Improving Tuberculosis Treatment

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Layout and printed by: Optima Grafische Communicatie (www.ogc.nl)
ISBN: 978-94-6361-556-3

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Verbetering van de behandeling van tuberculose

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. F.A. van der Duijn Schouten

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

woensdag 9 juni 2021 om 10.30 uur

door

Elise Dagmar Pieterman
geboren te Nijmegen

Erasmus University Rotterdam

PROMOTIECOMMISSIE

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INTRODUCTION

Tuberculosis (TB) is an ancient disease, that has plagued people around the world for thousands of years. Its causative agent *Mycobacterium tuberculosis* (Mtb) was discovered in 1882 by Robert Koch. TB spreads by coughing and subsequent inhalation of infected aerosols. As a consequence, many people (an estimated one third of the world population) are (latently) infected. Only 10% of these people will fall ill, of which 60% in the first two years after infection and 40% later in life (1). Although healthy people can develop active TB, the progression to active disease is often caused by weakening of the immune system by diseases such as HIV, diabetes mellitus and malignancies or by the use of immune suppressive medication (1). The most common symptom of active TB is productive coughing, caused by lung inflammation. Nevertheless TB can affect the entire body (which is more common in immune deficient patients) with a wide variety in symptoms (2). Even though TB treatment is available, TB has been the top infectious disease killer in the world for the last years (3). In 2018 an estimated 10 million people fell ill with TB and an estimated 1.2 million patients died due to this disease in the same year (3), underlining the importance of treatment improvement.

In the next chapters we will address how TB can be diagnosed, treated and modelled.

Diagnosis

Active TB can be diagnosed by (liquid) culturing through measuring the time-to-positivity, which is regarded as the gold standard. However, since it can take 2-6 weeks before the culture results are known (due to the long replication time of Mtb of 20 hours), it is necessary to also implement other diagnostics. By microscopic evaluation with auramine staining a mycobacterial infection can be detected when the bacterial load is high enough. This should be followed up by a molecular analysis to exclude an infection with other mycobacteria, particularly non-tuberculous mycobacteria. Since 2010 the WHO endorsed the use of the GeneXpert MTB/RIF for the diagnostics of TB and the screening for rifampicin resistance (4). This PCR test can diagnose a TB infection and genotypical rifampicin resistance in 2 hours (5). This is a substantial advantage compared to phenotypical resistance assessment (the gold standard), which can only be performed on Mtb cultures, which can take up to 6 weeks to culture.

Another useful new clinical application is whole genome sequencing, which can be used for diagnosis, assessment of drug resistance, surveillance and source investigation by complete DNA sequencing of the Mtb genome (6).

For the diagnostics of latent TB no gold standard test is available. However, it can be indirectly shown by detecting (memory) T-cell reactivity in response to Mtb antigens. The oldest test for latent TB is the tuberculin skin test (TST). By the injection of a purified protein derivate of tuberculine under the skin, an immune reaction of latently

infected patients will cause induration of the skin. However, as there is cross reactivity with the BCG-vaccine and other mycobacteria, this test is not very specific (7). Therefore the interferon-gamma release assays (IGRAs) have been developed. These blood tests (QuantiFERON and T-SPOT.TB) measure the interferon-y (INF-y) release of T-cells in response to Mtb specific antigens (ESAT-6 and CFP-10) in case of previous Mtb exposure. A disadvantage of both the TST and IGRAs is their inability to distinguish between recent and old infections as recent infections provide a substantial higher risk of progression to active disease and justify latent TB treatment (8, 9). Therefore in the latest version of the QuantiFERON test not only the CD4+ T-cell INF-y release is measured, but also the INF-y release of CD8+ T-cells, as a higher CD8+ T-cell response has been related to recent Mtb exposure (10). Another disadvantage of both latent TB tests is their inability to differentiate between active and latent disease, making treatment decisions even more complex. Based on this element several studies used active TB patients and patients with a low TB risk as surrogates to calculate the sensitivity and specificity of these latent TB tests. Consequently, the overall estimated sensitivity of for example the QFT-plus of 95.3% is the sensitivity for the diagnosis of active TB instead of latent TB (11).

Treatment

The standard treatment of active TB consists of isoniazid, rifampicin, pyrazinamide and ethambutol (HRZE) and takes at least 6 months. The long treatment duration and high pill burden hamper treatment compliance with a risk of the emergence of drug resistance and worse treatment outcomes as a result (treatment success rate of 85% globally in 2017) (3, 12). When Mtb is resistant to the first-line drugs isoniazid and rifampicin it is called multidrug resistant (MDR) TB. In 2018, there were about half a million new cases of rifampicin-resistant TB (of which 78% MDR-TB). Approximately 23,000 of these MDR-TB patients had additional resistance to fluoroquinolones and at least one of the three second line injectables, referred to as extensively drug resistant (XDR) TB and even cases of totally resistant tuberculosis (Mtb resistant to all available TB-drugs) have been described (13). When patients are diagnosed with drug resistant TB the treatment duration has to be increased to at least 9 months, but more often to more than 1.5 years. Also the amount of drugs that has to be taken increases and it can take up to 14,600 pills to treat one person with drug resistant TB. The treatment success rate of MDR-TB is poor with 54% and drops down to even 30% in case of XDR-TB (3). Based on these numbers it is clear that the therapy has to be improved to increase the treatment success and besides shortening the treatment duration and lowering the pill load is highly required.

Several strategies can be followed to achieve this goal. First of all, given the increased prevalence of drug resistant TB and the existence of even totally resistant TB, there is an urgent need for new drugs. Nevertheless, in the last decades only two novel TB-drugs came on the market; bedaquiline in 2012 and delamanid in 2013. Secondly, new regimens

with promising combinations of TB-drugs should be developed. In TB patients Mtb can be present as fast, slow and non-multiplying Mtb, with the last subpopulation being especially difficult to treat due to changes in the cell wall composition and their low metabolism compromising the efficacy of TB drugs (14, 15). These non-multiplying mycobacteria are considered the main reason for the prolonged treatment duration and are held responsible for disease relapse due to their hidden persistence (16). Therefore new regimens should ideally be based on combinations of drugs with different working mechanisms, to be active against all metabolic states of Mtb. Another way of therapy improvement could be the optimization of TB-drugs dosages. Most TB-drugs have been used for decades in the same dosage, while choices for the specific dose were not always based on optimal dose finding, but instead were financially driven, or based on the fear of adverse events (17). A different approach to increase the activity of drugs is trying to increase the intrabacterial drug concentrations. There is accumulating evidence that efflux pumps by the extrusion of TB-drugs facilitate the emergence of drug resistance (18, 19). By blocking these efflux pumps by efflux pump inhibitors such as verapamil, the intrabacterial drug concentration might increase and subsequently reduce the emergence of resistance (20).

Pre-clinical modelling

Good pre-clinical models are essential to assess the activity and efficacy of these new drugs (combinations) and to optimize drug dosages. However, the translation of the results of these pre-clinical models to clinical outcomes in TB patients is difficult, which has been shown in for example the ReMOX trail (21). This trial aimed for a treatment reduction of drug-susceptible TB from 6 to 4 months by the replacement of isoniazid or ethambutol by moxifloxacin which was based on promising *in vitro* and *in vivo* results (22-25). However, in patients it appeared to be unsuccessful (21). In order to prevent such future mismatches several international consortia, as for example PreDiCT-TB and ERA4TB, have been working on the development of an optimal pre-clinical research path (26, 27). By using a combination of different models, many different characteristics of TB-drugs can be assessed such as drug activity against different Mtb subpopulations, as well as intracellular drug activity, driving pharmacokinetics and pharmacodynamics (PK/PD) parameters and drug resistance mechanisms. Pre-clinical drug activity assessment against non-multiplying Mtb is important since this subpopulation is held responsible for the long treatment duration of TB in order to prevent relapse (15). However, drug activity on this subpopulation is difficult to assess. As this subpopulation is not replicating, it will not grow on solid culture plates, making this population difficult to identify (28, 29). Besides, creation of non-replicating Mtb is a lengthy process (at least 6 weeks) of Mtb growth inhibition by for example nutrient or oxygen depletion (30, 31). An alternative dormancy model is the 18b dormancy model (32). This model uses the 18b Mtb strain which is dependent on streptomycin for growth and without streptomycin it becomes non-multiplying. In

this non-multiplying state comparable "dormancy" genes are up regulated as in other dormancy models. Furthermore, the advantage of the 18b strain compared to these other dormancy models is that it becomes non-multiplying in only 10 days and has the additional benefit that it can be cultured on culture plates containing streptomycin (33). In this way the activity of drugs against fast- and non-multiplying Mtb subpopulations can be determined simultaneously. Consequently, this strain can be easily used in for example time-kill-kinetics assays. These assays could be a useful tool to assess concentration and/or time dependent activity against different mycobacterial subpopulations (34). In time-kill kinetics assays, static concentrations are used, while in humans drugs concentrations vary depending on its absorption rate and half-life time. In a hollow fiber infection model, these pharmacokinetic parameters can be simulated. Besides, more frequent sampling for assessment of the bacterial load and drug concentrations is possible compared to other *in vitro* and even *in vivo* models (35). In this way the driving PK/PD parameters can be revealed (36), providing tools for optimal dose finding. In addition to these *in vitro* models, there is an important role for *in vivo* murine TB-models for the assessment of drug efficacy, allowing assessment of culture negativity as well as relapse (cure), which could be followed by mathematical modelling in order to improve the predictive value and thus the translation into clinical practice (37).

THESIS OUTLINE

The overall aim of this thesis is improving treatment of latent TB by better determination of the indication for treatment by a new diagnostic test (chapter 2), as well as improving the treatment of active TB (chapter 3 & 4).

In chapter 2 the validation of the (at the time) new QuantiFERON-TB Gold Plus assay against its predecessor QuantiFERON-TB Gold In Tube is described. Besides, the additional value of CD8+ T-cell activity measurement to distinguish between recent and distant infection in the new QuantiFERON test was assessed in this study. This distinction is relevant as a recent TB infection provides a higher risk of progression to active disease and therefore could be used as a justification for treatment.

In chapter 3 the treatment shortening potential of different combination regimens for the treatment of active TB was explored. The new combination of the youngest TB-drugs bedaquiline, delamanid and linezolid was assessed in our mouse TB-model, by using a two-weekly relapse assessment and additional mathematical modelling (chapter 3.1). In the second murine TB study the additional value of the use of an efflux pump inhibitor was investigated, by adding verapamil to the combination of moxifloxacin and linezolid (chapter 3.2).

The chapter 4 focuses on the improvement of the predictably of two *in vitro* models for the treatment of active TB. The first study described improvement of the 18b dormancy model by the addition of time-to-positivity assessment and measurement of differences in area under the curve of time-kill-kinetics assays to allow better comparison of TB drug activity against multiplying and non-multiplying 18b (chapter 4.1). The second study assessed whether the hollow fiber infection model is a useful tool to evaluate dosage increase of rifampicin and rifapentine (chapter 4.2).

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Chapter 2

Improving diagnostics of latent TB infection

Chapter 2.1

A multicentre verification study of the QuantiFERON®-TB Gold Plus assay

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Tuberculosis (Edinb). 2018 Jan;108:136-142

ABSTRACT

Objectives: The aim of this verification study was to compare the QuantiFERON®-TB Gold Plus (QFT-Plus) to the QuantiFERON®-TB Gold In Tube (QFT-GIT). The new QFT-Plus test contains an extra antigen tube which, according to the manufacturer additionally elicits a CD8+ T-cell response above the CD4+ T-cell response. We assessed the value of this tube in detecting recent latent tuberculosis infections.

Methods: Between May 2015 and December 2016, 1031 subjects underwent the QFT-Plus and QFT-GIT test. Overall agreement between both tests and performance for different test indications and/or immune states was assessed. A difference of >0.6 IU/mL interferon-γ release between the two antigen tubes of the QFT-Plus assay was considered a true difference and used as estimation for CD8+ T-cell response.

Results: Analysis of the QuantiFERON tests resulted in an overall agreement between assays of 95%. Subjects considered to be recently exposed to tuberculosis had significantly more often a true difference in interferon-γ release compared to all other subjects (p=0.029).

Conclusion: Results of QFT-Plus are highly comparable to QFT-GIT. Although there is an indication that a true difference in interferon-γ release between the antigen tubes is associated with recent latent tuberculosis infection, the QFT-Plus could not be used to exclude recent exposure.

INTRODUCTION

Tuberculosis is still a major global health problem, with an estimated 10.4 million people suffering from this disease worldwide in 2015 (1). Moreover, about one third of the world population is latently infected with *Mycobacterium tuberculosis* (Mtb) and as such there is a vast reservoir (2). Once latently infected, the estimated lifetime risk to develop active tuberculosis is 5-15% (3). Therefore, the goal of the World Health Organisation to effectively eliminate tuberculosis by 2050 is only feasible if we can identify and eliminate latent tuberculosis infections (LTBI) (4).

LTBI are difficult to diagnose, because there is no gold standard test. The diagnosis is based on a positive tuberculosis skin test (TST) and/or interferon-γ (IFN-γ) release assay (IGRA) in the absence of active tuberculosis. The TST is based on an immune reaction in the skin towards purified protein derivate of tuberculine. The main limitation of this test is the cross reactivity with Bacillus Calmette-Guérin (BCG) vaccination and infection with other mycobacteria (5). In order to overcome this limitation, the IGRAs have been developed. Until recently two IGRAs were commercially available; the QuantiFERON®-TB Gold In-Tube (QFT-GIT) and the T-SPOT®.*TB*. By using Mtb derived specific peptides (ESAT-6, CFP-10 and TB7.7, the last one only in QFT-GIT) located in the region of difference (RD-1 and RD-11) of Mtb, IGRAs could elicit a more specific immune reaction compared to TST, characterized by IFN-γ production in case of previous Mtb exposure (6). Nonetheless, also the IGRAs show cross reactivity with some other mycobacteria that contain the RD1 region (e.g. *Mycobacterium marinum, Mycobacterium kansasii* and *Mycobacterium szulgai*) (7). The disadvantages of IGRAs compared to the TST are their alleged reduced sensitivity for the detection of infections in the distant past as well as the higher costs (8, 9). The TST and IGRAs both rely on an appropriate immune reaction and have therefore theoretically a reduced sensitivity in immunocompromised patients (10, 11). Moreover, all these tests are unable to distinguish between recent and old latent infections (12). Such a differentiation is of importance, since the risk of developing active disease is highest in the first two years after infection, justifying LTBI treatment (13).

In 2015 a new QuantiFERON test has been introduced: the QuantiFERON®-TB Gold Plus (QFT-Plus) which will replace the currently used QuantiFERON-TB Gold in Tube (QFT-GIT). For this new test the peptide TB7.7 is removed and it contains an extra antigen tube (TB2) compared to its predecessor QFT-GIT. The additional tube contains adapted peptides, developed to target not only CD4+ T-cells, as the antigen tube of QFT-GIT and the TB1 antigen tube of QFT-Plus, but also CD8+ T-cells (14, 15). A previous study showed a higher CD8+ T-cell response in persons recently exposed to tuberculosis compared to exposure in the distant past (16). Therefore the QFT-Plus test might be capable of differentiating between recent and remote LTBI, which might help in the decision to start LTBI treatment.

In this study we evaluated the performance of the QFT-Plus assay in the daily practice of 16 laboratories in the Netherlands and Belgium. Besides, we assessed the value of the additional tube of QFT-Plus in differentiating between recent and old LTBI.

MATERIALS AND METHODS

Study design and participants

We conducted a comparative verification study in 16 clinical laboratories in the Netherlands and Belgium. From May 2015 till December 2016 subjects referred for QuantiFERON testing to one of the participating laboratories were enrolled in this study. Participants underwent the regular QFT-GIT (Qiagen, Hilden, Germany) as well as the new QFT-Plus test (Qiagen, Hilden, Germany). Information on age, gender, immune state and test indication was collected by the local laboratory and forwarded anonymously for data analysis.

QFT-GIT and QFT-Plus diagnostics

Whole-blood samples of all participants were (according to manufacturer's protocol) either collected directly in QuantiFERON test tubes or pre-tubed in heparin tubes. The tests were performed and interpreted following regular laboratory procedures, according to the manufacturer's guidelines (14). The test was recorded positive if the IFN-γ release in the antigen tube (TB for QFT-GIT, TB1 and/or TB2 for QFT-Plus) minus the negative control (nil tube) was ≥0.35 IU/mL and ≥25% of the negative control. A test was considered negative if the IFN-γ release of the antigen tube minus the negative control was <0.35 IU/mL or <25% of the negative control provided that the positive control (mitogen tube) was ≥0.5 IU/mL. A test was considered indeterminate if the IFN-γ release of the negative control was >8 IU/mL or the antigen tube minus the negative control was <0.35 IU/mL or <25% of the negative control with a positive control of <0.5 IU/mL (14).

Statistical analysis

The agreement of both tests was assessed by calculating the percentage of concordant results and by computing the Cohen's Kappa. We performed chi square tests to analyse whether there were any significant differences in agreement of both tests according to different immune states and test indications. Data are expressed as mean ± standard deviation (SD) or as median and interquartile range (IQR).

We performed the Wilcoxon signed rank test to evaluate whether there was a significant difference in IFN-γ release between both tests. Bland-Altman plots were created to identify any systematic differences in IFN-γ release among both tests. Reference lines show mean and 95% limits of agreement (average difference ± 1.96 SD of the difference)

(17). Linear regression analyses were performed to assess the correlation of IFN-γ release between both tests. The same analyses were performed to assess the differences between TB1 and TB2 results of the QFT-plus assay. For all analyses IFN-γ release was calculated as the release of the antigen tube (TB, TB1 or TB2) minus the nil tube as the nil value for both tests could be different.

To evaluate whether IFN-γ release of CD8+ T-cells is related to recent exposure to tuberculosis, we subtracted the IFN-γ release in antigen tube TB1 from antigen tube TB2. A difference in IFN-γ release of >0.6 IU/mL was considered a true difference instead of intra-test variability based on the expected normal range of within-subject variability of IFN-γ release on retesting (18). We calculated the percentage of subjects with an estimated true difference of IFN-γ release within the positive test results, for each test indication. In addition, we performed chi square analyses to assess whether the differences between test indications were statistically significant.

Statistical analyses were performed using IBM Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA; version 22.0). The statistical significance level adopted was $p < 0.05$.

Medical ethical approval

All data were retrieved by the local diagnostic laboratory and forwarded anonymously for data analysis. No informed consent is needed for such data.

RESULTS

The patient characteristics are shown in table 1. A total of 16 laboratories participated in this study and the samples of 1031 subjects were analysed. The majority of the subjects was immunocompetent (56%), 17% was immunocompromised and for 27% of the cases the immune state was unknown. The most frequent test indication was screening before immunotherapy (33%), followed by tuberculosis infection in differential diagnosis (25%), periodic check (every half year) by occupational health services of health care workers working with tuberculosis patients or possibly *Mycobacterium tuberculosis* infected materials (18%) and contact investigation (12%).

Table 1. Patient characteristics in numbers and percentage or mean ± SD of the total population of 1031 subjects.

Gender	N (%)
Male	414 (40%)
Female	615 (60%)
Unknown	2 (0.2%)
Age [1] (years)	44 ± 18
Immunocompetent	N (%)
Yes	574 (56%)
No	178 (17%)
Unknown	279 (27%)
Test indication	N (%)
Tuberculosis infection in differential diagnosis	263 (25%)
Contact investigation	127 (12%)
Screening before immunotherapy	337 (33%)
Periodic check by occupational health services	189 (18%)
Other [2]	57 (5.6%)
Unknown	58 (5.6%)

[1] *N=1029*

[2] *Screening of immigrants 31 (3%), screening of homeless 5 (0.5%), employment medical examination 10 (1%), other 11 (1.1%)*

QFT-GIT versus QFT-Plus

In 981 of the 1031 tests performed, QFT-GIT results were similar to QFT-Plus which resulted in an agreement of 95% and a Cohen's kappa of 0.828 (table 2a). The results of QFT-Plus, divided by TB1 and TB2, compared to QFT-GIT showed an agreement of 95% and a Cohen's kappa of 0.806 and 0.835, respectively. No significant differences in agreement of QFT-GIT and QFT-Plus for different immune states or test indications were observed (p=0.554 and p=0.308, respectively). In table 2b, the discordant results are specified according to immune state and test indication. Since these were all small numbers, no statistical analysis has been performed. The discordancy was two-sided and 60% was in the borderline range of 0.25-0.8 IU/ml (18).

Table 2a. Results QFT-GIT and QFT-Plus

	QFT-Plus positive	**QFT-Plus negative**	**QFT-Plus indeterminate**	**Total**
QFT-GIT positive	131	22[2]	0	153
QFT-GIT negative	19[1]	835	5[4]	859
QFT-GIT indeterminate	0	4[3]	15	19
Total	150	861	20	981/ 1031

QFT-GIT: QuantiFERON®-TB Gold In-Tube, QFT-Plus: QuantiFERON®-TB Gold Plus. Bold numbers are concordant results. Discordant results are highlighted in different colours and the numbers in superscript refer to table 2b.

Table 2b. Discordant results QFT-GIT versus QFT-Plus

	1 QFT-GIT negative QFT-Plus positive (N=19)	2 QFT-GIT positive QFT-Plus negative (N=22)	3 QFT-GIT indeterminate QFT-Plus negative (N=4)	4 QFT-GIT negative QFT-Plus indeterminate (N=5)
Immune state				
Immunocompromised (17%)	4 (21%)	3 (14%)	1 (25%)	3 (60%)
Immunocompetent (56%)	11 (58%)	12 (55%)	3 (75%)	2 (40%)
Unknown (27%)	4 (21%)	7 (21%)	0	0
Test indication				
Tuberculosis infection in differential diagnosis (25%)	9 (47%)	6 (27%)	3 (75%)	1 (20%)
Contact investigation (12%)	3 (16%)	4 (18%)	0	0
Screening before immunotherapy (33%)	4 (21%)	1 (5%)	1 (25%)	3 (60%)
Check by occupational health services (18%)	3 (16%)	7 (21%)	0	0
Other (5.6%)	0	2 (9%)	0	0
Unknown (5.6%)	0	2 (9%)	0	1 (20%)

The median IFN-γ release of QFT-GIT positive results in TB 2.460 IU/mL (IQR: 0.810 - 7.398) was significantly higher (p<0.001) compared to the median IFN-γ release of QFT-Plus TB1 2.010 IU/mL (IQR: 0.385 - 6.195) and significantly lower (p<0.001) compared to the median IFN-γ release of QFT-Plus TB2 2.470 IU/mL (IQR: 0.570 - 6.070). However, there was no significant difference (p=0.673) in the median IFN-γ release of QFT-GIT negative results of TB 0.000 IU/mL (IQR: -0.010 - 0.020) compared to the median IFN-γ release of QFT-Plus TB1 0.000 IU/mL (-0.010 - 0.020). The median IFN-γ release of TB2 0.005 IU/mL (IQR: -0.010 – 0.030) was significantly higher (p=0.003) compared to the median IFN-γ release of TB of QFT-GIT negative results. The Bland Altman plots showed a minor positive systematic difference in IFN-γ release of the QFT-GIT compared to the QFT-Plus (TB1 0.122 IU/mL, 95% limits of agreement: -1.528 - 1.772; TB2 0.067 IU/mL, 95% limits of agreement: -1.582 - 1.714) (figure 1A and 1C). Linear regression analyses showed a good correlation between both tests (TB1 R^2=0.896, TB2 R^2=0.869) (figure 1B and 1D).

