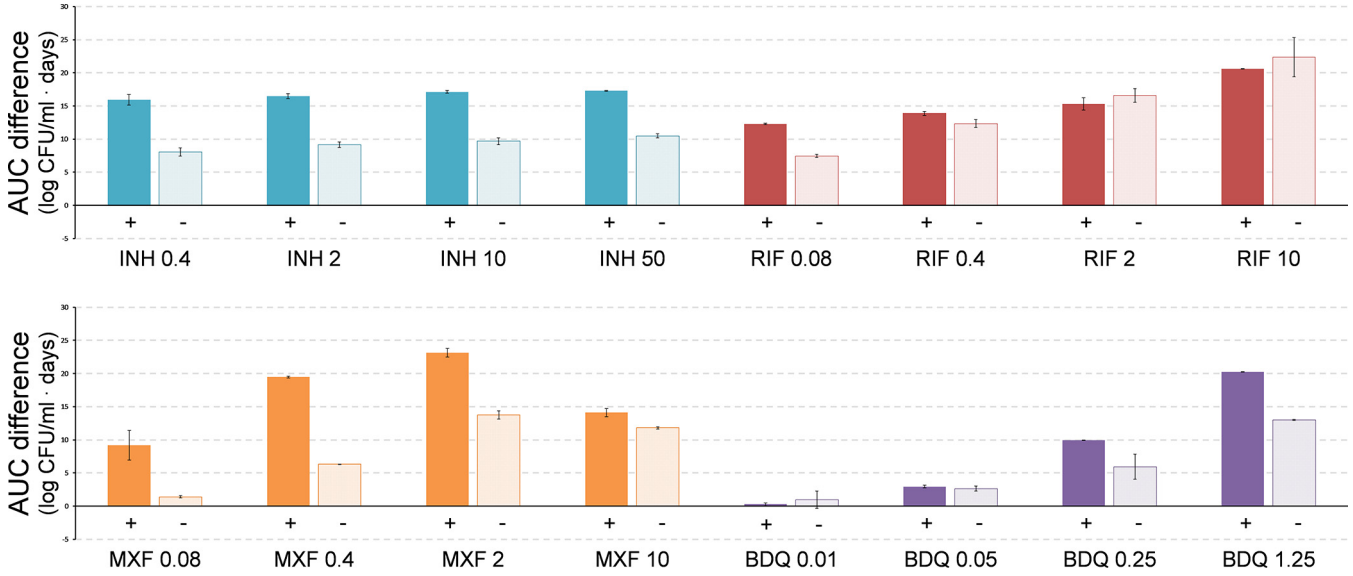

FIG 2 Mean differences of the area under the curve of CFU data between TB drugs and the respective controls. RIF, rifampin; INH, isoniazid; MXF, moxifloxacin; BDQ, bedaquiline; +, STR-exposed; -, STR-starved. Concentrations are indicated in μg/ml.

all control samples, only low levels of STR independence were observed, ranging from 0.0004% at day 0 to 0.18% at day 6 in STR-starved cultures.

To enable the comparison of the TB drug activity against STR-starved 18b relative to STR-exposed 18b, we used the difference in AUC (AUC of the control [unexposed] curve minus the AUC of the TB drug-exposed curve). Assessing AUC differences allows for a more solid comparison between TB drug activity against both mycobacterial populations, as this method takes into account the increase in the STR-exposed control observed at day 6 as opposed to the rather unchanged STR-starved control at day 6 compared to day 0. This way, an overestimation of TB drug activity against the STR-starved population is precluded.

The mean differences in AUC between TB drug-exposed mycobacteria and the control samples are reported in Fig. 2. Isoniazid and moxifloxacin caused a higher difference in AUC on STR-exposed 18b compared to STR-starved 18b, confirming that these drugs were more active against mycobacteria in log-phase growth, with the exception of 10 μg/ml moxifloxacin, which showed almost no difference in AUC between both states. The activity of rifampin and bedaquiline against both mycobacterial populations was generally comparable, with the exception of the highest concentration of bedaquiline, which showed more activity against STR-exposed 18b (Fig. 2).

Time to positivity. We added a liquid culturing method, since this might identify an additional mycobacterial subpopulation which cannot be cultured on solid culture plates. To assess the TTP, the control (unexposed to TB drugs) culture was added to a mycobacterial growth indicator tube (MGIT) supplemented with streptomycin at day zero. This was repeated at day 6 for the control culture and washed samples of all drug-exposed cultures (STR-exposed and -starved). The TTP was automatically recorded.

The TTP after 6 days of exposure to the different TB drugs is shown in Fig. 3. The control sample of the STR-exposed culture exhibited an expected decrease in TTP from an average of 86.5 h to 28.5 h. The control sample of STR-starved 18b showed a small increase in TTP from, on average, 98 h on day 0 to 129 h on day 6, compatible with the small decrease in CFU observed in the time-kill kinetics assay.

As for isoniazid activity, the same concentration independence was observed against both the STR-exposed and the STR-starved mycobacterial populations, except for the longer TTP observed at 50 μg/ml in the STR-starved population. Rifampin,

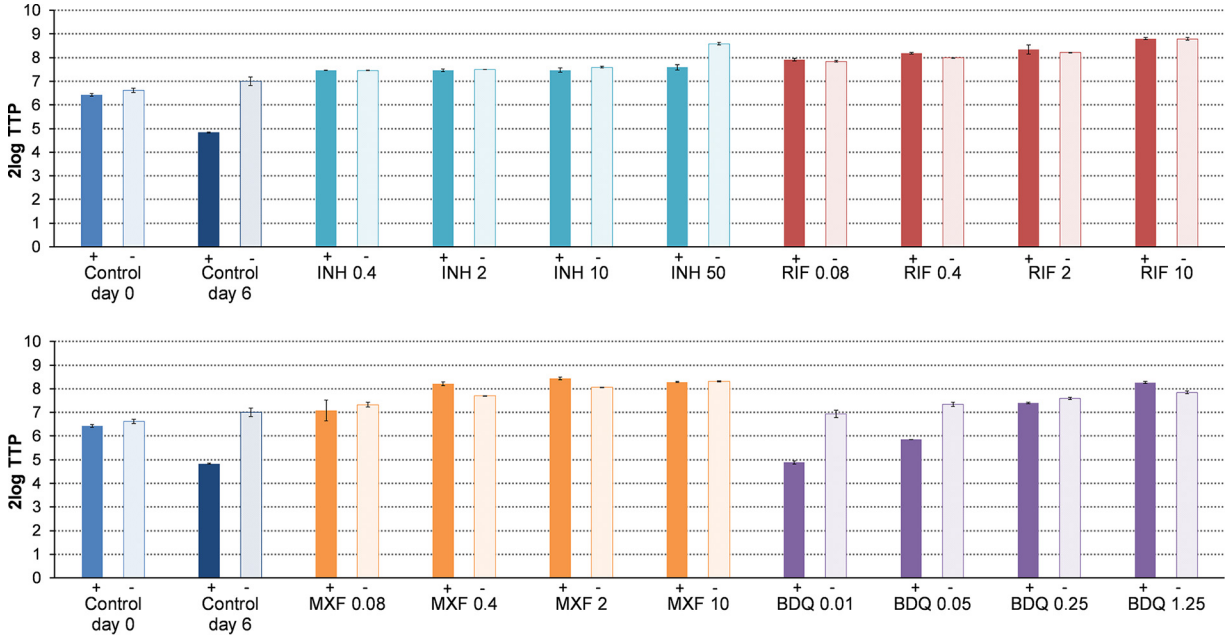

FIG 3 Bactericidal activity of rifampin, isoniazid, moxifloxacin, and bedaquiline against 18b assessed by the Bactec MGIT through time to positivity (TTP). Data are shown as 2 log TTP mean with range ($n = 2$). Samples were taken at day 6 of the corresponding experiments. INH, isoniazid; RIF, rifampin; MXF, moxifloxacin; BDQ, bedaquiline; +, STR-exposed 18b; -, STR-starved 18b. Concentrations are indicated in μg/ml.

moxifloxacin, and bedaquiline showed concentration-dependent activity for all concentrations within the TTP assay, in both the STR-exposed and STR-starved states, with the exception of 10 μg/ml moxifloxacin (Fig. 3).

Strikingly, the MGIT results revealed hardly any difference in TTP between the STR-starved and STR-exposed cultures when exposed to rifampin and isoniazid, while the CFU counts of the STR-starved cultures were on average 2 logs lower in the time-kill kinetics (TKK) assay.

DISCUSSION

In this *in vitro* study, we explored the applicability of the time-kill kinetics 18b dormancy model for assessment of TB drug activity against different *M. tuberculosis* subpopulations by using different detection methods.