TB1 versus TB2 in the QFT-Plus assay

In 1002 out of 1031 tests, outcomes were similar in TB1 and TB2, resulting in an agreement of 97% and a Cohen's kappa of 0.893 (table 3a). In table 3b, the immune state and test indication of the subjects with discordant results are specified. 33% of the subjects who had a negative result in TB1 and a positive result in TB2, was tested in the context of contact investigation, compared to only 12% in the overall population. In the same group only 10% was tested in the context of screening before immunotherapy, while in the total population this was 33%. Since these are small numbers no statistical analysis has been performed. However, we did not find a significant difference in agreement of TB1 and TB2 according to different immune states or test indications (p=0.531 and p=0.327, respectively). None of the TB1 positive / TB2 negative results had a difference in IFN-γ release of >0.6 IU/mL, however 8 out of 18 TB1 negative / TB2 positive results had a difference in IFN-γ release of >0.6 IU/ml.

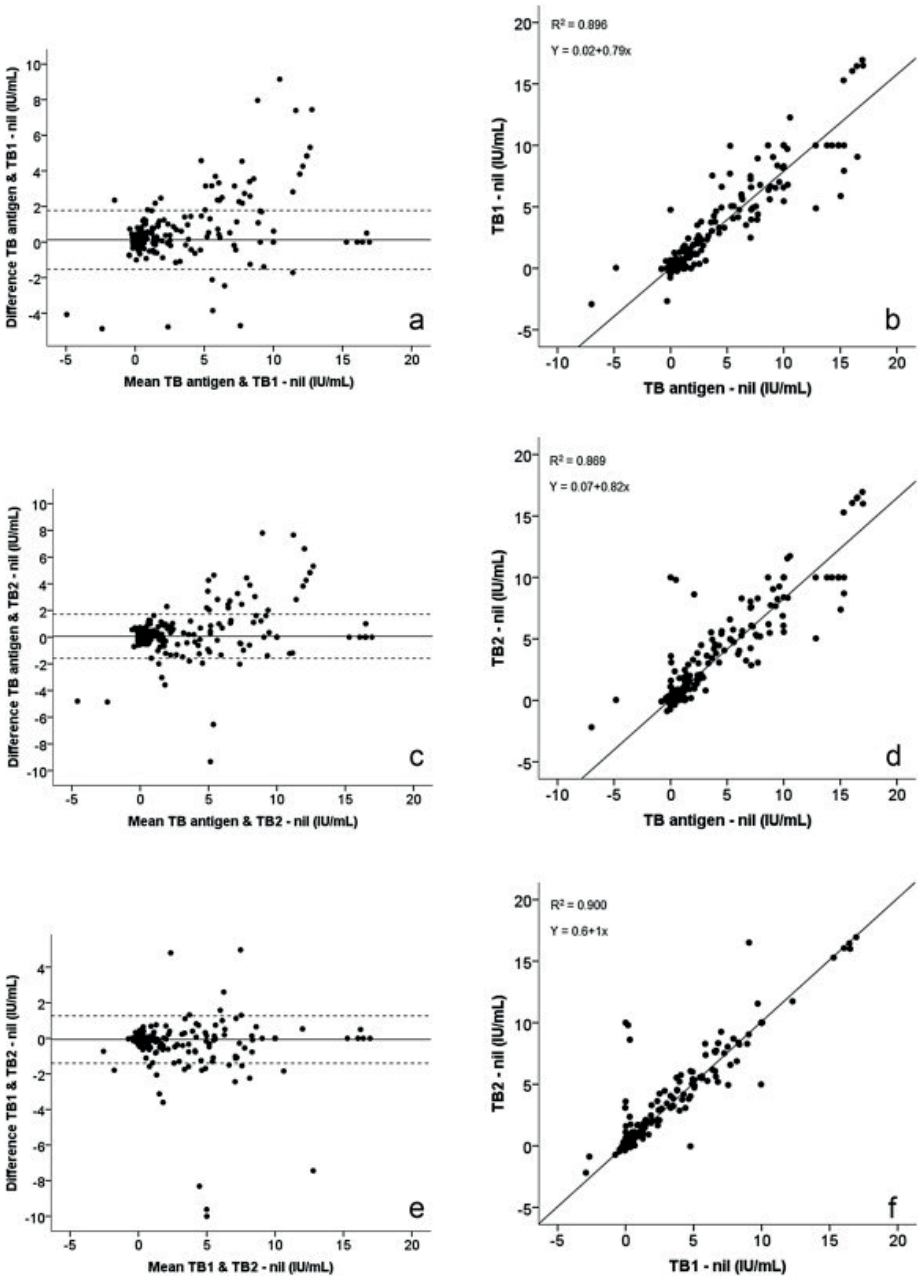

Figure 1. Bland Altman plot (A, C, E) reference lines showing mean difference and 95% limits of agreement and regression plot (B, D, F) of IFN-γ release in TB antigen-nil vs TB1-nil (A, B), TB antigen-nil vs TB2-nil (C, D) and TB1-nil vs TB2-nil (E, F)

Table 3a. Results QFT-Plus TB1 and TB2

	TB2 positive	TB2 negative	TB2 indeterminate	Total
TB1 positive	121	10[2]	1[3]	132
TB1 negative	18[1]	861	0	879
TB1 indeterminate	0	0	20	20
Total	139	871	21	1002/ 1031

QFT-GIT: QuantiFERON®-TB Gold In-Tube, QFT-Plus: QuantiFERON®-TB Gold Plus. Bold numbers are concordant results. Discordant results are highlighted in different colours and the numbers in superscript refer to table 3b.

Table 3b. Discordant results TB1 versus TB2

	1 TB1 negative TB2 positive (N=18)	2 TB1 positive TB2 negative (N=10)	3 TB1 positive TB2 indeterminate (N=1)
Immune state			
Immunocompromised (17%)	4 (22%)	3 (30%)	0
Immunocompetent (56%)	10 (56%)	6 (60%)	0
Unknown (27%)	4 (22%)	1 (10%)	1 (100%)
Test indication			
Tuberculosis infection in differential diagnosis (25%)	5 (28%)	4 (40%)	1 (100%)
Contact investigation (12%)	6 (33%)	1 (10%)	0
Screening before immunotherapy (33%)	2 (11%)	4 (40%)	0
Check by occupational health services (18%)	3 (17%)	1 (10%)	0
Other (5.6%)	1 (6%)	0	0
Unknown (5.6%)	1 (6%)	0	0

In 33% of the positive tests performed in the context of either contact screening or periodic check by occupational health services, the IFN-γ release was >0.6 IU/mL higher in TB2 compared to TB1 (table 4). In the tuberculosis infection in differential diagnosis and screening before immunotherapy group this was only 17% and 11 %, respectively. These results were not significantly different (p=0.224). However, we observed a statistically

Table 4. Difference in IFN-γ between TB1 and TB2 >0.6 IU/mL in positive results

Test indication	**IFN-γ >0.6 IU/mL (% within positive results)**
Tuberculosis infection in differential diagnosis	7 (17%)
Contact investigation	18 (33%)
Screening before immunotherapy	2 (11%)
Periodic check by occupational health services	3 (33%)
Other*	2 (15%)
Unknown	4 (33%)
Total	36

**Screening of immigrants, screening of homeless, employment medical examination, other*

significant higher number of true differences in IFN-γ release when the assumed recently exposed LTBI subjects (contact investigation group and periodic check by occupational health services group) are clustered and compared to the total of other test indication subgroups (p=0.029).

The IFN-γ release of TB2 was higher (p<0.001) compared to TB1 in the QFT-Plus negative results with a median of 0.050 IU/mL (IQR: -0.010 - 0.030) versus 0.000 IU/mL (IQR: -0.010 - 0.020). Within the positive results, a higher IFN-γ release of TB2 was observed as well (p=0.001), with a median of 3.04 IU/mL (IQR: 0.848 – 6.288) versus 2.345 IU/mL (IQR: 0.577 - 6.423). The IFN-γ release in the indeterminate tests was not significantly different (p=0.218). Similarly, the Bland Altman plot showed a positive systematic difference of the IFN-γ release of TB2 compared to TB1 (0.066 IU/mL, 95% limits of agreement: -1.263 - 1.395) (figure 1E). The linear regression analysis showed a good correlation (R^2= 0.900) (figure 1F). The discordant results showed median values of IFN-γ release around the cut-off of positive results, namely TB1 positive / TB2 negative; TB1 0.395 IU/mL (IQR: 0.377-0.607), TB2 0.178 IU/mL (IQR: 0.0085-0.283), TB2 positive / TB1 negative; TB1 0.248 IU/mL (IQR: 0.015-0.300), TB2 0.565 IU/mL (IQR: 0.398-3.218).

DISCUSSION

To our knowledge this is the largest verification study of QFT-Plus performed so far, including different subject groups. In the present study executed in the Netherlands and Belgium, two low burden countries (19), QFT-Plus results were highly comparable with QFT-GIT. No significant differences between both QFT-tests were observed for different immune states and test indications. The discordant results were two-sided and the majority was in the borderline range, suggesting that these discordant results might be due to test variability instead of true differences between tests (18).

The most striking observation of this study was that one third of all positive tested contact screening subjects had a true difference in IFN-γ release between TB1 and TB2 as well as one third of the positive tested subjects screened in the context of a periodic check by occupational health services. This was considerably more than for other subgroups. As we considered subjects in both screening categories are more likely to represent recent LTBI patients, these results might indicate that a higher IFN-γ release in TB2 compared to TB1 is associated with recent tuberculosis contact. An Italian study among contact investigation subjects showed similar results and found a true difference in IFN-γ release in 26% of all QFT-Plus positive tested contact screening subjects (20). Besides, in the same study a true difference between TB1 and TB2 was associated with a higher proximity

to the index case. Both findings are (indirect) indications that the TB2 is indeed associated with recent LTBI.

However, these results are in contrast with a study of Petruccioli *et al.* in which no higher IFN-γ release in TB2 for TB contacts was observed (15). Furthermore, even a lower IFN-γ release in TB2 compared to TB1 was measured in recent LTBI subjects. These differences in results might be due to the low number (N=12) of recent LTBI subjects enrolled in their study.

In this study we observed 10 discordant results which were TB1 positive and TB2 negative. Based on the assumption that TB1 only elicits a CD4+ T-cell reaction and TB2 a CD4+ and a CD8+ T-cell reaction, these observations seem to contradict this. However, none of these results had a difference in IFN-γ release of >0.6 IU/mL and might therefor be due to test variability (15, 18).

In the present study we observed a significant lower IFN-γ release of positive QFT-GIT tests in TB1 tubes compared to the antigen tube of QFT-GIT. A possible explanation for this higher IFN-γ release in QFT-GIT compared to TB1, might be the removal of the peptide antigen TB 7.7 in the new QFT-Plus test, which was present in QFT-GIT. Similar to QFT-Plus, a previous generation of the QFT-GIT (QuantiFERON®-TB Gold test) containing only ESAT-6 and CFP-10 peptide antigens, released significant less IFN-γ compared to QFT-GIT (21). The IFN-γ release in TB2 was higher compared to the antigen tube of QFT-GIT. This might be the result of the adapted peptides added to TB2 to elicit a CD8+ T-cell reaction besides the CD4+ T-cell reaction. These observations were comparable to previous studies also observing a significantly lower IFN-γ release in QFT-Plus compared to QFT-GIT and a lower IFN-γ release in TB1 compared to TB2 (22, 23).

In this study we did not include active tuberculosis patients since IGRAs are not developed for the diagnosis of active tuberculosis. Therefore, associations between active TB and a higher IFN-γ release in TB2 compared to TB1, as described in the majority of the previously published QFT-Plus studies, could not be studied. As mentioned, due to the absence of a gold standard sensitivity and specificity of QFT-Plus could not be assessed in a true matter. However, whether a real sensitivity and specificity could be obtained in the absence of a gold standard is debatable. Previous studies, performed with active tuberculosis patients as positive controls and low risk subjects used as negative controls, obtained a sensitivity of 89-90% and a specificity of 84-98% (22-25). This might indicate that the sensitivity and specificity remains approximately equal to QFT-GIT (90-94% and 82-99% respectively) (22-24).

In conclusion, the present study showed an equal performance of QFT-Plus compared to QFT-GIT in a large multicentre study with various test indications and different immune states. Although there is an indication that a higher IFN-γ release in TB2 compared to TB1 is associated with recent LTBI, the QFT-Plus could not be used to exclude recent exposure.

Acknowledgements

We would like to thank Qiagen for providing the QTF-plus kits.

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Chapter 3

Improving TB treatment efficacy

Chapter 3.1

Superior efficacy of a bedaquiline, delamanid and linezolid combination regimen in a mouse-TB model

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The Journal of Infectious Diseases, 2021; jiab043

ABSTRACT

Background: The treatment success rate of drug-resistant tuberculosis (DR-TB) is alarmingly low. Therefore, more effective and less complex regimens are urgently required.

Methods: We compared the efficacy of an all oral DR-TB drug regimen consisting of bedaquiline (25 mg/kg), delamanid (2.5 mg/kg) and linezolid (100 mg/kg) (BDL) on the mycobacterial load in the lungs and spleen of TB infected mice during a treatment period of 24 weeks. This treatment was compared to the standard regimen of isoniazid, rifampicin, pyrazinamide and ethambutol (HRZE). Relapse was assessed 12 weeks post-treatment. Two logistic regression models were developed to compare the efficacy of both regimens.

Results: Culture negativity in the lungs was achieved at 8 and 20 weeks of treatment with BDL and HRZE, respectively. After 14 weeks of treatment only one mouse relapsed in the BDL group, while in the HRZE group relapse was still observed at 24 weeks of treatment. Predictions from the final mathematical models showed that a 95% cure rate was reached after 20.5 and 28.5 weeks of treatment with BDL and HRZE, respectively.

Conclusion: The BDL regimen was observed to be more effective than HRZE and could be a valuable option for the treatment of DR-TB.

INTRODUCTION

It is estimated that in 2018 10 million people developed tuberculosis (TB), of which 500,000 contracted drug-resistant TB (DR-TB) (1). The treatment duration of DR-TB is at least 9 months, and frequently more than 1.5 years, requiring a combination of many different drugs. Even with this intensive therapy, the treatment success rate of DR-TB is only 54% in case of multi-drug resistant TB (MDR-TB) and 30% for extensively-drug resistant TB (XDR-TB) (1). Therefore shorter, less toxic and more effective regimens are required.

Despite the urgent need for new treatments, only three new TB-drugs have become commercially available in the last four decades. Bedaquiline is the first member of a new class of drugs called diarylquinolines. Its mechanism of action relies on blocking a proton pump of *Mycobacterium tuberculosis* that is required for ATP synthase, resulting in the loss of energy production and cell death (2). Delamanid and pretomanid belong to another novel class of drugs, the nitroimidazoles. Exposure to delamanid blocks methoxy-mycolic and keto-mycolic acid synthesis causing destabilization of the mycobacterial cell wall (3). In addition to these new TB-drugs, linezolid has recently been repurposed as a core second line agent for MDR-TB and belongs to the oxazolidinones, which inhibit protein synthesis (4).

Each of the drugs mentioned above possess different mechanisms of anti-mycobacterial action. Therefore, by combining the three orally administered drugs bedaquiline, delamanid and linezolid (BDL) we aimed to provide further evidence that this regimen can shorten DR-TB treatment duration. Experiments were performed in our previously validated mouse TB model using a drug-sensitive clinical *M. tuberculosis* strain (5, 6), and compared it with the standard regimen of isoniazid, rifampicin, pyrazinamide and ethambutol (HRZE). We used a drug-sensitive strain as no 'gold standard' DR-TB regimen has yet been defined, enabling the comparison of the performance of the BDL regimen to that of the classical TB drug regimen HRZE as well as to other studies in this field (7). The treatment-shortening potential of the BDL regimen is supported by a hollow fibre model study, indicating the synergistic activity of the combination of bedaquiline and linezolid against *M. tuberculosis* in various metabolic states (8). A previous *in vivo* study investigated the combination of bedaquiline, pretomanid and linezolid (BPaL) in a mouse TB-model, showing good efficacy (9). Subsequently, this particular combination was assessed in the Nix-TB trial, with promising results regarding efficacy and safety in patients with either MDR- or XDR-TB (10). Studying the efficacy of delamanid in this DR-TB drug regimen (as a replacement for pretomanid) is of interest as some mutations in the deazaflavin-dependent nitroreductase (ddn) enzyme are associated with resistance to pretomanid, but do not seem to affect susceptibility to delamanid (11). This is particularly relevant as such a mutation was already found to be present in a clinical *M. tuberculosis* Beijing strain

without the isolate ever being exposed to either pretomanid or delamanid (11). In this respect, delamanid could be a viable alternative for pretomanid in the BPaL regimen in cases of pretomanid resistance.

METHODS

Animals

Specified pathogen-free female BALB/c mice were obtained from Charles River (Les Oncins, France). Only female mice were used since some studies have shown different pharmacokinetic profiles between the sexes of mice (12). Animals were 13-15 weeks old at the start of the experiments, with experimental protocols adhering to the rules specified in the Dutch Animal Experimentation Act - concordant with the EU animal directive 2010/63/EU (license numbers 117-14-04 and AVD1010020173687).

Bacterial strain and TB-drugs

Experiments were performed using the drug-sensitive *M. tuberculosis* Beijing VN 2002-1585 genotype strain (6) with MICs (mg/L) of isoniazid 0.125, rifampicin 0.25, ethambutol 5 (13), bedaquiline 0.06, delamanid 0.015 and linezolid 0.25. MICs were determined according to Clinical and Laboratory Standards Institute standards (14). The antibiotic drugs used were prepared as previously described (supplement 1) (5, 15).

Experimental set-up

Using previously described power calculations (supplement 2) (5), a total of 378 mice - 210 for efficacy assessment and 168 for pharmacokinetic analysis - were needed. Mice were infected with *M. tuberculosis* suspensions as previously described (16). In short, a suspension of *M. tuberculosis*, stored at -80°C was defrosted at room temperature and centrifuged for 10 min at 14,000xg. The mycobacterial pellet was resuspended in phosphate-buffered saline (PBS) and centrifuged again for one minute at 1,900xg to eliminate any aggregated bacteria. The mycobacterial suspension was then diluted in PBS to obtain the intended bacterial load. Mice under anaesthesia were infected via intra-tracheal instillation with a suspension containing 1.2 (range 0.8-2.0) x 10^5 colony forming units (CFU) of *M. tuberculosis*, followed by inhalation to ensure the formation of a bilateral TB infection. Therapy was started two weeks after infection. Mice were checked daily, and were euthanized when humane endpoints (instability, dark eyes, decreased response to stimuli) were reached.

To assess the dose-response of monotherapy, mice were exposed to 0.5, 1 or 2 times the human pharmacokinetic equivalent doses (HED) of bedaquiline (i.e., 12.5, 25 or 50 mg/kg), delamanid (1.25, 2.5 and 5 mg/kg) or 0.25, 0.5 or 1 time the HED of linezolid (25, 50 and 100 mg/kg).

Mice intended for combination therapy were divided into two groups. The first group received a combination of 25 mg/kg bedaquiline, 2.5 mg/kg delamanid and 100 mg/kg linezolid (BDL). The second group received standard (HRZE) therapy i.e., 25 mg/kg isoniazid, 10 mg/kg rifampicin, 150 mg/kg pyrazinamide and 100 mg/kg ethambutol. All drugs were administered orally 5 times per week, using a feeding cannula and a total volume of 0.2 mL drug combination per day.

Pharmacokinetic analyses

For quantification of the drug concentrations two blood samples per mouse were taken via venous tail puncture. Samples were taken after 4 weeks of treatment and for a period of 24 hours. These 24 hour samples were taken in duplicate at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 hours post drug administration. Blood was collected in microcentrifuge tubes containing EDTA and centrifuged at 10,000*xg* for 5 minutes to obtain plasma, which was stored at -80°C. Methods for drug quantification by LC-MS/MS can be found in supplementary file 3.

PK/PD-parameters were determined using Prism 8 (GraphPad software, San Diego, CA, USA).

Mycobacterial load (efficacy) assessment

Mice receiving bedaquiline monotherapy were euthanized 0, 1, 2 or 4 weeks after start of treatment (n=3 per dosage, per time point). Mice receiving delamanid or linezolid monotherapy were euthanized at 0, 1, 2 or 3 weeks after the start of treatment (n=3 per dosage, per time point) since they reached humane endpoints before week 4 due to severe TB infection. After euthanasia the mycobacterial load was measured by CFU counting for assessment of early bactericidal activity (EBA). For CFU counts, the lungs and spleen were removed aseptically and homogenized, followed by serial dilution and culture. In order to prevent drug carry-over, therapy was stopped 72 hours before euthanasia of the mice, with samples being cultured on 7H10 Middlebrook agar containing activated charcoal.

Mice receiving combination therapy (total 90 mice) were euthanized at the start of treatment (n=3) and - to assess the CFU count immediately after treatment - after a treatment duration of 8, 12, 16, 20 or 24 weeks (n=3 per time point). In order to assess relapse, mice were also euthanized 12 weeks after treatment was stopped, after a treatment duration of 8, 10, 12, 14, 16, 18, 20, 22 or 24 weeks (n=3 per time point) as described previously (supplement 4) (5).

Statistical analysis

CFU counts per mL were log10 transformed before analysis and multiplied by 2.3 and 2.1 for lung and spleen, respectively. Group mean CFU counts were compared 3 or 4 weeks after the start of monotherapy (along with the control), using a one-way analysis of vari-

ance with a Dunnett's post-test to assess mono-activity. Differences in CFU count between the BDL and HRZE control groups were assessed at the start of treatment using unpaired two-sample *t*-test. The difference in cumulative time to relapse between both combination therapy groups was assessed using the log-rank test. The statistical significance level adopted was $p < 0.05$. Analyses were performed using Prism 8 (GraphPad software).

A logistic regression model was used to predict the treatment lengths required to reach an 85%, 90% and 95% cure rate in mice treated with either the BDL or the HRZE regimen. CFU counts were transformed into a binary outcome variable, describing 'relapse' or 'no relapse'. 'Relapse' was defined as a positive culture 12 weeks after discontinuation of treatment and 'no relapse' (i.e. cure) as a negative culture result. In order to describe the relationship between probability of relapse and treatment length, two logistic regression models, one for each regimen, were developed. During model development all parameters were estimated simultaneously using NONMEM software (version 7.4) (17). Model development is described in supplement 5.

In order to compare the efficacy of BDL and HRZE regimens, the cure rate at any treatment length, as well as the minimum treatment length needed to reach 85%, 90% and 95% cure, were predicted based on the final model for each regimen. Since the observed data consisted of n=3 mice per time-point we could only create cure rates of 0%, 33%, 67% and 100%, mathematical modelling was used to generate continuous cure rates between 0% and 100%. While in the logistic regression model the probability of relapse was modelled, the probability of no relapse, i.e. cure was used in the simulations.

Since the probability of cure is the complementary probability to probability of relapse, the probability of cure (P_{cure}) was obtained by subtracting the probability of relapse ($P_{relapse}$) from 1, as described in equation 1 in supplementary file 5. The proportion of cured mice was then simulated at each time-point. Predicted cure rates were simulated for treatment lengths between 0-30 weeks in 0.5 weeks increments for 1,000 mice per arm and time-point in order to achieve a high resolution in the predictions. Samples were randomly drawn from a uniform distribution using Monte Carlo sampling.

RESULTS

Pharmacokinetics analyses

Plasma concentration-time profiles and pharmacokinetic parameters of the BDL combination and the metabolites of bedaquiline and delamanid are shown in figure 1 and table 1.

Table 1. Results of the pharmacokinetic analysis (n=2 per time point). C_{max} is reported as the range of the maximum plasma concentration. The $AUC_{(0\text{-}24h)}$ is shown as mean with the standard error of the mean.

Drug (mg/kg)	T_{max} (hours)	C_{max} (ng/mL)	$AUC_{(0\text{-}24h)}$(ng/mL*h)
Bedaquiline (25)	1.00	1,920-1,310	19,661 (1,121)
***N*-desmethyl-Bedaquiline**	0.75	5,960-8,700	111,273 (6,312)
Delamanid (2.5)	0.75	864-1,080	11,234 (841.7)
DM-6705	0.25	18.9-23.7	352.4 (34.9)
Linezolid (100)	2.00	90,500*	251,269 (14,931)

**The drug concentration in only one mouse was assessed.*

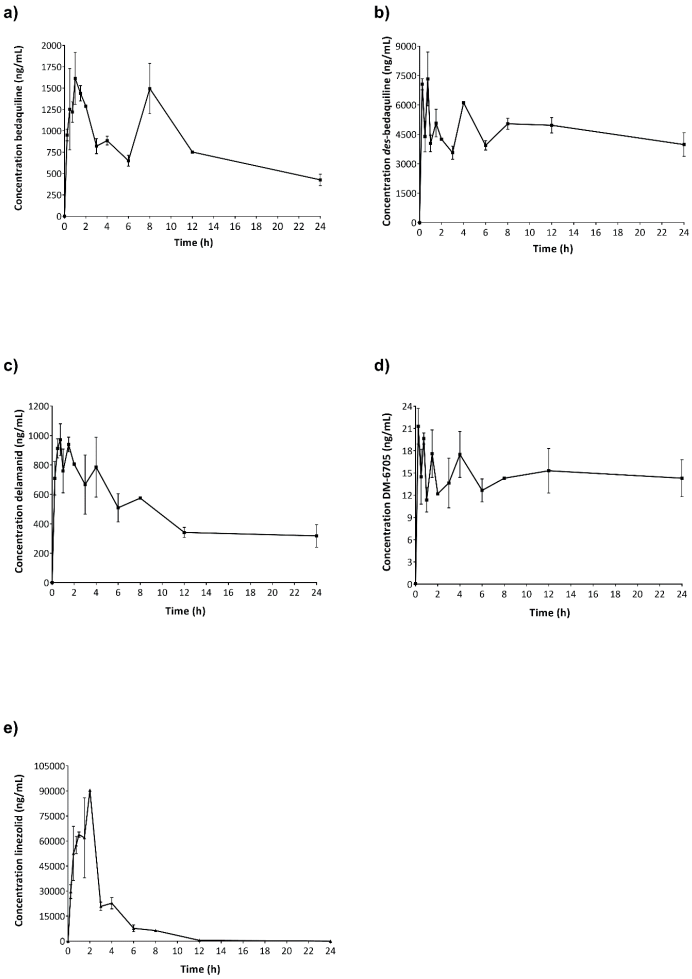

Figure 1. Mice were treated with 25 mg/kg bedaquiline, 2.5 mg/kg delamanid and 100 mg/kg linezolid 5 times per week. Bedaquiline (A), *N*-desmethyl bedaquiline (B), delamanid (C), DM-6705 (D) and linezolid (E) plasma concentration-time profiles after 4 weeks of BDL therapy in TB-infected BALB/c mice. Plasma concentrations are plotted as mean ± SEM of two mice per study drug per time point.

Pharmacodynamics analyses - Mycobacterial load assessment

During monotherapy, bedaquiline was well tolerated. However, mice treated with delamanid or linezolid showed stress during therapy and all mice receiving monotherapy of 25 mg/kg linezolid were euthanized before 3 weeks of treatment as humane endpoints were reached. The mycobacterial load in the lungs during 3 or 4 weeks of treatment with bedaquiline (a), delamanid (b) and linezolid (c) are presented in figure 2. The median total amount of mycobacteria in the lungs at the start of treatment with bedaquiline was 7.89 $\log_{10}$ CFU (range: 7.87-7.92), which declined significantly (p <0.01) after 4 weeks of treatment to 4.96, 4.55 and 5.28 $\log_{10}$ CFU, at 0.5x, 1x and 2x HED, respectively. Delamanid significantly reduced the CFU count in the lungs of mice after 3 weeks of treatment and at all doses tested (approximately 2 $\log_{10}$ CFU (p <0.001) were observed for all doses). Linezolid did not have a significant effect on the CFU count after 3 weeks of treatment (p =0.633). Results in the spleen were comparable for all drugs (data not shown).

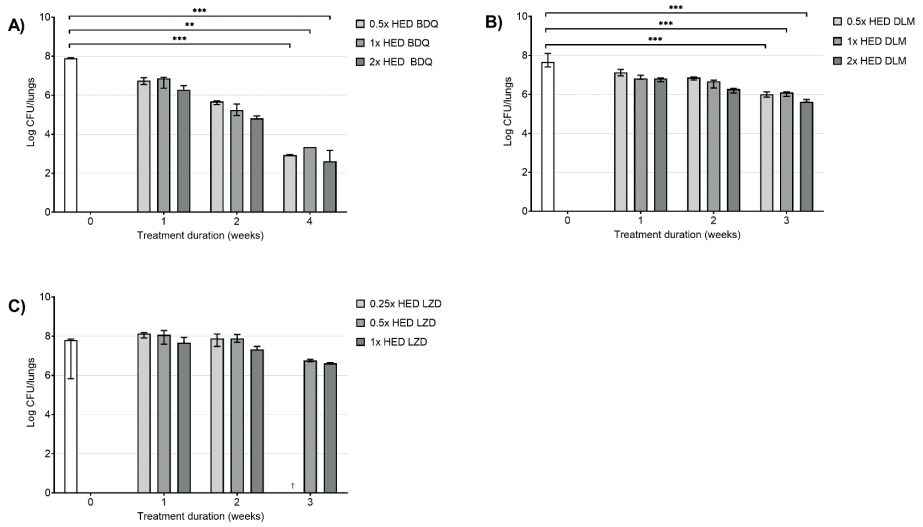

Figure 2. Mycobacterial load in lungs expressed as median ± range (error bars) of the colony forming units (CFU) (n=3), at week 0, 1, 2 and 3 or 4 weeks post treatment with bedaquiline (A), delamanid (B) or linezolid (C). The white bar represents the control mice, the bars from light to dark grey represent mice treated with 0.5, 1 and 2x HED, respectively. ** p < 0.01, *** p < 0.001 (one-way ANOVA). † 3 mice were euthanized before planned as humane endpoints had been reached. Results of one mouse treated with 0.5x HED, two mice treated with 1x HED and one mouse treated with 2x HED bedaquiline are missing in week 4, since no undiluted samples were analyzed and 10 times diluted samples of lungs and spleen did not show any CFUs.

Both BDL and HRZE combination treatments were well-tolerated. The mycobacterial load in lungs and spleen are presented in figure 3. The median total amount of mycobacteria in the lungs at start of BDL treatment was 7.80 $\log_{10}$ CFU (range: 7.66-8.07) and 8.03 $\log_{10}$ CFU (range: 7.89-8.22) at start of HRZE treatment (p =0.256). At 8 weeks

of treatment with BDL culture negativity in the lungs and spleen was achieved. As of 16 weeks of treatment, no relapse in the BDL group was observed, with the exception of one mouse in week 20. In HRZE treated mice, culture negativity in the lungs was achieved after 20 weeks of treatment. However, mice in this HRZE treatment arm relapsed (even) after 24 weeks of treatment. Results for the spleen showed a similar pattern. There were significantly fewer relapse episodes observed in the BDL group compared to the HRZE group (p <0.001) (Figure 4).

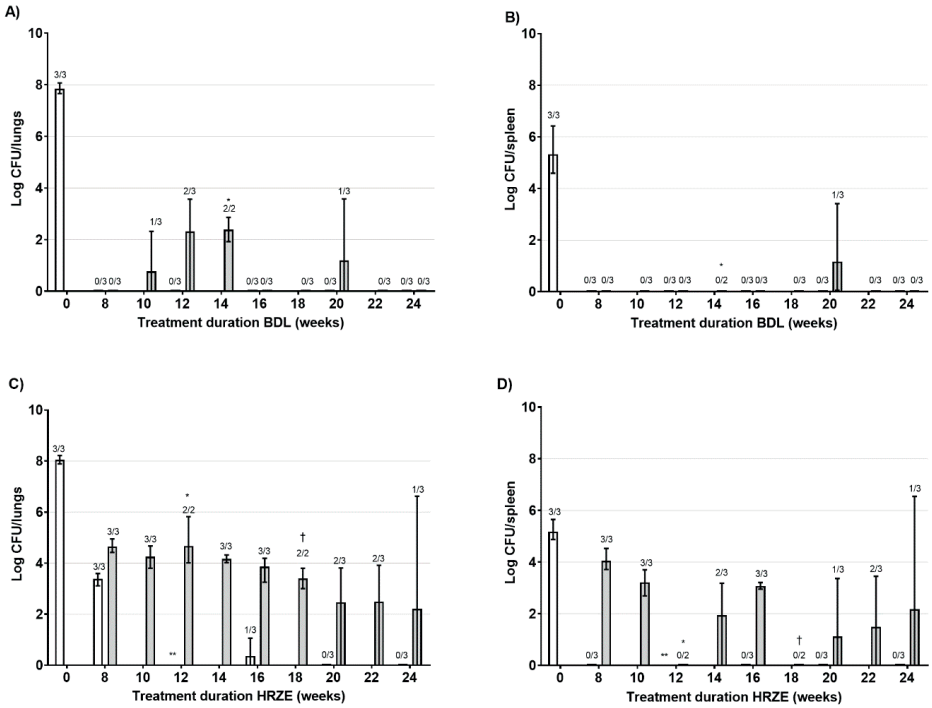

Figure 3. Mycobacterial load in lungs (A, C) and spleen (B, D) expressed as median ± range (error bars) of the colony forming units (CFU) per organ. Results at weeks 8, 12, 16, 20 and 24 of treatment are expressed as white bars and relapse assessment was performed 12 weeks after a treatment duration of 8, 10, 12, 14, 16, 18, 20, 22 and 24 weeks and expressed as grey bars. Mice were treated with BDL (A, B) or HRZE (C, D). Numbers above bars are the numbers of culture-positive mice out of total numbers of mice at that time point. * CFU counting was not performed since the culture plates were contaminated. † 1 mouse reached humane endpoints before planned euthanasia.

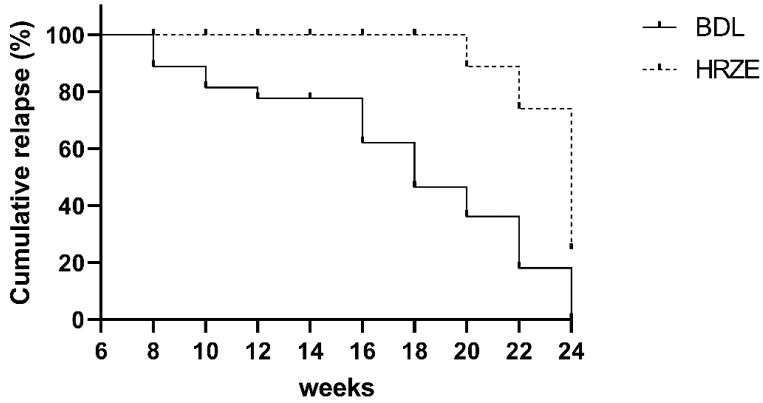

Figure 4. Cumulative relapse of mice treated with BDL or HRZE between 8 and 24 weeks. The solid line represents the BDL group and the dotted line the HRZE group.

Pharmacodynamics analyses - Modeling

For the BDL regimen, none of the assessed functions that related treatment length and probability of relapse (linear, E_{max} and sigmoidal E_{max} function (supplementary file 6)) led to a significant improvement in model fit compared to the base model (p <0.05). Therefore, a 'surge' function was evaluated (in addition to the already assessed functions) as graphical analysis revealed an initial increase, followed by a decrease, in the probability of relapse as a function of treatment length (figure 3c). This use of a 'surge' function resulted in a statistically significant improvement in model fit compared to the base model (p <0.001) and was consequently retained in the final model relating length of BDL treatment with the probability of relapse. For the HRZE regimen, the probability of relapse in relation to different treatment lengths was best described by a sigmoidal E_{max} relationship. The model parameter estimates and parameter uncertainties are described in supplementary file 6.

The required treatment length of the BDL regimen to reach cure rates of 85%, 90% and 95%, was predicted to be 17.5, 18.5 and 20.5 weeks, respectively. For the HRZE regimen, simulations of the predicted cure rates at different treatment lengths showed that a 95% cure rate was reached after 28.5 weeks of treatment and for cure rates of 85% and 90%, 26.0 and 27.0 weeks, respectively. A 95% cure rate was thus predicted to be reached 8 weeks faster in mice treated with the BDL regimen compared to the HRZE regimen, which corresponds to a 28.1% decrease in treatment length. The predicted cure rates at different treatment lengths for both BDL and HRZE regimens are shown in table 2 and figure 5.

Table 2. Comparison of model-predicted minimal treatment lengths required to reach 85%, 90% and 95% cure rates for both regimens. B = 25 mg/kg bedaquiline, D = 2.5 mg/kg delamanid and L = 100 mg/kg linezolid; H = 25 mg/kg isoniazid; R = 10 mg/kg rifampicin; Z = 150 mg/kg pyrazinamide; E = 100 mg/kg ethambutol.

	Minimal treatment length required to achieve a cure rate of…(weeks)		
Regimen	85%	90%	95%
BDL	17.5	18.5	20.5
HRZE	26.0	27.0	28.5

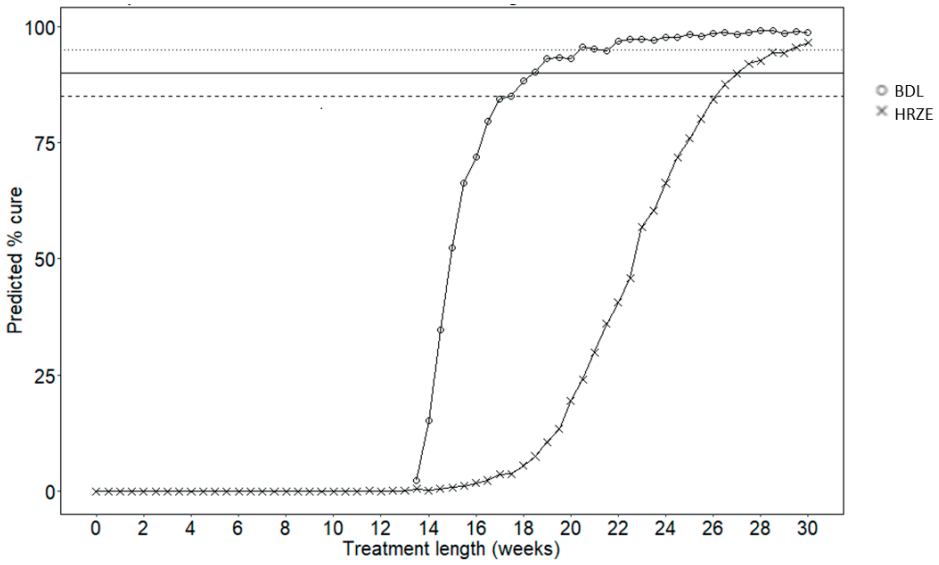

Figure 5. Model predicted cure rates at different treatment lengths for both BDL and HRZE regimens. The dashed line represents 85%, the solid line 90% and the dotted line 95% cure rate. H = 25 mg/kg isoniazid; R = 10 mg/kg rifampicin; Z = 150 mg/kg pyrazinamide; E = 100 mg/kg ethambutol; B = 25 mg/kg bedaquiline, D = 2.5 mg/kg delamanid and L = 100 mg/kg linezolid.

DISCUSSION

The current study indicated a higher efficacy for the BDL combination treatment regimen, as compared to the standard HRZE regimen, when using a mouse TB-model and the clinically relevant *M. tuberculosis* Beijing genotype strain (6). In the early phase of treatment, efficacy appeared to be mainly bedaquiline driven, as illustrated by the EBA results obtained using the three drugs separately as monotherapy. The observed dose dependent activity of bedaquiline was comparable to other murine studies (18, 19). Delamanid showed only minor bactericidal activity and linezolid no bactericidal activity in the first three weeks of monotherapy and mice were consequently euthanized one week before schedule. The limited EBA of delamanid is in line with other published murine studies. For example, one study showed a 2.5 $\log_{10}$ CFU/mL decrease after 28 days of treatment

with a dose of 2.5 mg/kg delamanid (3) and in another study a dose of 100 mg/kg (40 times as much as in our study) reduced the CFU count in the lungs by 1 $\log_{10}$ after 4 weeks of treatment (20). In line with the present study, linezolid showed no bactericidal activity in previous murine studies (21).

Our results indicated that a minimal treatment length of 20.5 weeks was predicted to be required in order to reach a 95% cure rate using the BDL regimen. This was 8 weeks shorter than the HRZE regimen. Interestingly, two other murine studies assessed the efficacy of the combination of bedaquiline, pretomanid and linezolid (BPaL), showing good results for this combination regimen (9, 22). Xu *et al.* showed that the addition of pretomanid increased the bactericidal activity of the bedaquiline-linezolid combination and prevented the emergence of bedaquiline resistance (22). Although the individual contribution of delamanid to the total efficacy of the BDL regimen was not assessed, it could be speculated that the same effect holds true for the contribution of delamanid in the current combination regimen. Further, the additional value of delamanid is supported by a study in MDR-TB patients showing increased sputum conversion when delamanid was added to a backbone treatment regimen (23). Moreover, the combination of bedaquiline and delamanid also appears to be promising in TB-patients. In studies reviewing the activity of these drugs in combination, 84% sputum conversion after 4 months of treatment (24) and 88% culture negativity after 24 weeks of treatment was observed when both drugs were added to a backbone regimen (92% of these patients also received linezolid) (25). Therefore, it is reasonable to assume that the BDL combination may also be a potent TB-drug regimen for patients, which would be in line with the first results of the Nix-TB trial studying the efficacy of BPaL in patients with MDR- and XDR-TB (10).

As such, delamanid seems to be a reasonable alternative to pretomanid in specific situations such as pretomanid resistance(11, 26). Other murine studies showed robust efficacy of the combination of bedaquiline and pretomanid combined with moxifloxacin and pyrazinamide (BPaMZ) with a relapse free survival of only 2 months of treatment (22, 27). Given the results of our present study, it would be interesting to study whether the substitution of pretomanid for delamanid in the BPaMZ regimen results in similar efficacy.

In the present study, the EBA of linezolid as monotherapy was low, prompting questions about the contribution of this drug to the total efficacy of the BDL regimen. However, Tasneen *et al.* revealed the treatment shortening potential of linezolid in the BPaL regimen by showing that the addition of linezolid to the combination of bedaquiline and pretomanid resulted in no relapse in mice after 3 months of treatment, while mice treated with only bedaquiline and pretomanid relapsed after 4 months of treatment (9). These findings further strengthen the hypothesis that EBA should not be solely used as a guide for regimen efficacy (28). In addition, the efficacy of linezolid was assessed in patients with XDR-TB. The treatment success rate in the linezolid therapy group was

significantly higher (70%) compared to the non-linezolid group (34%) (29). Therefore, despite the disappointing performance of linezolid in terms of EBA, it appears to be a valuable component of the drug combination studied in this publication.

Our pharmacokinetic results for bedaquiline, delamanid and linezolid fell within the range of those reported in previous murine TB studies(3, 30-32) and were also comparable to human pharmacokinetics (23, 33-35). Our bedaquiline concentration versus time curve showed a remarkable second peak after 8 hours of treatment. This second peak has not been observed in other murine studies (3, 30), although it has been reported in a human pharmacokinetics study (36). In this clinical study the double peak was explained by better absorption after food intake, which has also been shown in another study for rifapentine (37).

We also assessed the concentration versus time curves of the major metabolites of bedaquiline and delamanid, *N*-monodesmethyl bedaquiline (M2) and DM-6705 (M1), respectively. In humans, it is assumed that M2 does not have a significant effect on the total efficacy of bedaquiline since its exposure is 4-5 times lower, and the antimicrobial activity is 3-6 fold lower, compared to the parent compound (19). However, Rouan *et al.* showed that the exposure of M2 in mice (in terms of AUC) was three times higher compared to bedaquiline (19) and in our study it was actually five times higher. Since the AUC is the driver of efficacy for bedaquiline, and M2 plus bedaquiline has an additive effect, the efficacy of bedaquiline in mice might be overestimated when translated to humans. However, this effect is assumed to be limited due to the lower antimicrobial activity of M2 (38). In our study the exposure of M1 was 32 times lower compared to delamanid, which is in line with the study of Sasahara *et al.* (32). Their study showed that, after repeated drug administration, metabolite exposure in humans was much higher compared to mice and rats. However, in our study, we reasoned that this finding did not lead to an underestimation of the effect of delamanid, since antimicrobial activity of the three major metabolites of delamanid (DM-6704, 6705 and 6706) was previously reported to be poor, with MICs ranging from 6.25 to 50 mg/L (39).

A limitation of this study was that it was powered on three mice every four weeks for CFU assessment immediately after treatment and three mice every two weeks to asses relapse, as based on previous modelling experience (5). Unexpectedly, we observed zero relapse after week 8, while in the following 6 weeks the proportion of relapsing mice increased. Subsequently after 14 weeks of treatment no mice relapsed, with the exception of one mouse in week 20. Although this could be a sampling artefact, this event resulted in an incomplete fit of the BDL regimen to the sigmoidal E_{max} model. This issue was overcome by using a Surge model, which enabled the assessment of expected treatment duration of the BDL regimen in relation to HRZE. Another limitation was the use of a drug sensitive strain for the assessment of this DR-TB regimen. However, many previous studies have also utilized drug sensitive TB strains to model DR-TB treatment regimens(9,

22, 27) and are now evaluated in clinical trials, and some of them already showing a good translation to clinical practice (10).