The observed time-dependent activity of isoniazid and the concentration and time dependency of rifampin, moxifloxacin, and bedaquiline in our time-kill kinetics assay against the active, fast multiplying state is in line with previous reports of the activity of these drugs against other mycobacterial strains in log phase (13–15). This suggests that there is no interaction with the streptomycin added to the bacteria. The activities of isoniazid and rifampin against STR-starved 18b is in line with our previous study assessing the activity of these drugs against low metabolically active *M. tuberculosis* (H37Rv) (16). This indicates that these drugs act similarly against *M. tuberculosis* in both slow- and nonmultiplying states.

In our study, moxifloxacin concentrations of 0.4 μg/ml and 2 μg/ml showed higher potency against STR-exposed 18b than 10 μg/ml, which is comparable to a previous *in vitro* study of moxifloxacin against Beijing VN 2002-1585, in which maximum activity was reached at 0.5 μg/ml (14). This phenomenon is known as the "Eagle effect" and was first described by Eagle et al. (17), who observed that there was less activity at higher exposure to penicillin for several bacteria. Interestingly, this Eagle effect was not observed in experiments with STR-starved 18b or in acid-phase and oxygen-starved H37Rv in the hollow fiber infection model (18, 19). Surprisingly, Wu et al. did observe the Eagle effect in nutrient-starved *Mycobacterium smegmatis* (20). However, this effect was almost absent when these *M. smegmatis* cultures were pretreated with chloram-

phenicol to stop protein synthesis. This suggests that protein synthesis plays an important role in this phenomenon and might explain its absence in various dormant cultures, including our STR-starved 18b cultures. The Eagle effect of moxifloxacin was also not observed in our previous *in vivo* experiments (H. I. Bax and J. E. M. de Steenwinkel, unpublished data) or in published *in vivo* TB studies (21). Therefore, the true clinical value of this effect seems even more difficult to understand.

In this study, we introduced an AUC-based approach to compare the TB drug activity under different mycobacterial conditions. This novel method provides additional information to CFU counts and is central for appropriate assessment of TB drug activity on different subpopulations, since it corrects for the increasing STR-exposed control population (unexposed to TB drugs), while the STR-starved control stays at similar levels. This knowledge will be important when in the future more targeted therapy will be used for which it might matter whether the TB drug is more active on the nonmultiplying state than on log-phase growth or vice versa. In fact, although the CFU counts of the STR-starved *M. tuberculosis* exposed to isoniazid were lower than those of the STR-exposed population at day 6, a greater AUC difference between the control and the isoniazid-exposed samples was observed in the STR-exposed bacteria, confirming that isoniazid is more active on STR-exposed 18b than STR-starved 18b, instead of equally active, as might have been concluded based on the similar absolute CFU decline observed. As such, this also serves as a back-validation of our adapted 18b model. Also, we were able to show that rifampin displayed the most potent activity against STR-starved 18b compared to STR-exposed 18b, as there was no difference in AUC between the STR-starved and the STR-exposed populations. Similar to isoniazid, a larger AUC difference was observed for moxifloxacin in the STR-exposed bacilli, except for 10 μg/ml, which might be caused by the Eagle effect, which was more pronounced in the STR-exposed state than in the STR-starved state. As a result, the difference in activity between multiplying and nonmultiplying subpopulations might be (artificially) reduced. The fact that the AUC differences for bedaquiline were similar in the lower concentrations tested should be interpreted with caution, as in both metabolic states, almost no activity against 18b was observed. The AUC differences increased in the higher concentrations, and it would be of interest to assess whether the activity against log-phase growth and nonreplicating bacteria would indeed change when exposed for a longer period of time, as other *in vitro* studies reported that it takes time for bedaquiline to display bactericidal action (22). Overall, these findings are in line with the expected performances of these drugs and confirm the value of including AUC difference assessments in the newly developed 18b time-kill kinetics assay.