In conclusion, BDL seems to be a promising combination for the treatment of DR-TB. Furthermore, since all three drugs are commercially available, this combination could be readily implemented in clinical practice after assessment in clinical studies and might be a good alternative for BPaL when pretomanid is not available for inclusion in combined TB treatment regimens.

Acknowledgements

The authors thank John Hays for his writing assistance and Gerjo de Knegt, Marian ten Kate and Heleen van der Spek for their technical assistance. Research was conducted on behalf of the PreDiCT-TB Consortium (http://predict-tb.eu).

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SUPPLEMENT 1.

Isoniazid (Erasmus MC Hospital Pharmacy, Rotterdam, The Netherlands), rifampicin (Rifadin®; Aventis Pharma B.V., Hoevelaken, The Netherlands), pyrazinamide and ethambutol (both purchased from Sigma Chemical Co., St. Louis, MO, USA) were dissolved in distilled water. Bedaquiline was kindly provided by Janssen Pharmaceuticals (Johnson and Johnson, Machelen, Belgium) and dissolved in 20% cyclodextrine (Sigma Chemical Co) and distilled water and adjusted to a pH of 2.5. Delamanid was kindly provided by Otsuka Pharmaceutical Development & Commercialization (Rockville, USA) and linezolid was kindly provided by Janssen Pharmaceuticals. Both were dissolved in 20% cyclodextrine (Kleptose, Roquette NL BV, Hoofddorp, the Netherlands) and adjusted to a pH of 2.5. The combination of bedaquiline, delamanid and linezolid was dissolved in 20% cylcodextrine and pH adjusted to 2.5.

SUPPLEMENT 2.

This is a complete citation of the text in Mourik et al.(5).
"Statistical power analysis

Aim

To determine the appropriate sample size in order to detect a statistically significant difference in potency between different treatment regimens.

Methods

The statistical power to detect a statistically significant difference, at the 5% significance level, in potency of 50% between different treatments were conducted at different sample sizes (n=2-5 animals per time point). Included time points were 2 to 6 months with 0.5 month intervals (9 time-points in total). The power calculations assumed a sigmoidal E_{max} model with p_{base} of 1, E_{max} of 1, γ of 10 and a T_{50} of 2 months for treatment A (T_{50},A) and T_{50} of 4 months for treatment B (T_{50},B). One thousand simulations were performed at each sample size. The simulated data were re-estimated with one true model which included a difference in T_{50} between arms and an alternative model with no difference in T_{50}. The proportion of re-estimated models where the true model (including difference between treatments) was significantly better than the alternative model (without difference between treatments) was the power at each sample size. In addition, the precision in the estimated parameters for the true model were also summarized. The estimated parameters were γ, T_{50},A and T_{50},B. E_{max} and p_{base} were both fixed to 1.

Results

The power to detect a significant difference in potency was: 87.9, 100, 100 and 100% for sample size of 2, 3, 4 and 5, mice per time point, respectively. This indicates that a sample size of 3 or more mice is sufficient to achieve 100% power to detect a statistically significant difference in potency between different treatments. The parameter precisions for n=3 expressed as relative standard error were 13.0, 0.8 and 0.2 % for γ, T_{50},A and T_{50},B, respectively which indicates that the expected parameter precision is reasonably high at n=3.

Conclusion

A sample size of n=3 mice per time point is sufficient to detect a 50% difference in potency between different treatments and also gave reasonably high precision in model parameters (i.e. low parameter uncertainty)."

SUPPLEMENT 3.

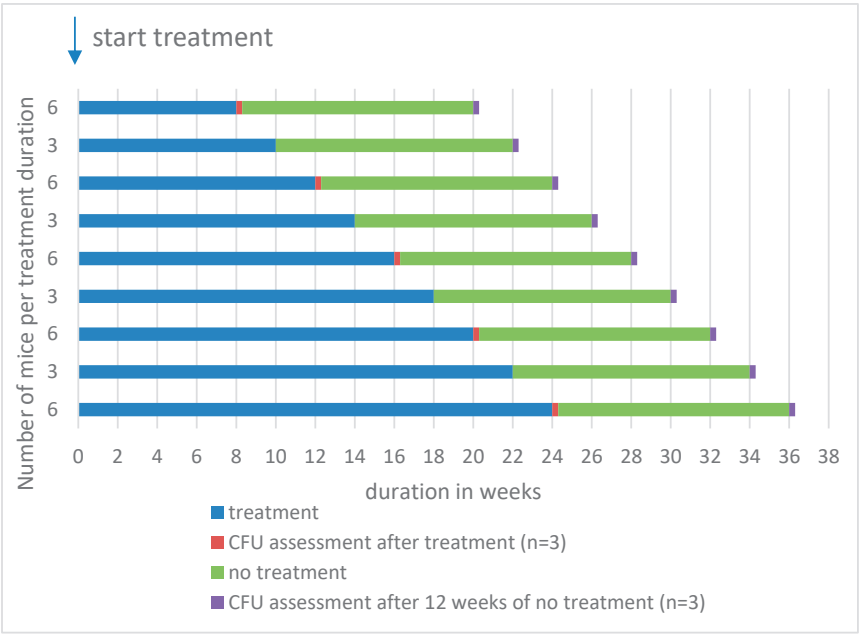
LC-MS/MS analysis was performed on a Sciex Applied Biosystems Qtrap 6500+ triple-quadrupole mass spectrometer coupled to an Shimadzu Nexera 2 HPLC system to quantify each drug in plasma. Delamanid and metabolites were quantified as published previously (40). Bedaquiline standard was received from Chemshuttle. *N*-monodesmethyl bedaquiline was purchased from Clearsynth. Linezolid was purchased from Sigma-Aldrich. Neat 5 mg/mL DMSO stocks for bedaquiline, *N*-monodesmethyl bedaquiline, and linezolid were serial diluted in 50/50 acetonitrile/water to create standard curves and quality control (QC) spiking solutions. Standards and QCs were created by adding 10 µL of spiking solutions to 90 µL of drug free plasma (CD-1 K2EDTA Mouse, Bioreclamation IVT). 20 µL of control, standard, QC, or study sample were added to 200 µL of acetonitrile/methanol 50/50 protein precipitation solvent containing internal standards (10 ng/mL BDQ-d6 and 100 ng/mL LIN-d8). Extracts were vortexed for 5 minutes and centrifuged at 4000 rpm for 5 minutes. 100 µL of supernatant was transferred for HPLC-MS/MS analysis and diluted with 100 µL of Milli-Q deionized water.

Bedaquiline, *N*-monodesmethyl bedaquiline, and linezolid chromatography was performed on an Agilent Zorbax SB-C8 column (2.1x30 mm; particle size, 3.5µm) using a reverse phase gradient. Milli-Q deionized water with 0.1% formic acid was used for the aqueous mobile phase and 0.1% formic acid in acetonitrile for the organic mobile phase. Multiple-reaction monitoring of parent/daughter transitions in electrospray positive-ionization mode was used to quantify the analytes. The following MRM transitions were used for bedaquiline (555.20/58.20), *N*-monodesmethyl bedaquiline (541.30/480.30), linezolid (338.00/235.00), BDQ-d6 (561.26/64.20), and LZD-d8 (346.15/304.20).

Sample analysis was accepted if the concentrations of the quality control samples were within 20% of the nominal concentration. Data processing was performed using Analyst software (version 1.6.2; Applied Biosystems Sciex).

SUPPLEMENT 4.

Study design combination therapy week 0-36, per regimen (BDL/HRZE).



SUPPLEMENT 5.

In a first step, the probability of relapse independent of treatment length was estimated for each regimen. This probability has been defined in equation 1 as base probability of relapse (P_{base}) and can take any value between 0 and 1. In equation 1, $P_{relapse}$ and P_{cure} are the probabilities of relapse and no relapse, i.e. cure.

$$P_{base} = P_{relapse} = 1\text{-}P_{cure} \qquad \textit{(Eq. 1)}$$

To assess the effect of treatment length on the probability of relapse, the following modelling strategy was applied to both regimens separately. First, a function describing the probability of relapse linearly decreasing with treatment length was applied to the data for

each regimen, as defined in equation 2, where *Slope* is the linear increase in probability of cure with treatment length and *T* is the treatment length in weeks.

$$P_{relapse} = 1\text{-}P_{Cure} = P_{base}*(1\text{-}Slope*T) \quad \textit{(Eq. 2)}$$

Next, an E_{max} function, relating the probability of relapse in a non-linear manner to treatment length, was tested (equation 3).

$$P_{relapse} = 1\text{-}P_{Cure} = P_{base}*(1\text{-}\frac{E_{max}*T}{T_{50}+T}) \quad \textit{(Eq. 3)}$$

In equation 3, *T* represents the treatment length in weeks, E_{max} the maximum reachable probability of cure, and T_{50} the time in weeks where the probability of cure is half-maximal.

In addition, a sigmoidal E_{max} model was evaluated (equation 4).

$$P_{relapse} = 1\text{-}P_{Cure} = P_{base}*(1\text{-}\frac{E_{max}*T^{\gamma}}{T_{50}^{\gamma}+T^{\gamma}}) \quad \textit{(Eq.4)}$$

In the sigmoidal E_{max} relationship the Hill factor γ describes the steepness of the E_{max} relationship at the T_{50} value.

Based on the results of the model evaluation and investigation of the raw data, we concluded that none of the above described relationships were appropriate to describe the probability of relapse dependent on treatment length for the BDL regimen. Therefore, a surge function describing the relationship between probability of relapse and treatment length was tested in addition for the BDL regimen (Equation 5).

$$P_{relapse} = \frac{SA}{(\frac{T\text{-}PT}{SW})^{\gamma}+1} \quad \textit{(Eq. 5)}$$

The surge function includes the surge amplitude (*SA*), which defines the maximum probability of relapse, the treatment lengths in weeks (*T*), the peak time (*PT*), which describes the time at which the maximum probability of relapse occurs in weeks, the surge width (*SW*), which defines half of the surge width in weeks, and a shape parameter (γ), that can take on even numbers.

During model development the likelihood of a model to fit, i.e. describe, the observed data was evaluated and compared between different applied models. The -2log likelihood for a model given the observed data, called objective function value (OFV), was compared to a reduced model using the likelihood ratio test (LRT). A statistically significant improvement, at the 5% significance level, of the model fit using a full model was indicated by a drop in OFV by at least 3.84 points for one degree of freedom, corresponding to the χ^2 – distribution. In addition, all models were evaluated based on parameter uncertainties as well as visual predictive checks (VPCs). In a VPC, the observed data, here the propor-

tion of mice that relapsed 12 weeks after stop of treatment, was graphically compared to data simulated from the model.

The model building process and parameter estimation, as well as all simulations were conducted in NONMEM 7.43 (ICON plc, Gaithersburg, MD, USA)(17). Parameter estimation was performed using the conditional likelihood estimation method. Data management and visualization were carried out in R statistical software version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria)(41). Visual predictive checks (VPCs) were created using PsN 4.9.1 (Department of Pharmaceutical Biosciences, Uppsala University, Uppsala, Sweden) and Xpose4(42).

Table E1. Final logistic regression model parameter estimates for the probability of relapse. H = 25 mg/kg isoniazid; R = 10 mg/kg rifampicin; Z = 150 mg/kg pyrazinamide; E = 100 mg/kg ethambutol; B = 25 mg/kg bedaquiline, D = 2.5 mg/kg delamanid and L = 100 mg/kg linezolid; CV = coefficient of variance

Regimen	Parameter	Description	Parameter estimate	Standard error (% CV)
HRZE	P_{base}	Baseline probability of relapse	1 FIX	-
	E_{max}	Maximum reachable probability of cure	1 FIX	-
	T_{50} (weeks)	Time at which E_{max} is half-maximal	22.6	6.4
	γ	Hill-factor	12.08	44.5
BDL	N	Shape parameter	2 FIX	-
	SA	Surge amplitude, defines maximum probability of relapse	1 FIX	-
	PT (weeks)	Peak time, time at which maximum probability of relapse occurs	13.28	3.7
	SW (weeks)	Surge width, half of surge width	1.68	27.4

SUPPLEMENT 6.

The probability of relapse in relation to different treatment lengths for the HRZE regimen was best described by a sigmoidal E_{max} relationship. The model relating treatment length linearly to the base probability of relapse did not show a significant effect (p <0.05) of treatment with the HRZE regimen on relapse rate compared to the base model (drop in OFV = 3.16 for one degree of freedom), and was thus rejected. Also an E_{max} relationship did not describe the data significantly better (p <0.05) compared to the base model with a decrease in OFV of 2.80 for one degree of freedom. A sigmoidal E_{max} relationship however was able to describe the data statistically significantly (p <0.001) better than the base model. Compared to the base model without the effect of treatment length, the OFV dropped by 10.04 for two degrees of freedom when relating treatment length to probability of relapse in a sigmoidal E_{max} relationship. Therefore, the sigmoidal E_{max} model was

kept as the final model for the HRZE regimen. Due to the very steep relationship between treatment length and probability of relapse and the consequently high estimated Hill factor of 12.08, a drug effect could only be described with the sigmoidal E_{max} relationship. Since the base probability of relapse was estimated to 0.99 and therefore close to 1 it was fixed to 1, which allowed the parameter uncertainties to be obtained. The final model parameter estimates and parameter uncertainties are provided in supplementary table E1.

For the BDL regimen, none of the above described functions relating treatment length and probability of relapse led to a significant (p <0.05) improvement in model fit compared to the base model. Therefore, a surge function was evaluated in addition since the graphical analysis revealed an initial increase in probability of relapse followed by a decrease in probability of relapse as a function to treatment length (figure 3A). Due to numerical instability, the maximum probability of relapse (SA) was fixed to 1, since it was estimated very close to 1. The parameter N which describes the steepness of the surge function can take on even numbers. Different values for N (0, 2, 4, 6, 8, 10, 12, 14) were evaluated and the value leading to the lowest OFV was chosen. Compared to the base model, the OFV dropped by 10.00 points, which is statistically significant (p <0.001) for two degrees of freedom. The surge function was thus kept as final model relating length of treatment with the BDL regimen to probability of relapse.

VPCs of both final models are presented in Supplementary figure E1. As shown by the graphs, the observed proportion of relapse lies within the 95% confidence interval of the simulated data based on 1000 simulation datasets.

SUPPLEMENTARY FIGURES

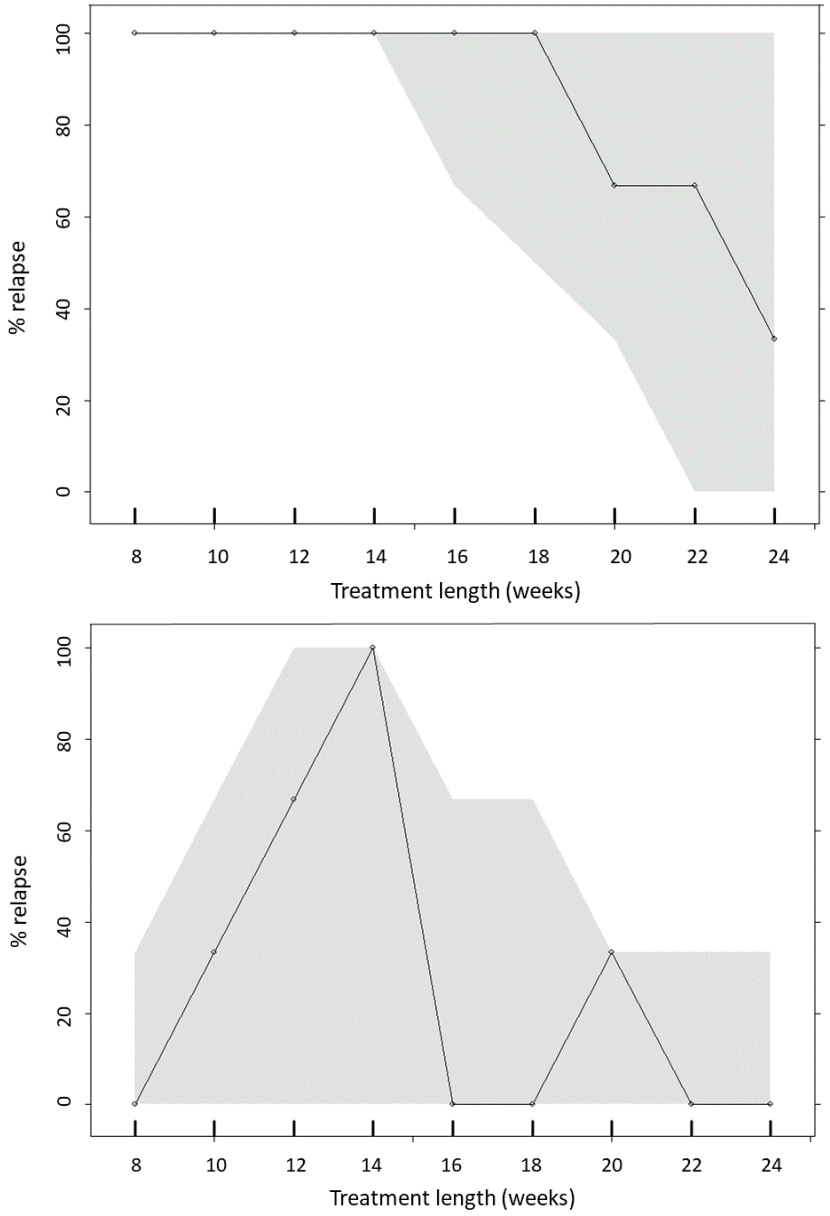


Figure E1. Visual predictive checks (VPCs) of the final models. The open circles connected with the solid line represent the observed proportion of relapse and the shaded area is the 95% confidence interval of the model-predicted relapse rates at different treatment lengths. Panel a) represents the HRZE regimen: H = 25 mg/kg isoniazid; R = 10 mg/kg rifampicin; Z = 150 mg/kg pyrazinamide; E = 100 mg/kg ethambutol. Panel b) represents the BDL regimen: B = 25 mg/kg bedaquiline, D = 2.5 mg/kg delamanid and L = 100 mg/kg linezolid.

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Chapter 3.2

Assessment of the additional value of verapamil to a moxifloxacin and linezolid combination regimen in a murine tuberculosis model.

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Antimicrob Agents Chemother. 2018 Aug 27;62(9):e01354-18

ABSTRACT

Background: Favourable treatment outcome of multidrug resistant tuberculosis (TB) is only 54% and therefore new drug regimens are urgently needed. In this study, we evaluated the additional value of the efflux-pump inhibitor verapamil to a backbone of moxifloxacin and linezolid in a murine TB model.

Methods: BALB/c mice infected with Mycobacterium tuberculosis were treated with human-equivalent doses of moxifloxacin (200 mg/kg) and linezolid (100 mg/kg) with or without verapamil (12.5 mg/kg) for 12 weeks. Pharmacokinetic parameters were collected during treatment at steady state.

Results: After 12 weeks of treatment, the mycobacterial load in the lungs declined 5.0 Log colony forming units (CFU) without verapamil and 5.9 Log CFU with verapamil ($p<0.001$) but sterilization was not achieved yet and the effect on relapse rates could therefore not be evaluated. The spleens of all mice were culture-negative after 12 weeks of treatment with both treatment modalities and addition of verapamil caused a significant reduction in relapse (14/14 positive spleens without versus 9/15 with verapamil, $p=0.017$).

Conclusion: The addition of verapamil did not significantly increase the effectiveness of a regimen consisting of moxifloxacin and linezolid in our mouse TB model, but data on relapse warrant follow-up studies with a longer treatment duration.

INTRODUCTION

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 rifampicin resistant or multidrug resistant (MDR) TB (1). Currently patients with MDR-TB need to undergo up to two years of treatment with toxic drugs, while only 54 percent are cured (1). Novel treatment regimens that are both more effective, short and safe need to be developed to treat this devastating disease.

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

In the current WHO treatment guideline, linezolid (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 and extensively drug resistant (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 six month treatment for XDR-TB (Nix-TB) trial(11) and a phase 3 study evaluating various doses and treatment durations of linezolid, bedaquiline and pretomanid in multi-drug resistant (MDR)- and XDR-TB patients (ZeNix trial) (12).

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 as well as oxazolidinones (13, 14). 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 (15).

Verapamil was the first discovered inhibitor of P-glycoprotein mediated drug efflux (16) and showed to be able to increase the accumulation of P-glycoprotein substrate drugs in macrophages (17). Another study showed that macrophage-induced tolerance of moxifloxacin could be reversed upon exposure to verapamil (13). Furthermore, verapamil does not only affect macrophage efflux pumps, but has also been identified as an inhibitor of *M. tuberculosis* efflux systems (18-21).

In the present study, we evaluated the additional value 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 + linezolid (group 1) and moxifloxacin + linezolid + verapamil (group 2) combination treatments were well tolerated, although mice showed mild distress during the first three weeks of treatment in both groups.

In figure 1, the mycobacterial load in lungs and spleen during 12 weeks of treatment and after a 12 weeks post-treatment period is presented. The median total amount of mycobacteria in the lungs at start of treatment was 8.18 Log CFU (IQR: 7.96 - 8.19). During the 12 weeks of treatment the mycobacterial load in the 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 start treatment. No significant difference between group 1 and 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 treatment. The addition of verapamil was associated with a significant reduction in relapse after three 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 figure 2 and table 1.

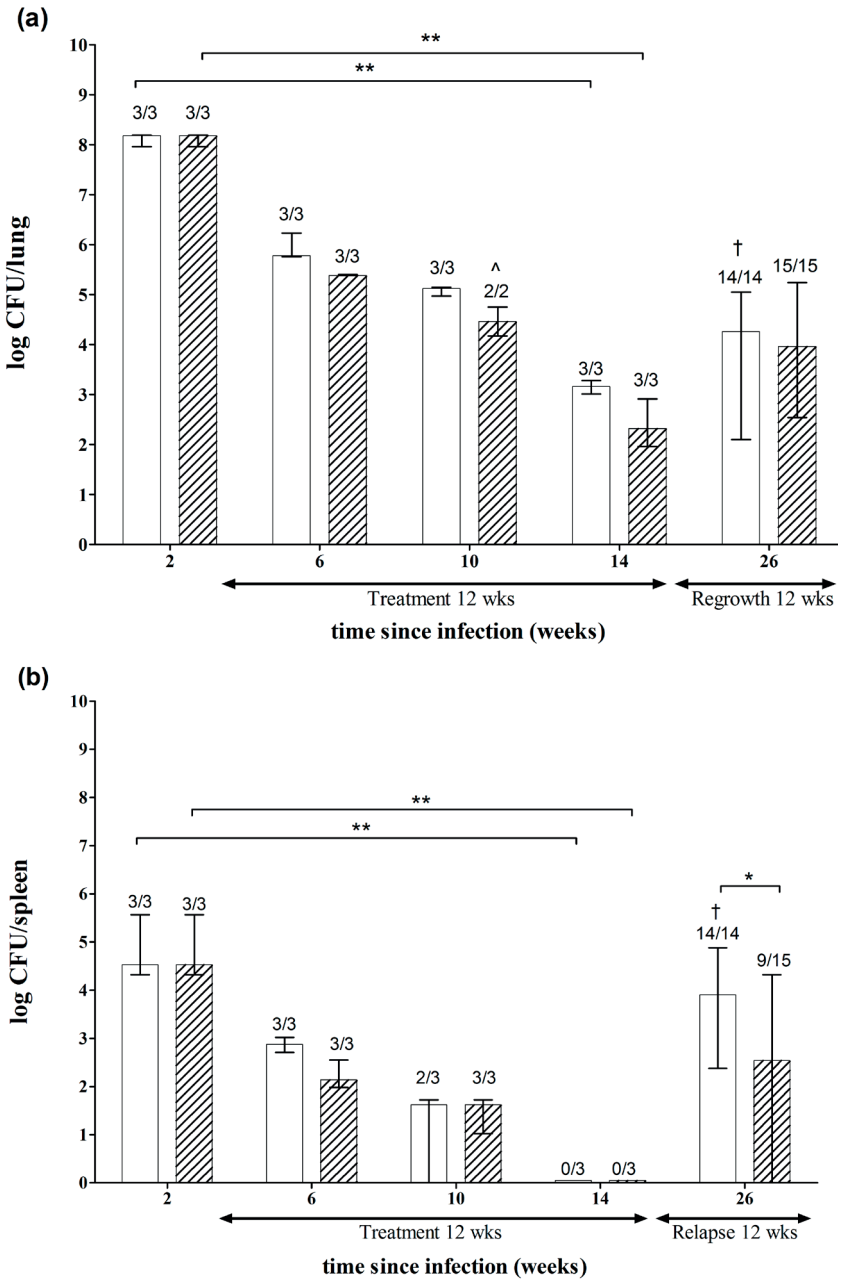

Figure 1. Mycobacterial load in lung (a) and spleen (b) expressed as median ± range (error bars) of the colony forming units per organ, at week 2, 6, 10 and 14 and at 12 weeks post treatment (week 26). Grey bars are mice treated with moxifloxacin and linezolid, striped bars are mice treated with moxifloxacin, linezolid and verapamil. Numbers above bars are the numbers of culture-positive mice out of total numbers of mice at that time point. Significance is noted as (**) p <0.0001 and (*) p < 0.05. † 1 mice became moribund before planned dissection.
CFU, colony forming units; LZD, linezolid; MXF, moxifloxacin; VPM, verapamil.

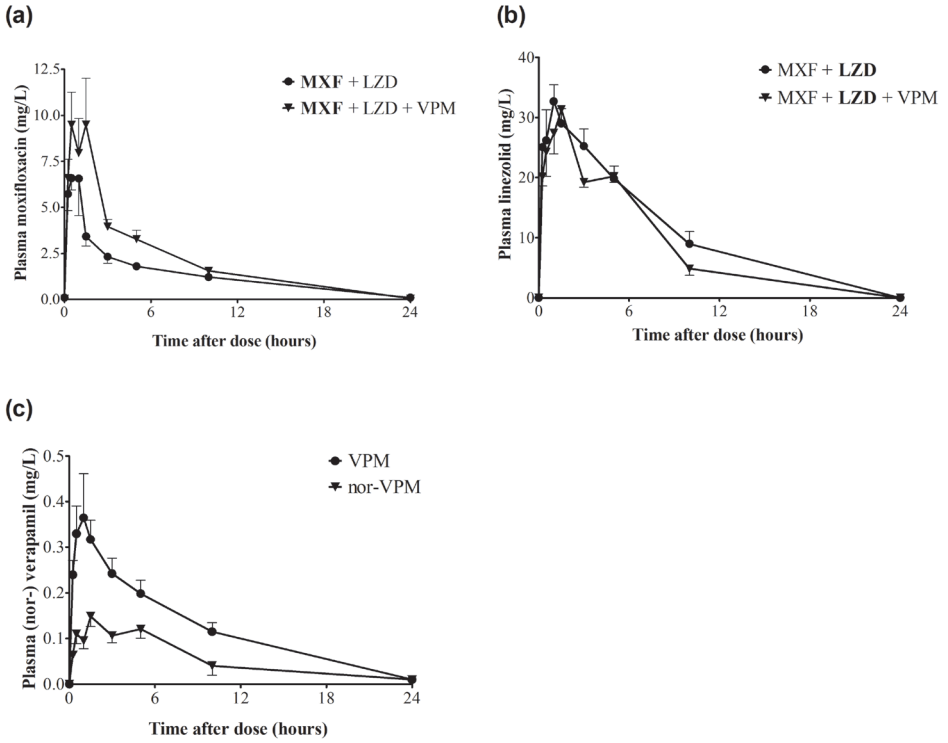

Figure 2. Moxifloxacin (a), linezolid (b) and (nor-)verapamil (c) plasma concentration-time profiles at steady state following an oral dose of 200 mg/kg moxifloxacin, 100 mg/kg linezolid and/or 12.5 mg/kg verapamil in TB-infected BALB/c mice. Plasma concentrations are plotted as mean ± SEM of three mice per study drug per time point.
CFU, colony forming units; LZD, linezolid; MXF, moxifloxacin; VPM, verapamil.

Table 1. Pharmacokinetic parameters at steady state of moxifloxacin and linezolid and (nor-) verapamil in plasma following oral administration in TB-infected BALB/c mice.

Drug	Dose (mg/kg)	$AUC_{0-\tau}$ (mg/L*h)	C_{max} (mg/L)	Cl/F (L/h)	Vd/F (L)	$t_{½}$ (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	2670[a]	365[a]	0.00010	0.00067	4.5
nor-VPM (+ MXF + LZD)	NA	1387[a]	149[a]	NA	NA	NA

Pharmacokinetic parameters are based on 8 time points; concentrations at each time point were based on plasma samples of 3 mice.
*[a] For verapamil and nor-verapamil $AUC_{0-\tau}$ and C_{max} were expressed in µg/L*h and µg/L, respectively*
$AUC_{0-\tau}$, area under the plasma concentration-time curve within the dosing interval; Cl, clearance; C_{max}, maximum plasma concentration; F, bioavailability; LZD, linezolid; MXF, moxifloxacin; Vd, volume of distribution; VPM, verapamil.

The total exposure to moxifloxacin was 53% higher in the mice that received verapamil and also the peak concentration (C_{max}) was 44% higher with addition of verapamil (9.5 versus 6.6 mg/L). In contrast, the area under the plasma concentration-time curves (AUC_{0-24}) of

linezolid was slightly (17%) lower in mice that received verapamil, whereas the linezolid peak concentrations in the two groups were approximately similar (31.3 and 32.7 mg/L). The total exposure of verapamil and its active metabolite nor-verapamil was 2670 µg/L*h and 1387 µg/L*h, respectively, with a C_{max} of 365 µg/L and 149 µg/L, respectively.

DISCUSSION

In the present study we evaluated whether verapamil could increase the treatment efficacy of a combination regimen consisting of moxifloxacin and linezolid in a murine TB model. The addition of verapamil did not significantly increase the treatment efficacy of this backbone. This is in line with the results of our *in vitro* study in which no additional effect of verapamil to moxifloxacin plus linezolid was observed (22). We hypothesized that applying this regimen to mice would result in a more prominent effect of verapamil compared to our *in vitro* study, since verapamil is supposed to affect not only the *M. tuberculosis* efflux pumps, but also the efflux pumps of macrophages. Such an effect on macrophages was shown by Adams *et al.* in an *in vitro* macrophage model where the addition of verapamil to moxifloxacin reduced tolerance against moxifloxacin (13). In addition, other studies showed promising effects of the use of verapamil in combination with several anti-TB drugs. For example Gupta *et al.* showed that verapamil could increase the treatment efficacy of a standard TB drug regimen consisting of rifampicin, isoniazid and pyrazinamide in mice (23). Similarly, it was shown for the new drug bedaquiline, that sub-inhibitory dosing of bedaquiline in a murine TB model could become as effective as regular dosing after the addition of verapamil (24). Both murine TB studies showed a significant difference of ± 1 Log CFU between regimens with and without verapamil on different time-points (23, 24). Gupta *et al.* also showed that the addition of verapamil could reduce the relapse rate of a standard TB regimen (23). In our study a significant reduction in relapse in the spleen was achieved in the mice that used verapamil. However, since all mice were culture positive it is difficult to assess the clinical value of this observation.

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 both drugs in humans (6, 25). Most studies on MDR-TB regimens containing linezolid in humans were either performed when moxifloxacin resistance was present or linezolid was added to a wide range of regimens (26, 27). However, previous *in vitro* studies provide some insight in the efficacy of combining both drugs. A pharmacokinetics-pharmacodynamics study of Deshpande *et al.* showed that the combination of moxifloxacin and linezolid could result in either a synergistic, indifferent or antagonistic effect based on their $AUC_{0\text{-}24h}$/MIC

ratios (28). The $AUC_{0\text{-}24h}$ of moxifloxacin and linezolid in the present study were neither in the synergistic or antagonistic range.

Another study showed that the interaction between moxifloxacin and linezolid was strain dependent, although in most cases a synergistic effect was observed (29).

In our study the combination of moxifloxacin and linezolid achieved a load reduction of 5 Log CFU in the lungs after 3 months of treatment and the mycobacterial load in the spleens was undetectable at that time point. The treatment duration of 3 months appeared to be too ambitious to achieve sterilization in the lungs and therefore relapse could not be assessed. Given the strong reduction of bacterial load in the lungs by the linezolid and moxifloxacin backbone, it would be interesting to apply this regimen to an MDR-TB mouse model for an extended period of time. By extending the treatment duration, relapse reduction mediated by verapamil, as observed by Gupta *et al.*, may possibly be replicated (23).

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 addition of verapamil. This effect could probably be ascribed to the inhibition of P-glycoprotein activity at the site of the intestines of the mice, as peak exposure was also amplified with 44% (30). However, this increased exposure did not result in a significant increased effect. In this context, it would be interesting to measure the drug concentrations in the macrophages to evaluate whether the use of verapamil also increased the drug concentrations intracellularly. Whether an increased intra-macrophage drug concentration will also lead to an increased effect is still unclear (15).

Next, we compared our observed $AUC_{0\text{-}24}$ to clinically relevant $AUC_{0\text{-}t}$ exposures observed in humans at steady state, to confirm exposure equivalence of the study drugs with human exposures. Total exposures to moxifloxacin ($AUC_{0\text{-}t}$ 30 mg/L*h) were comparable with the range of exposures found in humans ($AUC_{0\text{-}24h}$ 25-29 mg/L*h), which were obtained with the recommended 400 mg of moxifloxacin orally (31, 32). Similarly, total exposures to linezolid in our study were comparable to $AUC_{0\text{-}12}$ values found in pharmacokinetic studies in humans after an oral intake of 600 mg linezolid twice daily ($AUC_{0\text{-}12}$ 108-146 mg/l*h), assuming that $AUC_{0\text{-}24}$ is twice the $AUC_{0\text{-}12}$ (33, 34). Verapamil exposure in our study mimicked the range as observed in humans, namely an $AUC_{0\text{-}24h}$ of 3253 µg/L*h after daily oral intake of 240 mg of slow-release verapamil (35), and an $AUC_{0\text{-}8h}$ of 1999 µg/L*h after thrice daily oral intake of 120 mg immediate-release verapamil with a nor-verapamil total exposure of 2312 µg/L*h (36). Based on these results we can conclude that drug exposures in the present murine study were comparable with those achieved in humans. Although total exposures were comparable to humans, the C_{max} of moxifloxacin and linezolid in this study were higher compared to humans (6.6 mg/L vs 3.9 mg/L and 32.7 mg/L vs 20.4 mg/L, respectively)(32, 33). Since the AUC/MIC ratio is considered

to be the driver of efficacy for moxifloxacin and linezolid (37, 38) and moxifloxacin C_{max}/MIC ratio showed a poor correlation in efficacy in BALB/c mice (38), we assumed that this has limited effect on the translational value.

A possible limitation for implementation of a linezolid containing regimen, is the known toxicity of linezolid. Therefore, it may be worthwhile to consider to replace linezolid with a new oxazolidinone, such as sutezolid. Sutezolid showed promising results in an *in vitro* study, with a bactericidal effect against non-replicating *M. tuberculosis* (39). Furthermore, sutezolid seemed to be superior to linezolid and tedizolid (another new oxazolidinone) in murine models (39, 40). Besides, sutezolid was well tolerated without severe side effects in small human studies (41, 42). Also tedizolid seemed to have a better safety profile compared to linezolid in a randomized-controlled trial of a 6-days regimen for bacterial skin and skin structure infections (43), and showed to be effective in the treatment of TB in a mouse model (40). However efficacy seemed to be less compared to linezolid and sutezolid. Whether the safety profiles of tedizolid and sutezolid are also better compared to linezolid with increasing treatment duration remains to be determined. Besides, it might take several years before tedizolid and sutezolid will be available for routine use.

In conclusion, the present study in mice showed no added value of the addition of verapamil to a backbone of moxifloxacin and linezolid in terms of outcome after three months of treatment. Verapamil did prevent relapse in spleen tissue, whereas relapse in lung tissue could not be evaluated. Moreover, the backbone of moxifloxacin and linezolid showed a strong and steady CFU decline. A study performed in an MDR-TB mouse model for an extended treatment period (with and without verapamil) would be worth exploring.

MATERIALS AND METHODS

Animals

Specified pathogen-free female BALB/c mice were obtained from Charles River (Les Oncins, France). At the start of the experiments, animals were 13-15 weeks old and weighed 20-25 grams. Experimental protocols adhered to the rules specified in the Dutch Animal Experimentation Act and are in concordance with the EU animal directive 2010/63/EU. The Institutional Animal Care and Use Committee of the Erasmus MC approved the present protocols (117-12-14).

Experimental set-up

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 (figure S1) (44). In short, mice under anaesthesia were infected by intra-tracheal instillation with a suspension (40 µL) containing 2.9 x 10^5 colony forming units (CFU, 2.5 – 3.2 x 10^5) of the Beijing VN 2002-1585 genotype strain (45), followed by inhalation to ensure the formation of a bilateral TB infection (44). Two weeks after infection, three mice were used as control to determine infection efficacy and reproducibility in time. 48 mice were divided in two combination therapy groups; group one received 200 mg/kg moxifloxacin (Bayer, Leverkusen, Germany) plus 100 mg/kg linezolid (Sigma-Aldrich, Zwijndrecht, the Netherlands), the second group received the same backbone in combination with 12.5 mg/kg verapamil (Sigma-Aldrich, Zwijndrecht, the Netherlands). Selected doses were chosen based on previous *in vivo* / mouse studies (24, 40, 46). Dry powder moxifloxacin was dissolved in distilled water + 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 + linezolid suspension. Drugs were administered orally for 5 times per week, using a feeding cannula in a total volume of 0.2 mL per day.

Mycobacterial load (efficacy) assessment

Half of the infected mice receiving treatment (n=51) were sacrificed at the start and after 4, 8, and 12 weeks of therapy (n=3 per time point per regimen) and at 12 weeks post-treatment to assess relapse (n=15 per regimen). To prevent carry-over of TB-drugs, therapy was stopped 72 hours before sacrificing the mice and activated charcoal (0.4%) was added to the culture media. The lungs and spleen were removed aseptically and homogenized in M-tubes with the gentleMACS Octo Dissociator using the RNA program (Miltenyi Biotec BV, Leiden, the Netherlands) in 2 mL phosphate buffered saline. From each tissue homogenate 10-fold serial dilutions were performed 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 steady state after 4 weeks of treatment in the other half 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 hours 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 x*g* for 5 minutes to obtain plasma, which was stored at -80°C upon analysis.

Moxifloxacin, linezolid and (nor-)verapamil concentrations in plasma were analysed by validated high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) for human samples at the University Medical Centre Groningen, the Netherlands

(47, 48) (see Supplementary file). Assays were cross-validated for the measurement in murine plasma (see Supplementary file). All measured concentrations were total (i.e. protein-bound plus unbound) drug concentrations. Pharmacokinetic parameters were assessed using standard non-compartmental methods in Phoenix WinNonlin version 6.4 (Pharsight Corporation), as described previously (49).

Statistical analysis

CFU counts were log10 transformed before analysis. Group mean CFU counts after 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 the Fisher's exact test. The statistical significance level adopted was $p < 0.05$. Analyses were performed using Prism 5 (GraphPad software, San Diego, CA, USA).

Acknowledgements

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

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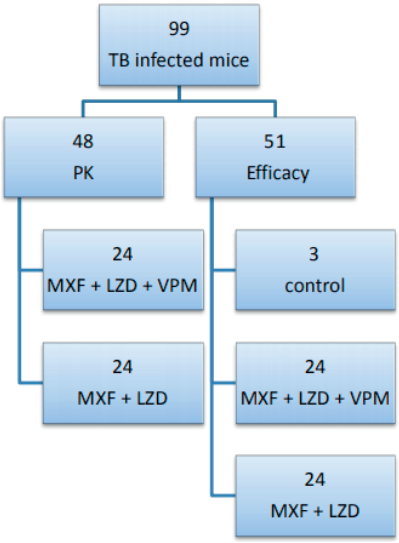
SUPPLEMENTARY FILE 1.

Moxifloxacin concentrations in plasma were analyzed with a LC-MS/MS assay, validated for human plasma, at the University Medical Center Groningen, the Netherlands (1). Crossvalidation of the assay for human plasma to murine plasma was performed by comparing responses of fivefold measurements of Quality Control (QC) moxifloxacin samples (concentrations 0.5, 2.5 and 5.0 mg/L) in human plasma with those 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 88-97% and the within-run CV amounted to 4.0-6.2%. Linezolid concentrations in plasma were analysed by a validated high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay at the University Medical Center Groningen, the Netherlands (2). The assay accuracy was 96.3-108.5%, the within-run CV ranged from 2.5-7.1% and the lower limit of quantitation (LLOQ) was 0.05 mg/L. Crossvalidation between human and murine plasma matrices was performed by comparing responses of three Quality Control (QC) linezolid concentrations (0.5, 15 and 30 mg/L in fivefold) in human plasma with those in murine plasma. The accuracy of murine relative to human plasma measurements was between 101-114%, with a within-run CV of 1.7-2.6%. Verapamil and nor-verapamil concentrations were analyzed on a Thermo Fisher (San Jose, USA) triple quadrupole LC□MS/MS with a FinniganTM Surveyor® LC pump and a FinniganTM Surveyor® autosampler. The mobile phase consisted of an aqueous buffer (containing ammonium acetate 5 g/L, acetic acid 35 ml/L and trifluoroacetic anhydride 2mL/L water), water and acetonitrile and had a flow of 0.3 mL/min. Samples were prepared by 100 µL serum or plasma and 750 µL precipitation reagent (mixture of methanol and acetonitrile (4:21, v/v) containing cyanoimipramin as internal standard), were vortexed for 1min, and subsequently centrifuged at 11,000×g for 5min. From the clear upper layer 5µL was injected onto the LCγMS/MS system. The FinniganTM TSQ® Quantum Discovery mass selective detector was operating in electrospray positive ionization mode and performed selected reaction monitoring. The mass transitions for verapamil were 455.3 m/z 165.1 m/z and for norverapamil 441.2 m/z- 165.0 m/z; a scan width of 0.5m/z was used. The calibration curves were linear within the concentration range of 18 to1800 ug/L for verapamil and 19 to 1900 ug/L for norverapamil; with a correlation coefficient (R2) of 0.997 and 0.999 resp. This method was precise and accurate: within day precision ranged between 1.0% and 5.5% for verapamil, 1.6% to 5.9% for norverapamil and between-day precision ranged from 1.5% to3.1% for verapamil and 0.0% to1.2% for norverapamil. The calculated accuracy ranged from 1.8% and 4.5% for verapamil and 0.2% to 2.0% for norverapamil.



Supplementary figure S1
LZD; linezolid, MXF; moxifloxacin, PK; pharmacokinetics, TB; tuberculosis, VPM; verapamil

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Chapter 4

Optimizing *in vitro* TB models for drug activity assessment

Chapter 4.1

Advanced quantification methods to improve the 18b dormancy model for assessing the activity of tuberculosis drugs *in vitro*

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Antimicrob Agents Chemother. 2020 Jun 23;64(7):e00280-20

ABSTRACT

One of the reasons for the lengthy tuberculosis (TB) treatment is the difficult to treat non-multiplying mycobacterial subpopulation. In order to assess the ability of (new) TB drugs to target this subpopulation, we need to incorporate dormancy models in our pre-clinical drug development pipeline. In most available dormancy models it takes long to create a dormant state and it is difficult to identify and quantify this non-multiplying condition.

The *Mycobacterium tuberculosis* 18b strain might overcome some of these problems, because it is dependent on streptomycin for growth and becomes non-multiplying after 10 days of streptomycin starvation, but still can be cultured on streptomycin-supplemented culture plates. We developed our 18b dormancy time-kill kinetic model to assess the difference in activity of isoniazid, rifampicin, moxifloxacin and bedaquiline against log-phase growth compared to the non-multiplying *M. tuberculosis* subpopulation by CFU counting including a novel 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 rifampicin 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 non-culturable 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.

INTRODUCTION

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 six months (1). This prolonged duration has an unfavorable effect on treatment compliance and shorter regimens are therefore highly required.

One of the reasons for this lengthy treatment is the difficulty in eradicating the non-multiplying *Mycobacterium tuberculosis* subpopulation (2). In the non-multiplying 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 non-multiplying bacilli when aiming at shortening TB treatment duration. In order to assess the ability of (new) TB drugs to target these bacteria, we need to incorporate dormancy models in our pre-clinical 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 non-multiplying mycobacterial subpopulation, since some of these mycobacteria are non-culturable on solid media (6, 7). Therefore, the 18b model could be an attractive alternative for studying TB drug potency against non-multiplying *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 non-multiplying state (9, 10). The up-regulation of 'dormancy regulon' and 'stationary-phase induced' genes in this state are 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) allow the identification of a mycobacterial subpopulation that is non-culturable 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, rifampicin, moxifloxacin and bedaquiline against both log-phase growth and

non-multiplying *M. tuberculosis* subpopulations. We performed time-kill kinetics assays, assessed CFUs and included a novel AUC based approach to compare drug activity against the non-multiplying (STR-starved) state relative to log-phase growth (STR-exposed). Besides 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 during 6 days to 4 concentrations of isoniazid, rifampicin, moxifloxacin and bedaquiline (1/25, 1/5, 1 and 5 times fC_{max}). The TB drugs were added at day 6 post inoculation to the STR-exposed cultures and at day 14 post inoculation 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 figure 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. Rifampicin 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 all control samples, only low levels of STR-independency were observed, ranging from 0.0004% at day 0 till 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.