In our study, we included TTP as a quantification method to assess the difference between solid and liquid culture medium for the detection of the mycobacterial subpopulation that is nonculturable on solid medium. Interestingly, we observed, especially for rifampin, that the TTP of the STR-starved cultures was on average shorter than would be expected based on the CFU counts. In fact, the TTP of the starved cultures exposed to rifampin was similar to that of the STR-exposed cultures at all concentrations tested, while the CFU counts of the STR-starved treated samples were considerably lower than those of the STR-exposed samples at day 6. A possible explanation for these results might be that some of the STR-starved mycobacteria are not able to grow on solid medium but can be recovered in STR-containing liquid medium. This is comparable to what has been observed *in vitro* in nonmultiplying *M. tuberculosis* cultures which could not be cultured on solid medium but where growth was observed in liquid medium even without the addition of resuscitating promoting factors when using the most probable number as the quantification method (12). Also, in human sputum samples, a nonmultiplying subpopulation was detected when CFU counts were compared to TTP measurements during treatment (11). Therefore, the TTP assay might give a better estimation of the total mycobacterial load and thus should be performed in addition to classical CFU counting.

The observed drug activity of rifampin, moxifloxacin, and bedaquiline in our study was comparable to previous 18b studies (10, 23). These studies were generally based on

the resazurin microplate assay (REMA) and luciferase for mycobacterial load assessments as opposed to the time-kill kinetics assay and CFU counting as used in the present study. Although these assays are relatively easy to perform, they cannot be used for the evaluation of the activity of cell wall inhibitors (23). This might explain why in these REMA and luciferase studies, hardly any effect of isoniazid on STR-starved 18b was observed, while we found a 3 log CFU/ml decrease after 6 days of treatment (23, 24). The reason that these assays cannot be used for the assessment of cell wall inhibitors might be that the increase in cell wall permeability allows for a more efficient conversion of resazurin in resorufin, which will cause an increase in fluorescence instead of the expected decrease (25). This limitation is very relevant, as cell wall inhibitors could be valuable components of short-course TB drug regimens (26), underlining the significance of the ability to assess the activity of this class of drug in our adapted 18b model. In addition, REMA is an endpoint analysis and not applicable to time course investigations, and although with the luciferase assay, time course investigation is possible, when drugs display autofluorescence, the luminescence signal is extinguished and impacts results (23). Therefore, as described in this paper, time-kill kinetics assays with CFU counting including the use of AUC differences as well as TTP measurements are useful additions to the REMA and luciferase assays in the assessment of drug activity against the 18b strain.

A review by Iacobino et al. showed the diversity in activity of several TB drugs in different dormancy models (27). Our time-kill kinetics results are most comparable to the results obtained in the hypoxic model of Wayne (5), which is in line with the comparable gene expression profiles observed in the STR-starved state in 18b and the hypoxia model (9). An explanation for the differences in TB drug response between the dormancy models might be that every model uses a different stress condition to lower mycobacterial metabolism, e.g., nutrient starvation, hypoxia, and low pH, which might be associated with different mycobacterial phenotypes and responses upon TB drug exposure (9, 27). Consequently, it is important to use different dormancy models in the assessment of TB drug activity in order to mimic the heterogeneity of the nonreplicating mycobacterial subpopulation present in TB patients. Besides, our study also confirmed the importance of incorporating more readouts (solid and liquid culturing), to be able to identify phenotypically diverse mycobacterial subpopulations. The advantages of the 18b dormancy model compared to other models are that the nonmultiplying state can be achieved in only 10 days, while it takes, for example, 6 weeks in the nutrient starvation model and 100 days in the 100-day stationary-phase model (4, 28). Furthermore, it is easier to maintain the dormancy conditions compared to hypoxia models, in which sampling and manipulating the samples requires additional measures to maintain the hypoxic state (5). As such, the 18b dormancy model in its current form can be a useful tool in the early phase of the drug development pipeline.

An important limitation of the 18b strain is the occurrence of STR-independent growth. This effect appeared to be limited in the time frame of our study. However, during our subsequent hollow fiber infection model (HFIM) experiments when mycobacterial cultures were unexposed to streptomycin for 4 weeks, complete STR independency was observed, and the amount of nonmultiplying bacteria declined fast and therefore became unusable (data not shown). The decline of the nonmultiplying population was also observed in other HFIM studies (19, 29). Therefore, the 18b strain can only be used in short-course experiments, including the time-kill kinetics assay as described in the present study.

Another limitation might be that experiments were performed in duplicate, and consequently, no statistical analyses have been performed. However, the small intertest variability and absence of outliers in our TKK assays is supporting evidence of the reliability of our results. This is strengthened by corresponding results in other studies on this subject, as discussed previously.

In summary, our results confirm the differences in activity of the different TB drugs against the active and nonmultiplying state that were expected based on the working mechanisms of these drugs (30, 31). As such, our model is "back-validated" as a useful

preclinical model to assess the activity of new compounds, where the mode of action and expected effect on the nonmultiplying state are not as clear. In addition, our results support the use of classical CFU counting as well as measurement of AUC differences and TTP in the analyses of drug activity against 18b.

MATERIALS AND METHODS

Bacterial strain and growth conditions. Frozen *M. tuberculosis* 18b samples were thawed and grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (OADC; Becton, Dickinson and Company [BD], Sparks, MD, USA), 0.5% glycerol (Scharlau Chemie S.A, Sentmenat, Spain), 0.05% Tween 80 (Sigma Chemical Co., St. Louis, MO, USA), and 50 μg/ml streptomycin for actively growing 18b under shaking conditions at 96 rpm at 37°C. For STR-starved 18b cultures, no streptomycin was added to the broth. Cultures on solid medium were grown on Middlebrook 7H10 agar (Difco) supplemented with 10% OADC, 0.5% glycerol, and 50 μg/ml streptomycin for 28 days at 35°C with 5% CO_2.

Antimicrobials. Isoniazid, rifampin, moxifloxacin, and streptomycin were all purchased from Sigma (Sigma-Aldrich, Zwijndrecht, The Netherlands). Bedaquiline was kindly provided by Janssen Pharmaceuticals (Johnson and Johnson, Belgium). All dry powder drugs were dissolved in Middlebrook broth, with the exception of rifampin, which was first dissolved in dimethyl sulfoxide (DMSO). As to antimicrobial stability, 50% degradation in Middlebrook medium has been observed after 14 and 7 days for isoniazid and rifampin, respectively (32, 33). Given the fact that the MGIT liquid culture system using the same broth and incubation time (6 days) is an FDA-approved method for susceptibility testing of isoniazid and rifampin, we consider that the stability of those drugs during our time-kill kinetics assay is not a major concern. Moxifloxacin and bedaquiline have been shown to be stable for at least 25 days and 14 days, respectively (34, 35). Streptomycin showed a minor activity loss after 4 weeks (32). Since in our study, mycobacteria were only exposed to streptomycin for a maximum of 12 days, we assumed the impact of the activity loss to be limited.

Time-kill kinetics assay. TB drugs were added at day 6 postinoculation (log-phase growth) in STR-exposed experiments and at day 14 postinoculation (nonmultiplying) in the STR-starved experiments. The cultures were exposed to TB drugs for 6 days at 37°C under shaking conditions at 96 rpm. In the absence of TB drugs, the STR-exposed mycobacterial population increased from mean log 6.7 (range, 6.5 to 6.8) to mean log 8.3 (range, 8.2 to 8.3) CFU/ml. As expected, the STR-starved population did not increase.

TB drug concentrations were based on the peak free serum concentration (fC_{max})—1/25, 1/5, 1, and 5 times fC_{max}. On days 0, 1, 4, and 6 after the addition of TB drugs, samples were taken, centrifuged at 14,000 $\times$ *g* and washed to avoid carryover, serially diluted, and plated onto Middlebrook plates supplemented with 50 μg/ml streptomycin. To affirm persistence of the streptomycin-dependent phenotype, mycobacterial growth of the unexposed control sample was assessed at day 0 and day 6 on Middlebrook plates without streptomycin. Results were depicted as log reduction in mycobacterial load over time as well as difference between the area under the curve (AUC). The AUC of the non-TB drug-exposed 18b curves (controls) was determined based on CFU counts from 0 to 6 days and was subtracted by the AUC of TB drug-exposed 18b samples. In this way, we corrected for the inherent difference between the mycobacterial load of the STR-exposed and the STR-starved control at day 6, allowing for a solid comparison between the activity of TB drugs on STR-exposed and STR-starved 18b.

Time to positivity. At day 0, 200 μl of the control culture was added to a mycobacterial growth indicator tube (BBL MGIT; Becton, Dickinson and Company, MD, USA) in combination with 800 μl OADC enrichment, supplemented with 50 μg/ml of streptomycin. This was repeated at day 6 with 200 μl of the control culture and washed samples of all drug-exposed cultures (STR-exposed and -starved). Tubes were incubated in the BD Bactec MGIT 960 automated mycobacterial detection system (Becton, Dickinson and Company, MD, USA), and TTP was automatically recorded and depicted as 2 log TTP.

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