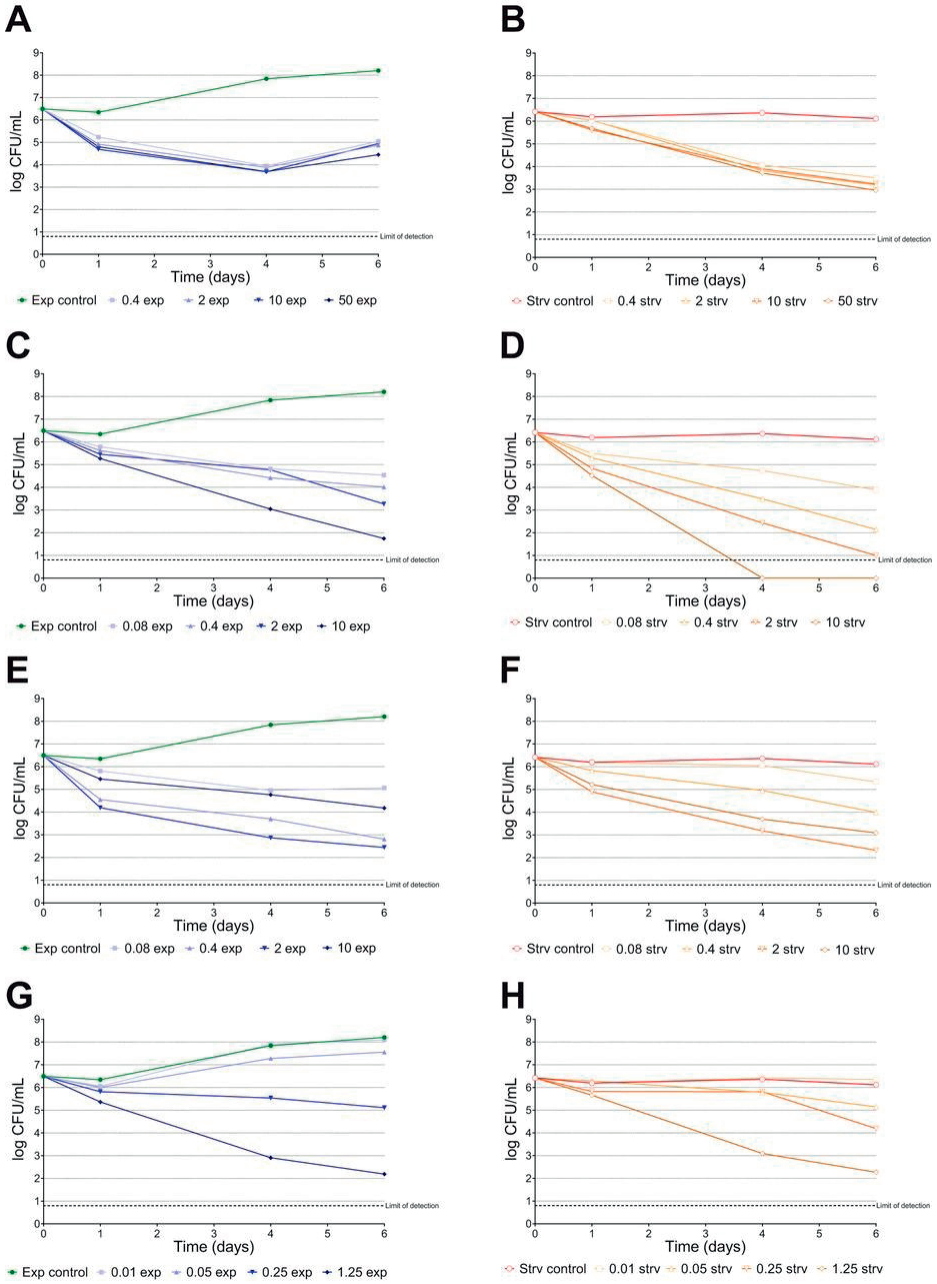

Figure 1. Bactericidal activity of 0.4, 2, 10 and 50 µg/ml isoniazid (A and B), 0.08, 0.4, 2 and 10 µg/ml rifampicin (C and D), 0.08, 0.4, 2 and 10 µg/ml moxifloxacin (E and F) and 0.01, 0.05, 0.25 and 1.25 µg/ml bedaquiline (G and H) against strain 18b in STR-exposed (A, C, E and G) and STR-starved state (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. RIF, rifampicin; INH, isoniazid; MXF, moxifloxacin; BDQ, bedaquiline; exp, STR-exposed; strv, STR-starved. Concentrations are indicated in µg/ml.

The mean differences in AUC between TB drug exposed and the control samples are reported in figure 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 exception of 10 µg/ml moxifloxacin, which showed almost no difference in AUC between both states. The activity of rifampicin and bedaquiline against both mycobacterial populations was generally comparable, with exception of the highest concentration of bedaquiline that showed more activity against STR-exposed 18b (figure 2).

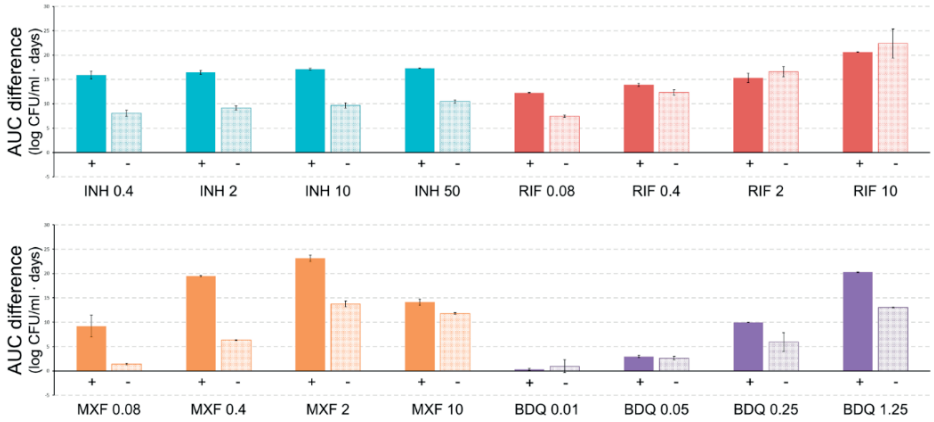

Figure 2. Mean differences of the area under the curve of CFU data between TB drugs and the respective controls. RIF, rifampicin; INH, isoniazid; MXF, moxifloxacin; BDQ, bedaquiline; +, STR-exposed; -, STR-starved. Concentrations are indicated in µg/ml.

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 six days of exposure to the different TB drugs is shown in figure 3. The control sample of the STR-exposed culture exhibited an expected decrease in TTP from an average of 86.5 hours to 28.5 hours. The control sample of STR-starved 18b showed a small increase in TTP from on average 98 hours on day zero to 129 hours on day 6 compatible with the small decrease in CFU as 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. Rifampicin, moxi-

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

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

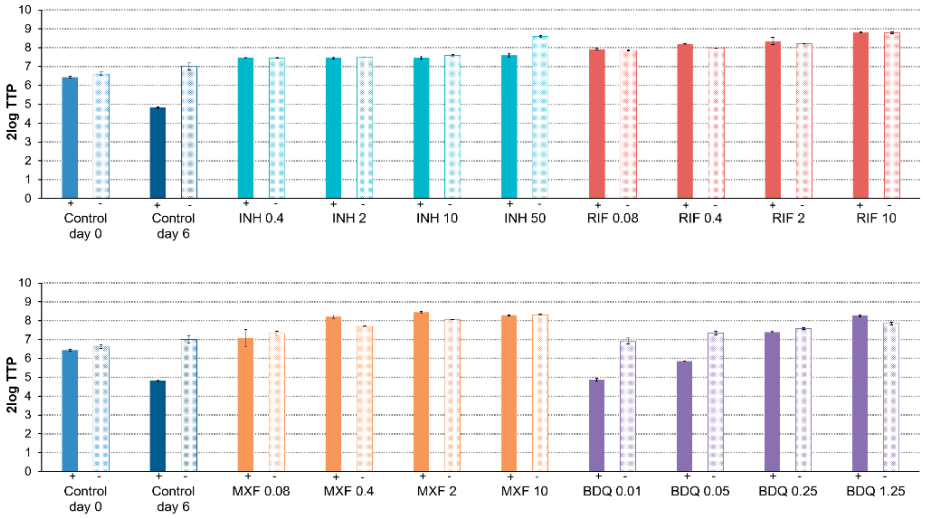

Figure 3. Bactericidal activity of rifampicin, isoniazid moxifloxacin and bedaquiline against 18b assessed by the BACTEC MGIT through Time to Positivity (TTP). Data are shown as 2log TTP mean with range (n=2). Samples were taken at day 6 of the corresponding experiments. INH, isoniazid; RIF, rifampicin;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

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 concentration- and time-dependency of rifampicin, 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 activitiy of isoniazid and rifampicin 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 non-multiplying states.

In our study moxifloxacin concentrations of 0.4 µg/ml and 2 µg/ml showed higher potency against STR-exposed 18b compared to 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 nor 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 pre-treated with chloramphenicol 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 (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 TB drug activity between 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 is 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 non-multiplying state compared to log-phase growth or vice versa. In fact, although the CFU counts of the STR-starved *M. tuberculosis* exposed to isoniazid were lower compared to 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 compared to STR-starved, 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 rifampicin 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 population. 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 compared to the STR-starved state. As a result, the difference in activity between multiplying and non-multiplying 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 non-replicating 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 non-culturable mycobacterial subpopulation. Interestingly, we observed especially for rifampicin 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 rifampicin was almost similar to the STR-exposed cultures at all concentrations tested, while the CFU counts of the STR-starved treated samples were considerably lower than 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 media, but can be recovered in STR-containing liquid medium. This is comparable to what has been observed *in vitro* in non-multiplying *M. tuberculosis* cultures which could not be cultured on solid media but where growth was observed in liquid media even without addition of resuscitating promoting factors, when using the most probable number as quantification method (12). Also, in human sputum samples a non-multiplying subpopulation was detected when comparing CFU counts 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 rifampicin, 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 drugs in our adapted 18b model. Besides, 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 of 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 (27), 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 a low pH, which might be associated with different mycobacterial phenotypes and response 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 non-replicating mycobacterial subpopulation present in TB patients. Besides, our study also confirmed the importance of incorporating more read outs (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 non-multiplying 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 more easy 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 during 4 weeks, complete STR-independency was observed and the amount of non-multiplying bacteria declined fast and became therefore unusable (data not shown). The decline of the non-multiplying 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.

In summary, our results confirm the differences in activity of the different TB drugs against the active and non-multiplying 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 non-multiplying state is 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 media 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, rifampicin, 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 rifampicin, 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 rifampicin, 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 rifampicin, 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 during 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 post inoculation (log-phase growth) in STR-exposed experiments and at day 14 post inoculation (non-multiplying) 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 6.5-6.8) to mean log 8.3 (range 8.2-8.3) 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 day 0, 1, 4 and 6 after the addition of TB drugs, samples were taken, centrifuged at 14000 *xg* and washed to avoid carry over, 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 0-6 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 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 zero, 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 2log TTP.

Acknowledgements

Research was conducted on behalf of the PreDiCT-TB Consortium (http://predict-tb.eu).

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Chapter 4.2

Higher dosing of rifamycins does not increase activity against *M. tuberculosis* in the hollow fibre infection model.

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Antimicrobial Agents & Chemotherapy 2021 Feb 8; AAC.02255-20

ABSTRACT

By improving the translational value of preclinical models, more successful and focussed research to shorten the treatment duration of tuberculosis can be performed. Although the hollow fibre infection model (HFIM) is considered to be a valuable addition to the drug development pipeline, its exact role is not fully determined yet. As increasing the dose of rifamycins is being evaluated for its treatment shortening potential, additional *in vitro* modelling is of importance. Therefore we assessed increased dosing of rifampicin and rifapentine in our HFIM 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/kg rifampicin as well as 600 mg and 1,500 mg rifapentine were assessed in our HFIM using the *Mycobacterium tuberculosis* H37Rv strain. Drug activity and emergence of drug resistance were assessed by CFU counting and subsequent mathematical modelling over 14 days, and pharmacokinetic exposures were checked.

Increasing rifampicin 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 clinical relevance of this observation is questionable. Moreover, the emergence of resistance to both drugs was unrelated to exposure.

In conclusion, in the most simplistic experimental set-up, the results of the HFIM did not fully correspond to pre-existing clinical data. Including additional parameters and read-outs in this pre-clinical model could be of interest for proper assessment of the translational value of the HFIM.

INTRODUCTION

Tuberculosis (TB) treatment is long and complex, leading to poor adherence, with emergence of drug resistance and low treatment success rates as a result. To shorten TB treatment duration it is important to focus on drug discovery, dose optimization and implementation of new drug combinations. Besides, it is necessary to improve the clinical predictability of pre-clinical models, to make this process more effective. This became apparent in the REMoxTB trial, aimed to shorten the treatment duration of drug-susceptible tuberculosis from 6 to 4 months by replacing ethambutol or isoniazid for moxifloxacin (1). Although previous *in vitro* and *in vivo* studies showed 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 pre-clinical models (both existing and new models) against the performance in clinical trials (6). They aim to develop an optimal research path by combining different preclinical models including pharmacokinetics/pharmacodynamics (PK/PD) modelling and a simulation framework to facilitate prediction of optimal drug combinations for assessment in clinical studies (6, 7).

A well-known PK/PD model is the hollow fibre infection model (HFIM). This method, approved by the European Medicines Agency (EMA), is developed to simulate human PK dynamics *in vitro*, while on every desired moment PD sampling of a liquid *Mycobacterium tuberculosis* culture can be performed (8). This is an improvement over traditional *in vitro* methods as for example the time-kill-kinetics (TKK) assay assessing 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 as ethical reasons. Although the HFIM showed to be valuable in the assessment of PK/PD indices (10), its exact role in the pre-clinical drug development pipeline is not fully determined yet.

For treatment improvement, dosing optimization of old as well as new TB drugs is gaining more interest (11, 12). Although rifampicin has been the cornerstone of TB-treatment since decades, its optimum dose is still unknown (11, 12). Since its introduction in 1971, rifampicin is given to patients as a 10 mg/kg dose, while this dose is presumed to be mainly based on fear for adverse events and price issues, instead of proper dose-finding studies (13). Already in 1980 a study evaluating different doses of rifampicin (5, 10 and 20 mg/kg) in a small population showed that increasing the rifampicin dose resulted in faster decline in colony forming units (CFU) during the first two days of treatment (14). Later *in vitro* and *in vivo* studies confirmed that increasing the rifampicin dose resulted in more activity and efficacy (15, 16). These observations led to the start of several clinical trials

assessing increased rifampicin dosing, which were all suggestive of a positive dose-response relationship (11, 12, 17).

Rifapentine, approved by the FDA in 1998, has a longer half-life time and a lower minimum inhibitory concentration (MIC) for *M. tuberculosis* than rifampicin (18) and was primarily intended to be used for intermittent therapy to lower the patients pill load (19). Later, both daily dosing, and increasing the standard 600 mg dose were explored in order to shorten TB treatment duration, both with promising results (20-22).

In this work we assessed the activity of standard and high dosing of rifampicin and rifapentine in our HFIM. We developed a PD model describing the observed change in CFU over time to test the effects of different dosages. We compared the results to 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 pre-clinical drug development pipeline.

MATERIALS AND METHODS

Strain

Experiments were performed using the H37Rv strain, with an MIC of 0.5 mg/L for rifampicin and 0.125 mg/L for rifapentine. MICs were determined according to CLSI standards (23). 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 enrichment (OADC, 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

Rifampicin (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. Rifampicin and rifapentine protein binding in human plasma of 80% and 97%, respectively were used to calculate the free drug concentrations (24). Target maximum concentrations (C_{max}) were based on results of previous clinical studies (11, 21). The doses simulated in the HFIM were 2.7, 10 and 50 mg/kg rifampicin and 600 mg and 1500 mg rifapentine (table 1). For rifapentine, no weight based dosage was chosen, since weight does not influence its plasma concentrations (21).

Hollow fibre infection model

Twenty mL samples of *M. tuberculosis* log-phase growth cultures (density ~2x10^6 CFU/mL) preconditioned with 7H9 broth at 37°C were inoculated in the peripheral compartment of the hollow-fibre cartridge (C3008 Medium Cellulosic Cartridge 5kd, FiberCell systems, New Market, USA). These cartridges consist of a peripheral compartment with inside semi-permeable hollow fibres with pores that allow drugs and nutrients to freely transfer in and out of the peripheral compartment, while keeping the *M. tuberculosis* in the peripheral compartment. Twenty-four hours after the cartridges were inoculated, the mycobacteria in each cartridge were exposed to different q24h dosing schemes of rifampicin or rifapentine during 14 days (table 1), at the same time one cartridge was unexposed to drugs and served as a control. All experiments were performed in duplicate.

Table 1. Simulated target drug concentrations in the hollow fibre infection model

Drug/Dose	fC_{max} (mg/L)	$fAUC_{(0\text{-}24h)}$ (mg/L*h)	C_{max} (mg/L)	$AUC_{(0\text{-}24h)}$ (mg/L*h)
Rifampicin				
2.7 mg/kg	0.74	4.19		
10 mg/kg	2.00	11.34	10.00*	60.28*
50 mg/kg	10.00*	60.28*	50.20	286.70
Rifapentine				
600 mg	0.47	7.60	15.70	257.40
1500 mg	0.99	14.80	33.00	544.30

**same experiment*

Drugs were administered by digitally controlled syringe pumps (World Precision instruments, Sarasota, Florida, USA) through a dosing port just before the central compartment, simulating the absorption rate of the drugs in humans (table 2) (24). By use of digitally controlled peristaltic pumps (Watson-Marlow, Cornwall, United Kingdom) fresh broth was pumped into the afferent port of the central compartment of the cartridge while drug-containing broth was iso-volumetrically removed from the efferent port of the system at rates programmed to simulate the half-life time of the drugs encountered in humans (table 2) (24).

Table 2. Pharmacokinetic parameters

	Rifampicin	Rifapentine
Volume (L)	0.349	0.349
Half-life (hours)	3	16
T_{max} (hours)	2	4
Protein binding (%)	80	97

PD samples (1 mL) were collected from each cartridge just before drug administration at day 0, 1, 2, 3, 6, 8, 10, 13 and 14. To prevent drug carryover samples were washed and serially diluted as described previously (25), before adding 200 µL on solid culture plates. All cultures were plated on 7H10 agar plates with and without 4 mg/L rifampicin or 1 mg/L rifapentine (4 times the clinical breakpoint), in order to assess total and resistant populations, respectively (26).

Pharmacokinetic assessment

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

A bio-assay was performed to assess the drug concentrations of rifampicin and rifapentine in the HFIM (27). *Sarcina lutea* was grown on blood agar plates (BD, Vianen, the Netherlands) during 48 hours at 35°C. Subsequently a suspension of 0.5 McFarland was made and plated on Mueller Hinton II plates (BD, Vianen, the Netherlands) in which wells with a diameter of 0.5 cm were punched. To create a standard curve, 100 µL of thawed known concentrations of rifampicin or rifapentine (8-4-2-1-0.5-0.25-0.125 µg/mL) were added in duplicate to the wells of the agar. In addition, 100 µL 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 hours 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 bio-assay the inhibition zones of rifapentine in the presence and absence of OADC were assessed with a standard curve of the previously described seven known concentrations, in order to assess any protein binding in the HFIM.

Modelling and statistics

The CFU data were analysed by developing a semi-mechanistic 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 analysed jointly and the structure of the model was inspired by previous work with TKK data (28). Since estimating both the number of resistant bacteria in the inoculum and the delay in 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 rate for the wild-type and resistant bacteria was assumed to be the same. Random inter-experiment variability was assumed to follow log-normal distributions. The effect of different rifamycin concentrations was tested on the kill-rate of the wild-type bacteria and the delay in start of growth

of resistant bacteria. Initial evaluation simply tested a difference between the arms (with different target C_{max} levels), while full dynamic concentration-effect relationships could be evaluated. The statistical significance was assessed with likelihood ratio tests using a 5% confidence level.

Plotting of the raw CFU data, PK analyses and AUC calculations was performed using Prism 5 (GraphPad software, San Diego, CA, USA). Modelling, data management, graphical evaluation and post processing of results were performed in R (Foundation for Statistical Computing, Vienna, Austria), partially using the Xpose package (Department of Pharmaceutical Biosciences, Uppsala University, Uppsala, Sweden). The model was developed in NONMEM 7.4 (Icon Development Solutions, Ellicott City, Maryland, USA), aided by PsN (Department of Pharmaceutical Biosciences, Uppsala University, Uppsala, Sweden) and Pirana (Pirana Software & Consulting, San Francisco, USA) (29).

RESULTS

The observed rifampicin and rifapentine C_{max} and total exposure ($AUC_{(0\text{-}24h)}$) of all doses tested are shown in table 3. The measured values agreed well with the (pre-set) targets for all dose levels. The presence of OADC did not change the inhibition zones of rifapentine in our bio-assay, suggesting that protein binding does not affect rifamycin activity in the HFIM (data not shown).

Table 3. Observed mean (±standard deviation) drug concentrations in the hollow fibre infection model, in steady state.

Drug/Dose	fC_{max} (mg/L)	$AUC_{(0\text{-}24h)}$ (mg/L*h)	C_{max} (mg/L)	$AUC_{(0\text{-}24h)}$ (mg/L*h)
Rifampicin				
2.7 mg/kg	0.69 (0)	3.29 (0.13)		
10 mg/kg	1.82 (0.08)	10.94 (0.77)	11.30 (0.45)*	62.61 (2.44)*
50 mg/kg	11.30 (0.45)*	62.61 (2.44)*	54.48 (2.80)	312.30 (31.60)
Rifapentine				
600 mg	0.50 (0.08)	7.71 (0.97)	15.27 (0.83)	240.00 (8.92)
1500 mg	1.00 (0.10)	14.64 (3.54)	33.18 (2.02)	496.20 (37.41)

**same experiment*

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 two days (days 13 and 14) even aggregation was observed. Mycobacterial killing and regrowth over 14 days of exposure to rifampicin or rifapentine are shown in figure 1a and 1c, respectively. Exposure to both rifamycins did not result in culture negativity at all concentrations tested. As for rifampicin, the lowest concentration resulted

in less bactericidal activity compared to the other concentrations reaching a mean of 3.5 log CFU/mL on day 10 and a mean of less than 2 log CFU/mL on day 8, respectively. Regarding rifapentine, the highest rifapentine concentration resulted in more bactericidal activity compared to the three lower concentrations at all time-points. Emergence of drug resistance was observed from day 6 onwards for rifampicin and from day 8 for rifapentine, resulting in regrowth (figure 1b and 1d). The emergence of resistance in the control cultures remained below 1.3 log CFU/mL.

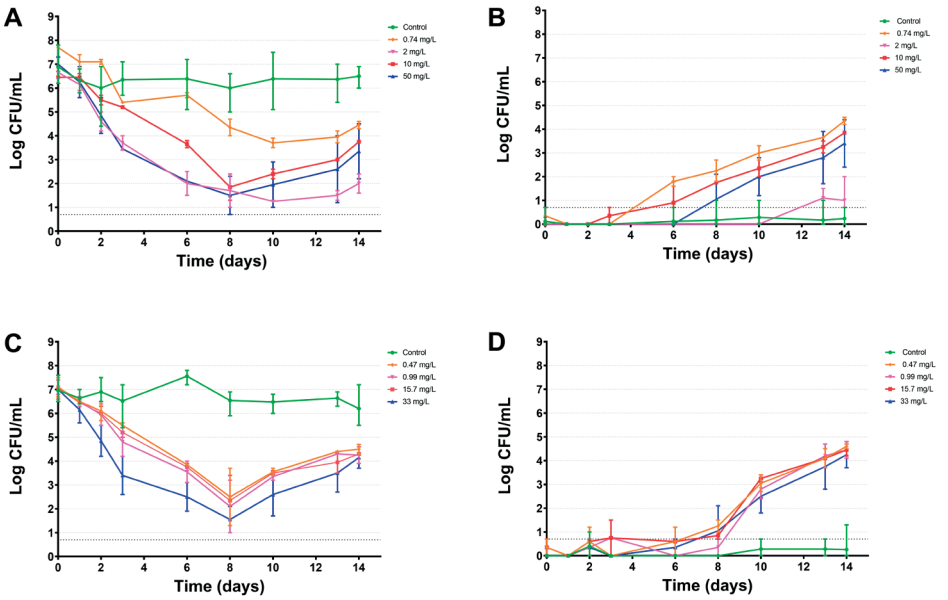

Figure 1. Activity and emergence of resistance of different simulated daily dosing of rifampicin and rifapentine (intended maximum concentrations are indicated in the legend) during 14 days towards initial log-phase growth H37Rv in the hollow fibre infection model (n = 2, mean ±range). A) Rifampicin activity. B) Emergence of resistance during rifampicin exposure. C) Rifapentine activity. D) Emergence of resistance during rifapentine exposure.

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 inoculum (I) for the WT and R compartments were defined as

$$I_{WT} = 10^{TVlogI_{WT} \times exp(\eta_I)}$$
$$I_R = I_{WT} \times 10^{-6}$$

where $TVlogI_{WT}$ is the typical WT inoculum on the log scale and η_I is the random inter-experiment variability drawn from a normal distribution with mean 0 and estimated variance ω_I^2. The maximal carrying capacity of the system (N_{max}), limiting the growth rate of the bacteria, was set to the total inoculum ($I_{WT} + I_R$) given that no growth was observes in the control arms. The growth rate was defined as

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

where the *kg(t)* is the growth rate at time *t* and kg_{base} is the growth rate in the unlimited state (T << N_{max}). The dynamics of the amounts of WT and R bacteria in the system was described by

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

$$\begin{cases} t \leq DELAY\ then\ \dfrac{dR(t)}{dt} = 0 \\ t > DELAY\ then \dfrac{dR(t)}{dt} = kg(t) \times R(t) \end{cases}$$

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

The k_{kill} was found to differ between the lowest rifampicin exposure (C_{max} 0.74 mg/L) and the other arms ($p < 0.005$). No additional effect was seen at higher rifampicin exposures. The k_{kill} was not statistically different between the three lower levels of rifapentine, but some additional effect was seen for the highest exposure (C_{max} 33 mg/L, $p < 0.005$). An exposure effect on the delay in 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 4. The model described the observed data well as illustrated by the visual predictive check included in Figure 2. The estimated growth rate of the unexposed mycobacterial

Table 4. Final parameter estimates including uncertainty (relative standard error, RSE)

Parameter (unit)		RSE
Typical values		
Inoculum wild type, $logI_{WT}$ (log10 CFU)	6.63	1.7%
DELAY RIF (days)[a]	4.94	21%
DELAY RPT (days)[a]	4.37	21%
Growth rate, kg_{base} /h	0.0307	11%
Kill rate, k_{kill} RIF /h	0.0555	6.0%
Kill rate, k_{kill} RPT /h	0.0761	6.5%
Extra kill RIF Cmax > 0.74 mg/L[b]	0.631	16%
Extra kill RPT Cmax > 15.7 mg/L[b]	0.264	32%
Inter experiment variability	CV%	
Inoculum wild type, $logI_{WT}$ (log10 CFU)	107%	19%
DELAY RIF	43%	22%
DELAY RPT	20%	73%
Kill rate, k_{kill} RIF /h	7.8%	23%
Kill rate, k_{kill} RPT /h	0 FIX	
Residual variability (Proportional error)	16.4%	9%

[a] *Estimated through the MTIME parameter in NONMEM*

[b] *Parameterized as k_{kill} high rifamycin = k_{kill}*(1+ Extra kill high rifamycin)*

population translated to a doubling time of 22.6 hours, which is in line with what is expected for *M. tuberculosis* (30). The model structure relied on certain assumptions, e.g. that there is a pre-existing small population of drug resistant bacteria and that the start of growth in this population is delayed. Alternatively one could for example assume no pre-existing resistant population, that the first resistant bacteria appears during the experiment and starts growing from that moment. This structure could probably also describe the data and would likely give the same results in terms of the drug effect evaluation. Both structures make sense theoretically, but with the information at hand we have no chance to discriminate which is the better.

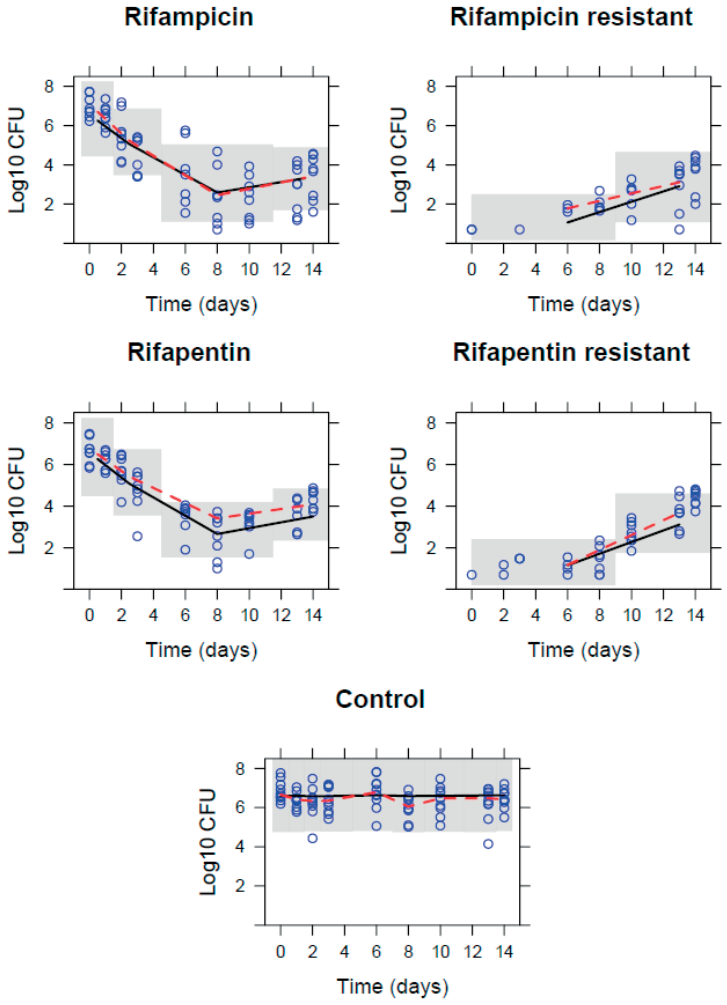

Figure 2. Visual predictive check of the final model. The blue rings represent the observed data, the red dashed line is the median of the observed data, the black line in the median model prediction and the grey area is the 90% confidence interval of the predicted median.

DISCUSSION

In the present study higher rifampicin exposure did not result in increased antibacterial activity, not even at extremely high concentrations. In line, increased activity was only observed for the highest rifapentine concentration, which is probably not representative for 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 (31, 32). 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 interpreting the relation between rifampicin 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 decrease after 6 days of mono-exposure. This is not expected to impact clinical efficacy as in patients, rifamycins are always combined with other antimicrobial agents. Previous HFIM studies also failed to show an exposure response relationship for rifampicin (33, 34) and to our knowledge, no other HFIM experiments with rifapentine have been described. Interestingly and similar to the present study, in those studies simulated high rifampicin dosages (ranging from 100-900 mg) were not enough to reach sterilization (33, 34).

These findings in the HFIM are in contrast to the results observed in our *in vitro* time-kill curves where no CFUs were detectable after 6 days of exposure to 8 mg/L rifampicin (35, 36), while this concentration has a lower C_{max} and $AUC_{0\text{-}24}$ compared to the highest given dose in the current study. The same holds true for rifapentine, as in our previous time-kill experiments with the *M. tuberculosis* Beijing-1858 strain rifapentine showed concentration and time-dependent activity, with 4 mg/L resulting in sterilising 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 compared to time-kill experiments given the significantly longer duration of HFIM experiments. Our previous time-kill studies of rifampicin showed that rifampicin activity was lower in low-metabolically active *M. tuberculosis*, which was associated with the emergence of drug resistance (35, 36). In line, Hu *et al.* showed that 16-fold higher concentrations of rifampicin were required to eradicate stationary phase growth cultures compared to log phase growth cultures (16, 36). Therefore, the presence of such a population in our HFIM might explain the reduced bactericidal activity observed in our HFIM compared to our time-kill experiments.

Previous *in vivo* studies on rifampicin and rifapentine did show that increasing the dose in *M. tuberculosis* infected mice resulted in higher efficacy (15, 37). Our group showed that an eight-fold increase in the standard rifampicin dose of 10 mg/kg allowed a treatment reduction from 6 to 2 months in a murine TB model (15). In addition, Rosenthal *et al.*

showed that when the rifampicin 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) (37). The same study showed dose dependant efficacy with doses up to 20 mg/kg rifapentine in combination with standard dosing of isoniazid and pyrazinamide (37). Generally, clinical studies were also suggestive of an exposure-response relationship for both rifamycins. Several studies in TB patients showed less early bactericidal activity (EBA) with low dosing of rifampicin (5 mg/kg) compared to the standard dose of 10 mg/kg (14, 38, 39). Which is comparable to our HFIM results, confirming that lower rifampicin exposure is indeed associated with lower success rates. However, two of these studies also showed an increased EBA of rifampicin when a 20 mg/kg dose was used (14, 39). Also studies assessing doses up to 35 mg/kg rifampicin were suggestive for increased effectivity of high rifampicin dosing when liquid culturing was applied (11, 40). For rifapentine an exposure-response relationship was shown when combining the results of the liquid cultures of two phase 2 trials assessing daily dosing ranging from 10-20 mg/kg and 450-1500 mg (21). However, whether these clinical observations would indeed lead to improved cure is still to be determined as only surrogate markers as time to culture conversion were used and the clinical follow up was maximum 12 months (17, 22, 40). It could be speculated that the discrepancy between these studies and our results might be caused by various factors present in *in vivo* models as well as in TB patients influencing rifamycin efficacy, which are absent in our HFIM. One could think of the presence of intracellular mycobacteria, variability in mycobacterial subpopulations, as well as different immunological factors and differences in drug tissue penetration. The clinical predictability of the HFIM might therefore be increased by including more metabolic populations (41), the use of macrophages (42) and other intra-cellular approaches (43) as well as different mycobacterial strains (18).

A remarkable observation is that results of liquid cultures as used in clinical trials were suggestive for increased efficacy of both rifamycins, whereas the results of solid cultures were less conclusive (11, 21, 40). It could be speculated that increased effect of higher exposure to the rifamycins is due to the effect on a mycobacterial subpopulation which is better detectable in liquid media. Given the fact that in the present study mycobacterial cultures were grown on solid media, this subpopulation might have been missed and thus an additional explanation for the lack of concentration dependent activity of rifamycins in our HFIM. The use of additional read-outs such as time-to-positivity and/or most-probable number assessment could therefore be of interest for future HFIM studies (44).

The reliability of our results is supported by the PK results which were in line with what was intended. On top of that the semi-mechanistic model described the observed data well, enabling statistical analysis of the differences in mycobacterial killing rate at different rifamycin concentrations. General concern for dose finding in the HFIM is the availability of clinical PK information as a requirement. As such, several important PK

parameters including drug half-life time, absorption rate, C_{max} and AUC have to be known beforehand. When these parameters are known, the HFIM could be a useful tool for dose-scheduling by finding the PK/PD parameter with optimal effect (45, 46).

To conclude, the results of the experiments performed in a 'standard' HFIM, did not fully correspond to pre-existing clinical data of high dosing of rifampicin and rifapentine. The use of additional parameters and read-outs in this model could be of interest to further determine the place of the HFIM in the drug-development pipeline.

Acknowledgements

Authors thank Virginia Ramos Martin and Adam Johnson for their technical advice and C. Roodbol-de Goeij and Marian ten Kate for their technical assistance. Research was conducted on behalf of the PreDICT-TB Consortium.

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Chapter 5

Summarizing discussion & future perspectives

SUMMARIZING DISCUSSION

This thesis was written with the intention to improve the treatment of tuberculosis (TB) as the treatment of TB is prolonged and complex and treatment success rates of drug resistant (DR) TB are still very low. We assessed a new diagnostic test for latent TB which has been developed to better determine the indication for treatment (chapter 2). Furthermore, we assessed new treatment regimens for drug resistant TB and the value of drug potentiators (chapter 3.1 and 3.2). At last we assessed the applicability of two *in vitro* TB models to predict the outcomes of new TB treatment options (chapter 4.1 and 4.2).

Latent tuberculosis diagnostics

In 2015 the most recent version of the QuantiFERON test was introduced in the Netherlands. As there is no gold standard test for latent TB it is challenging to clinically validate such a new test. Therefore we performed a verification study in which we compared the new QuantiFERON-TB gold plus (QFT-plus) to its predecessor QuantiFERON-TB gold in tube (QFT-GIT) (chapter 2). The QFT-GIT consists of 3 tubes for blood sampling; the nil-tube (negative control), the mitogen-tube (positive control) and the antigen tube (measuring interferon-y (INF-y) release of CD4+ T-cells). The QFT-plus is providing an extra antigen tube (TB2), which elicits besides a CD4+ T-cell reaction also a CD8+ T-cell reaction. The hypothesis was that these two antigen tubes could distinguish between recent and old TB infections, as CD8+ T-cell INF-y release has been associated with recent infection (1). The study included immune competent as well as immune compromised patients.

The test indications were categorized as contact investigation, periodic check by occupational health services and screening before immunotherapy. In addition, some of the tests were performed in the context of ruling out active disease although the test is actually not suitable to distinguish between active and latent infection. In our study the agreement between both QuantiFERON-TB tests was 95%. The discordant results were two-sided and the majority of the discordant results were in the borderline range of the test results, suggesting that these differences were due to inter-test variability instead of a true difference between both tests. After our study, similar verification studies have been performed in other countries with comparable outcomes (agreement range 86.8 - 98.3%) (2-8). There are no other studies evaluating the test accuracy in immunocompromised patients. In line with our study, in two studies assessing the performances of both INF-y release assays in the context of health care worker screening, the agreement between the tests was high (95.6 and 96.6 %) with almost all discordant results in the borderline range (6, 8). In the two studies assessing the agreement between both tests for contact investigation, significantly more positive QFT-plus results were observed (4, 9), which might suggest that the new test is better in detecting recent infections. To explore whether

this was the case and if the QFT-plus could indeed differentiate between old and recent infections we compared the test indications with a positive test and a true difference (>0.6 IU/ml) in INF-y release between TB1 and TB2. In this group, significantly more people who were expected to be more recently infected (based on their test indication) showed a true difference in antigen release of the antigen tubes, compared to subgroups representing more distant infections. However, only 33% of the total expected recently infected persons had a true difference in INF-y release. This was comparable to a previous study performed in Italy in which 26% of the positive tested contact investigation subjects had a true difference in INF-y release between TB1 and TB2 (9). Although more often a true difference of INF-y release between both tubes was measured in patients assumed to be recently infected, it does not answer the question whether we should treat a latent TB patient or not (given the low estimated sensitivity). In order to answer that question Gupta *et al.* performed a prospective cohort study to assess whether a difference in INF-y release between TB1 and TB2 resulted in a higher progression rate to active TB (10). In their study, with a median follow up of 1.93 years, 10 out of 492 patients progressed to active disease and no difference in progression to active disease was observed for patients with a higher TB2 versus TB1 INF-y release. In this respect it is reasonable to question whether the addition of the extra TB2 tube of QFT-plus is actually an improvement compared to its predecessor, as an extra blood sample has to be taken, which can be difficult in young children or other circumstances where blood sampling is more complex.

Improvement of drug-resistant TB treatment

We assessed the efficacy of two promising new DR-TB regimens in our murine TB-model. The first combination consisted of the new TB-drugs bedaquiline and delamanid combined with the recently added core second line agent linezolid (BDL) (chapter 3.1). In our study this combination achieved sterilization in the lungs of the mice already after 8 weeks of treatment, while mice treated with standard therapy of isoniazid, rifampicin, pyrazinamide and ethambutol (HRZE) were culture negative after 20 weeks of treatment. More importantly, no relapse was observed after 20 weeks of BDL treatment, while in the HRZE group mice still relapsed at the end of the study (24 weeks). Subsequent mathematical modelling showed that with BDL a 95% cure rate could be achieved after 20.5 weeks of treatment, while it was forecasted to take 28.5 weeks with HRZE. Although these results are promising, clinical studies should evaluate whether a treatment duration of less than 6 months is feasible for DR-TB.

In our study we did not assess the additional value of each drug separately. Based on the monotherapy results one might question the additional value of delamanid and linezolid as both drugs showed only a limited decrease in colony forming unit (CFU) , which is in line with the results of other murine studies (11-13). However, Xu *et al.* showed in a mouse TB model that the addition of pretomanid (belonging to the same drug family as delamanid,

the nitroimidazoles) to the combination of bedaquiline and linezolid prevented the emergence of bedaquiline resistance (14). These results might be extrapolated to our study as no relapse was observed after 20 weeks of treatment with the BDL regimen, while in the study of Xu *et al.* after 4 months of treatment with only bedaquiline and linezolid still 14 out of 15 mice relapsed (14). Tasneen *et al.* revealed the treatment shortening potential of linezolid by showing that the addition of linezolid to the combination of bedaquiline and pretomanid resulted in no relapse in mice after 3 months of treatment, while mice treated with only bedaquiline and pretomanid still relapsed after 4 months of treatment (15). Not only in mice delamanid and linezolid showed to have additional value. In humans faster sputum conversion was achieved when adding delamanid to a DR-TB backbone regimen (16). The combination of bedaquiline, delamanid and linezolid has so far not been evaluated in humans. However, a study reviewing the combination of bedaquiline and delamanid together with a backbone regimen as DR-TB treatment in humans observed that after 24 weeks of treatment in 88% of the cases sputum conversion was achieved and 92% of these patients also received linezolid (17). Furthermore, a randomized controlled trial in patients with XDR-TB showed that the treatment success rate in the linezolid therapy group was significantly higher (70%) compared to the non-linezolid group (34%) (18), supporting the positive outcomes of our study and its assumed clinical relevance. Besides, the results of the Nix-TB trial (a study assessing the combination of bedaquiline, pretomanid and linezolid (BPaL) in MDR and XDR-TB patients) were promising. *In vitro*, mutations have been identified that are associated with pretomanid resistance, but does not seem to affect delamanid susceptibility (19, 20). As such BDL might be a good alternative for BPaL in case of pretomanid resistance. Currently a subsequent clinical trial (ZeNIX) is being performed to assess whether linezolid doses can be lowered in order to limit toxicity (21). These results are also of interest for the clinical application of the BDL regimen. The WHO changed its recent guidelines concerning restricted use of the combination of bedaquiline and delamanid by reason of possible QT-prolongation and now permits its combined use when accompanied by electrocardiography surveillance as well as audiometry and specific biochemical tests (22). As such, the path for this powerful combination in the treatment of MDR- or even XDR-TB is reopened.

The second DR-TB regimen we assessed was the combination of moxifloxacin and linezolid, which showed a strong CFU reduction in the lungs (5 log CFU/lung) and sterilization in the spleen in our mouse TB-model in only 12 weeks of treatment (chapter 3.2). In order to assess whether the addition of an efflux pump inhibitor could increase the efficacy, we added the efflux pump inhibitor verapamil to this backbone regimen. The addition of verapamil to the moxifloxacin-linezolid combination only resulted in a modest additional CFU decline, but a significant reduction in spleen relapse was observed. Other studies showed similar effects in terms of CFU decrease with the use of verapamil in combination with rifampicin, isoniazid and pyrazinamide (23) and with bedaquiline (24).

One of these studies also showed a reduced relapse rate (23). However, the relevance of the reduced spleen relapse rates observed in our study is uncertain since culture positivity in the lungs was still observed at the end of treatment. To better evaluate the additional value of verapamil in reducing TB relapse rates when combined with moxifloxacin and linezolid, we suggest follow up studies with a longer treatment duration. Interestingly, in our previous *in vitro* study no increased activity with the addition of verapamil to the moxifloxacin-linezolid combination was shown (25). It might be that the effect observed *in vivo* was due to the effect of verapamil on the efflux pumps in macrophages as has been shown in a TB macrophage model before (26). Another reason could be the observed increased moxifloxacin exposure after the addition of verapamil, which has also been described when verapamil was combined with bedaquiline (27). As for the cause of this increase in AUC it was speculated that this is mediated through an effect of verapamil on mammalian drug transporters (27). As moxifloxacin is subject to P-glycoprotein-mediated efflux the increased AUC might be ascribed to the inhibition of P-glycoprotein mediated drug-efflux at the site of the mice intestines (28). It is important to further investigate the above described possible working mechanisms of efflux pump inhibitors. If the effect of verapamil in this study was indeed mediated by the mammalian drug transporters causing an increased pharmacokinetic exposure, this might increase side effects of the enforced drug. Despite the limited effect of verapamil in our study, the backbone of moxifloxacin and linezolid showed to be a promising combination in mice and a might be an interesting option for the treatment of DR-RB to further evaluate in clinical trials.

Optimization of *in vitro* TB models

To evaluate new drugs and regimens for the treatment of TB, decent pre-clinical models which allow a good translation to clinical practice are essential to further improve the treatment of TB. In chapter 4 we assessed the (translational) value of two *in vitro* TB models for TB-drug activity assessment and evaluated their place in the drug-development pipeline.

As one of the reasons for the long treatment duration of TB is considered to be the difficulty of eradicating dormant *Mycobacterium tuberculosis* (Mtb), it is important to assess whether drugs are active against this subpopulation. In this context, good pre-clinical dormancy models are essential. In chapter 4.1 we described the activity assessment of isoniazid, rifampicin, moxifloxacin and bedaquiline in our time-kill kinetics 18b dormancy model by different quantification techniques. This model uses the 18b Mtb strain which is dependent on streptomycin for growth. In the presence of streptomycin (STR-exposed) the bacteria will start multiplying (fast multiplying state), when not exposed to streptomycin (STR-starved) the bacteria will go in a non-replicating state comparable to dormant Mtb. The 18b dormancy model was previously mostly used as a fast screening method to assess whether a drug is active against fast and non-multiplying bacteria using

the resazurin microplate assay (REMA) (29) and luciferase for mycobacterial load assessments (30). However, these assays are limited in the assessment of cell wall inhibitors as those influence the conversion of resazurin in resorufin, which will cause an increase in fluorescence and therefore falsely influence the results (29). Besides, REMA does not allow time-course investigation as it is an end-point analysis. To overcome both limitations we used the 18b strain in a time-kill-kinetics assay during 6 days and performed CFU counting. We observed time-dependent activity of isoniazid and concentration- and time-dependency of rifampicin, moxifloxacin and bedaquiline against fast and non-multiplying 18b, which was expected based on our previous studies (25, 31, 32). To assess whether a TB-drug is more active on the non-multiplying bacteria compared to fast growing bacteria or vice versa, 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 fast multiplying control observed at day 6 as opposed to the rather unchanged non-multiplying control at day 6 compared to day 0. This way an overestimation of TB drug activity against the non-multiplying population is precluded. By applying this method we confirmed that isoniazid is more active on STR-exposed 18b compared to STR-starved, instead of equally active as might have been assumed based on the similar absolute CFU decline. As these findings were in line with the expected performances of the drugs (25, 31, 32), the use of AUC differences for TB-drug activity assessment in the 18b dormancy model could be a useful additional read-out in time-kill kinetics studies. As it has been shown that with liquid culturing addition mycobacterial subpopulations can be revealed (33, 34), we included the liquid bacterial load assessment method time-to-positivity (TTP) by MGIT as additional read-out. Interestingly, we observed that the TTP of the starved cultures exposed to rifampicin was almost similar to the TTP of the STR-exposed cultures at all concentrations tested, while the CFU counts of the STR-starved treated samples were considerably lower than the STR-exposed samples. This might be explained by the presence of a mycobacterial subpopulation, which is unable to grow on solid media, but can be recovered in STR-containing liquid medium. This phenomenon has been observed previously in *in vitro* as well as in clinical studies, where a non-multiplying subpopulation was revealed by using another liquid based read out (most probable number) or the TTP assay as quantification method (33, 34). This observation suggests that liquid culturing might give a better estimation of the total mycobacterial population present and is a useful addition to CFU counting, probably also for other *in vitro* (dormancy) models.

Overall, our results were in line with previous studies on the activity of these drugs in the 18b dormancy model, except for isoniazid, which was expected as this is a cell wall inhibitor and could not properly be assessed by REMA and luciferase assays (30, 35). When comparing our results to other dormancy models the results were comparable

to those of the hypoxic model of Wayne (36), which is in line with the similar gene expression profiles observed in the non-multiplying mycobacterial populations in both models (37). As drug activity varies substantially in different dormancy models (36), it is important to use different models and different quantification methods when assessing TB drug activity against non-multiplying mycobacterial subpopulations, which is underlined by the results of this study. The time-kill kinetics 18b model is relatively easy to use, with fast development of a non-replicating state which is relatively easy to maintain. As such this model has its place in the drug development pipeline as a useful preclinical model to assess the activity of novel compound using different read-outs.

Another interesting possible addition to the drug development pipeline is the Hollow Fiber Infection Model (HFIM). In this model human pharmacokinetics can be simulated and samples for mycobacterial load assessment can be taken on every desired moment. In chapter 4.2 we assessed the place of the HFIM in the drug development pipeline by assessing the activity of high dosing of rifampicin and rifapentine against drug sensitive Mtb. Subsequently, we back-validated our HFIM results to pre-existing data of clinical trials.

In our study higher rifamycin exposure did not result in increased anti-mycobacterial activity, not even with concentrations that were expected to give toxic side-effects (38). This observation is in line with previous HFIM studies and comparable to our study these studies did not reach sterilization of the cultures due to emergence of resistance (39, 40). In contrast, in our *in vitro* time-kill kinetics assays sterilization was achieved upon rifampicin exposure (31, 41). This might perhaps be explained by the presence of a lower metabolically active subpopulation in our HFIM due to the longer duration of these experiments. In our previous time-kill kinetics assay experiments rifampicin activity was lower in low compared to high metabolically active Mtb, which was associated with the emergence of rifampicin resistance (31, 41). This is supported by the observation of Hu *et al.* that much higher concentrations of rifampicin were required to sterilize stationary phase cultures compared to log-phase growth cultures (41, 42). In contrast to our HFIM study, *in vivo* studies showed an exposure response relationship for both rifamycins (43, 44). Clinical studies were also suggestive of such an exposure response relationship which could only be revealed when liquid culturing was applied (45-47). However, whether this increased effect of higher rifampicin dosing will result in a better clinical outcome has still to be evaluated, since the follow up of the clinical studies assessing higher rifamycin doses was short and only surrogate outcome markers have been used (46, 48-50). Recently, a study (study 31) assessing high dose rifapentine in combination with moxifloxacin, isoniazid and pyrazinamide during 4 months showed non-inferiority compared to standard HRZE of 6 months in all analyses, suggesting that higher rifapentine dosing is indeed more effective (51). Various factors present in *in vivo* models as well as in TB patients, but absent in our HFIM, might be the explanation for the discrepancy in results, such as different mycobacterial subpopulations, immunological factors, differences in drug tissue

penetration and the use of different read-outs. Other studies incorporated different metabolic subpopulations or Mtb infected macrophages and other intra-cellular approaches in the HFIM (52-54). These factors combined with the use of different mycobacterial strains and the use of additional read-outs such as TTP could perhaps contribute to improving the translational value of the HFIM.

An important limitation is that HFIM experiments require that several PK parameters as drug half-life time, absorption rate, C_{max} and AUC are known beforehand. When this information is known, it might be a useful tool for dose scheduling by assessing the PK/PD parameter with best effect (55, 56) and has as such its place in the drug-development pipeline.

FUTURE PERSPECTIVES

Latent TB diagnostics

In our study only 33% of the recently infected patients showed a real increase in interferon-γ release from the CD8+ cells. Therefore no distinction in recent and older infections could be made based on the new QFT-plus test. This is disappointing as a test that could predict these features might save a lot of unnecessary treatments. Therefore the development of future new latent TB tests should focus on improving this particular feature. Possibly the recently discovered biomarker ΔHLA-DR might be an interesting target, as this biomarker was able to identify recent TB infection and disease progression (57).

With regards to the current QFT-plus test, it is known that a lot of subjects periodically checked by occupational health services are tested false positive and frequently showed to be negative after retesting (8, 58). It would be worthwhile to re-assess the cut-off point used for this test in the Netherlands, or introduce a borderline-range in order to lower the number of false positive test results. A study performed in the USA with low risk health care workers showed that using a more conservative interpretation of the QFT-plus reduced 90% of the non-reproducible positive results (8). A similar study has been performed for T.SPOT.TB by Rego *et al.* (59) and might serve as a guide for a future study to assess a more optimized cut-off point or borderline range for the QFT-plus when testing low risk subjects.

Improvement of drug resistant TB treatment

With approximately 0.5 million people who fell ill with drug-resistant(DR) TB and only 56% of the treated patients recovered, future investments in studies aiming to increase the treatment success of DR-TB are highly required (60). In our mouse TB model we showed that the combination of the all oral TB drugs bedaquiline, delamanid and linezolid and also a backbone of moxifloxacin and linezolid are promising for the treatment of DR-TB,

with an expected relatively short treatment duration. Currently several clinical studies are ongoing to assess novel combinations of TB drugs (61). However not (yet) with our examined regimens, which will therefore be of interest.

Various murine TB studies including a study from our research group assessed the combination of bedaquiline, pretomanid and linezolid (BPaL) and bedaquiline, pretomanid, moxifloxacin and pyrazinamide (BPaMZ) (14, 15, 62). In line with the results of our study assessing BDL efficacy, these combinations also showed promising results and are already being evaluated in clinical studies. It would be of interest to assess whether the replacement of pretomanid by delamanid in these combinations would provide similar results. To our knowledge it has not been clinically evaluated whether pretomanid or delamanid has superior efficacy. This information might be useful as we should be aiming for the most effective regimen, with the least side-effects. In case of equal efficacy and side-effects they might be used as substitutes for each other.

In one of our murine TB studies we also evaluated the additional value of the efflux pump inhibitor verapamil to the combination of moxifloxacin and linezolid. We showed that the addition of verapamil resulted in only a modest additional CFU decline and in significantly less relapse in the spleen. However, as the lungs of the mice were still culture positive at the end of treatment, the clinical relevance of this finding is questionable. Previous murine studies of our group on the use of the efflux pump inhibitors thioridazine and SILA-421 showed disappointing results (63, 64). Other studies did show that the addition of verapamil to bedaquiline might reduce TB relapse rates (24). Importantly, in this study the addition of verapamil increased bedaquiline exposure similar to what was observed in our study for moxifloxacin. If increased efficacy is indeed related to the increased exposure to bedaquiline or moxifloxacin by verapamil addition, why not just increase the dose of these drugs instead of adding an extra drug which might provide extra side-effects.

Currently, the search for dose optimization is gaining more interest. In our HFIM we showed that increasing the dose of rifampicin and rifapentine did not result in increased activity, although clinical studies so far suggest otherwise (45-49). In this context, study 31 showed that higher dosing of rifapentine could reduce the treatment duration of drug susceptible TB with 2 months (51). Besides in the ZeNIX trial is currently assessed whether the dose of linezolid could be lowered in the combination with bedaquiline and pretomanid to limit toxicity while containing efficacy. These dose optimization studies are useful additions for the treatment optimization of TB and should be further explored as limited new drugs have entered the marked in the last decades or are expected in the near future.

Optimization of pre-clinical models

Several research consortia are aiming to provide an optimal research path assessing novel TB drugs and drug regimens by combining several pre-clinical models. We assessed the place of two of those pre-clinical models in the pre-clinical drug-development pipeline and showed that the 18b dormancy model in its current form is a useful tool to assess drug activity against fast and non-replicating Mtb in the early stage of drug development. The place of the HFIM in the drug development pipeline is more difficult to determine, due to its dependence on clinical PK/PD information. Furthermore, in our study the translational value of the HFIM results was limited. As previously suggested this translational value might increase by the use of additional outcome parameters and the inclusion of more parameters such as different mycobacterial subpopulations. In this context, we and other research groups used the 18b strain in the HFIM for assessment of drug activity against mycobacteria in the non-replicating state (52, 65). However, in these experiments the mycobacterial load of the control culture declined significantly during the experiments (52, 65) and we observed a substantial amount of regrowth of mycobacteria that became independent of streptomycin for growth (unpublished results). Therefore, the 18b strain should only be used for short course experiments. Consequently, we should search for a better dormancy model to use in the HFIM. As such, potassium or nutrient depletion might be good alternatives to assess in the future (66, 67). Also using lipid-rich medium in the HFIM might be an interesting option, as within this lipid-rich dormancy model the mycobacteria will represent a dormant phenotype with a dormancy gene expression profile, but are still culturable (68). Besides, it will be interesting to evaluate the additional value of incorporating macrophages and other intracellular approaches in the HFIM, in order to increase its translational value.

To increase the predictive value of preclinical models it is essential to include different mycobacterial subpopulations. In addition, it is important to include different read-outs for the optimal identification of the total mycobacterial population present. Several studies have shown that applying liquid culturing increased detection (33, 34, 69). Therefore it is recommended to include different outcome parameters in all pre-clinical models, to better estimate drug activity and efficacy on the total mycobacterial population. By gaining more knowledge about drug activity against different subpopulations, more targeted regimens can be made. For example by including in the first phase mainly drugs that act on the fast-multiplying population and in the later phase drugs that act against the non-multiplying population, to limit the pill load and side-effects. In this context we should aim for an outcome parameter that is able to reveal the total mycobacterial population. The mycobacterial load (MBL) assay, based on the detection of 16s ribosomal RNA, is expected not to be influenced by the metabolic state of mycobacteria and might therefore measure the bacterial load more precisely, which has been suggested in murine as well as clinical studies (69, 70).

Optimal pre-clinical drug development pipeline

Based on the findings of our research the optimal pre-clinical drug development pipeline might be filled in as follows:

After a new possible lead is found, one should start with assessment of the minimal inhibitory concentration (MIC). Based on the MIC a TKK assay with the clinical Beijing strain should be performed, in order to assess concentration and time-dependent activity and eventually early emergence of resistance. The next step would be a TKK assay with the use of the 18b strain including the use of our new AUC approach and TTP measurement, in order to assess the activity of the new drug against dormant *M. tuberculosis* and possible other subpopulations that can only be cultured in liquid medium. Based on these results EBA studies in our mouse TB model can be performed during 6 weeks, with different drug concentrations. Also in this phase it is important to include different outcome parameters, like CFU, TTP and MBL in order to assess the drug activity on different subpopulations. Also combination regimens can be assessed in our mouse TB model, where the focus should be on the relapse assessment. Subsequently mathematical modelling can be useful for treatment duration assessment as last step before studies in humans.

SUMMARY

In all, with this thesis we provided useful information and tools to improve the treatment of tuberculosis. Further research in this field should focus on:

- Preventing unnecessary treatment of latent TB by developing a test which can predict the chance of progression to active disease.
- Dose optimization should have a prominent place in the development of new regimens and pre-clinical models should be further improved for this assessment.
- Including different mycobacterial subpopulations and improving and using different methods to detect these subpopulations in pre-clinical models in order to better predict clinical outcomes.

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Chapter 6

Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

In dit proefschrift worden verschillende onderzoeken beschreven die als doel hebben de behandeling van tuberculose (TB) te verbeteren. Het is bekend dat patiënten met latente TB (besmet met *Mycobacterium tuberculosis* maar (nog) niet ziek), vooral baat hebben bij behandeling wanneer zij recent geïnfecteerd zijn. Dit komt doordat het risico op actieve TB na besmetting het grootst is in de eerste twee jaar na infectie. Echter het aantonen wanneer iemand besmet is geraakt is niet goed mogelijk. Daarom hebben we een nieuwe test voor de diagnostiek van latente TB onderzocht die is ontwikkeld om een onderscheid te maken tussen recente en oudere infectie om zodoende de behandelindicatie beter vast te kunnen stellen (**hoofdstuk 2**).

Het volgende probleem waarop het onderzoek in dit proefschrift gericht is, is dat momenteel de behandeling van actieve TB complex is met een behandelduur van minimaal 6 maanden met 4 verschillende middelen. Deze therapieduur moet nog eens verlengd worden tot minimaal 9 maanden en soms zelfs tot 2 jaar met heel veel verschillende antibiotica wanneer dit zorgt voor een hoge behandellast, bijwerkingen en afname van de therapietrouw. De behandeling is dan ook met name bij patiënten met resistente TB in veel gevallen niet succesvol. Daarom hebben we nieuwe behandelregimes onderzocht voor resistente TB met als doel de behandelduur te verkorten en het aantal middelen te verlagen. Ook hebben we gekeken naar de rol van zogeheten drug potentiators, middelen die de werking van antibiotica kunnen versterken (**hoofdstuk 3.1 en 3.2**).

Voor het onderzoeken van deze nieuwe regimes is het van belang om te beschikken over goede preklinische modellen met een hoge translationele (voorspellende) waarde, waarin deze nieuwe regimes getest kunnen worden. Daarvoor hebben we de toepasbaarheid van twee preklinische TB modellen onderzocht (**hoofdstuk 4.1 en 4.2**).

In **hoofdstuk 2** hebben we onderzocht of de resultaten van de nieuwe latente TB test QuantiFERON Gold Plus (QFT-plus) vergelijkbaar waren met zijn voorganger de QuantiFERON Gold In Tube (QFT-GIT), hetgeen in 95% van de testen het geval was. Deze nieuwe test is ontwikkeld om een beter onderscheid te kunnen maken tussen een recente TB infectie en een infectie die langer geleden opgelopen is. Hiervoor is er een extra antigeenbuis aan de QFT-plus toegevoegd die naast de interferon-y release van CD4+ T-cellen ook de interferon-y release van CD8+ T cellen meet, welke geassocieerd is met een recente infectie. Om te onderzoeken of deze test daadwerkelijk dit onderscheid tussen recente en oudere infectie kan maken hebben we bij alle positieve testen van ons onderzoek gekeken of er daadwerkelijk een verschil was in de gemeten hoeveelheid interferon-y tussen de twee antigeen buizen. Hierbij vonden we significant vaker een verschil in interferon-y release tussen de twee antigeenbuizen bij patiënten die vermoedelijk recent besmet waren. Dat betekent echter niet dat de QFT-plus gebruikt kan worden om het onderscheid te

maken tussen een recente dan wel oudere infectie aangezien dit slechts in 33% van de vermoedelijk recent geïnfecteerde patiënten het geval was.

In **hoofdstuk 3** hebben we de effectiviteit van twee nieuwe combinaties van antibiotica voor de behandeling van antibiotica resistente TB onderzocht. In **hoofdstuk 3.1** hebben we gekeken naar de effectiviteit van de combinatie van de nieuwe tuberculosemiddelen bedaquiline en delamanid samen met linezolid (BDL). Dit regime hebben we getest in ons muis-TB model, waarbij we gedurende 24 weken de effectiviteit van het regime getest hebben door tijdens behandeling elke 4 weken de mycobacteriële load in de longen en milt van 3 van de muizen te bepalen. Om de 2 weken hebben we de behandeling van 3 muizen gestaakt en vervolgens na 12 weken zonder behandeling de mycobacteriële load in de longen en milt bepaald. De BDL combinatie bleek erg effectief waarbij in een vroeg stadium (na 20 weken behandeling) geen TB relapse (12 weken na behandeling nog steeds geen *M. tuberculosis* aantoonbaar) meer gezien werd en er dus sprake was van genezing. De potentie van dit nieuwe regime is hoog vergeleken met de standaard TB behandeling (isoniazide, rifampicine, pyrazinamide en ethambutol) waarbij er zelfs na 24 weken behandeling nog steeds sprake was van relapse. Vervolgens hebben we data gemodelleerd om een uitspraak te kunnen doen over de te verwachten behandelduur met het nieuwe regime. Hieruit bleek dat de BDL behandeling naar verwachting 8 weken sneller tot genezing zal leiden dan de standaard behandeling. Antwoord op de vraag of de behandelduur van resistente TB met BDL daadwerkelijk korter zou kunnen zijn dan de huidige behandeling van niet-resistente TB zal nog uit klinische studies moeten blijken. Ook de combinatie van moxifloxacin en linezolid (**hoofdstuk 3.2**) leek effectief voor de behandeling van TB in ons muis-TB model, waarbij we een snelle afname van de mycobacteriële load (colony forming units (CFU)'s) zagen. De behandelduur van 14 weken in ons experiment was echter te kort om de mycobacteriën in de long te elimineren, waardoor we geen uitspraak over de mate van relapse konden doen. Omdat uit eerdere studies gebleken is dat effluxpompremmers mogelijk de werking van bepaalde tuberculosemiddelen kunnen versterken, door het remmen van de effluxpompen van macrofagen, maar ook die van *M. tuberculosis* zelf, hebben we gekeken of de effluxpompremmer verapamil de effectiviteit van de moxifloxacin-linezolid combinatie kon verbeteren. Dit leidde echter nauwelijks tot een grotere afname van het aantal CFU's. Om de exacte waarde van deze bevinding te onderzoeken zou de studie herhaald moeten worden met een langere behandelduur en follow up.

Om de activiteit en effectiviteit van nieuwe TB-middelen en -regimes te kunnen onderzoeken zijn goede preklinische modellen met een hoge translationele waarde van essentieel belang. *M. tuberculosis* kan zich afhankelijk van de omstandigheden snel delen, langzaam of helemaal niet. Wanneer de bacteriën niet meer delen noemen we deze metabole toestand dormant (slapend). Het is bekend dat wanneer *M. tuberculosis* dormant is, het veel minder gevoelig is voor antibiotica, wat ook één van de redenen is dat de

behandelduur van TB zo lang is. Om de therapieduur te verkorten ligt daarom de focus van veel preklinisch onderzoek op het effectiever behandelen van deze mycobacteriële populatie. Derhalve is het van belang om goede preklinische modellen te hebben die deze metabole toestand goed kunnen simuleren en daarmee ook betrouwbare voorspellingen te kunnen doen ten aanzien van de effectiviteit van de behandeling in patiënten. Het nabootsen van deze metabole toestand is in veel *in vitro* modellen een tijdrovende en complexe zaak. Er bestaat echter een *M. tuberculosis* stam (18b) die voor zijn groei afhankelijk is van de aanwezigheid van streptomycine. In afwezigheid van streptomycine, stopt deze *M. tuberculosis* binnen 10 dagen met delen en zal er een volledig dormant populatie ontstaan. Deze 18b stam hebben wij gebruikt om de activiteit van de middelen isoniazide, rifampicine, moxifloxacin en bedaquiline te onderzoeken, hetgeen in **hoofdstuk 4.1** beschreven staat. Hiervoor hebben we met de 18b stam in zowel dormant als actief delende toestand verschillende time-kill kinetics assays uitgevoerd. Hierbij hebben we de actieve en de dormant populatie gedurende 7 dagen aan verschillende concentraties antibiotica blootgesteld en op verschillende dagen samples genomen om de hoeveelheid CFU's te bepalen. Om een goede vergelijking te kunnen maken tussen het effect van de verschillende antibiotica op de actief delende en dormant 18b hebben we een nieuwe methode gebruikt. Hierbij is gekeken naar het verschil in de area under the curve (AUC) tussen de onbehandelde en de behandelde samples. Hiermee konden we aantonen dat rifampicine het meest actief was tegen dormant 18b, waarbij er vrijwel geen verschil in activiteit van rifampicine was tegen actieve en dormant 18b. Omdat sommige subpopulaties van *M. tuberculosis* beter identificeerbaar zijn in vloeibaar medium hebben we ook de samples van dag 0 en dag 6 in vloeibaar medium gekweekt om de tijd nodig tot groei te bepalen. De resultaten suggereerden dat er mogelijk een extra subpopulatie van *M. tuberculosis* binnen de dormant 18b populatie in de rifampicine behandelde groep aanwezig was, die alleen in vloeibaar medium aangetoond kon worden. Op basis van de resultaten van deze studie concluderen we dat onze 18b time-kill-kinetics assay een nuttig model is voor het evalueren van de activiteit van tuberculosemiddelen tegen *M. tuberculosis* in verschillende metabole toestanden. Daarnaast heeft ons onderzoek het belang laten zien van het meten met meerdere uitkomstmaten, aangezien dit meer inzicht geeft in het effect van de antibiotica op meerdere mycobacteriële subpopulaties.

In **hoofdstuk 4.2** hebben we de activiteit van hoge doseringen rifampicine en rifapentine getest in ons hollow fiber infection model (HFIM). Dit is een model waarin de menselijke farmacokinetiek nagebootst kan worden en op elk willekeurig moment samples genomen kunnen worden om de mycobacteriële load te bepalen. Om de plaats van dit model in het preklinisch onderzoek naar de potentie van TB middelen beter te kunnen bepalen hebben we de resultaten van onze experimenten vergeleken met de resultaten van klinische trials.

In ons HFIM zagen we vrijwel geen concentratie-afhankelijke effecten voor beide rifamycines. Dit komt overeen met de resultaten van andere HFIM studies, maar is in tegenspraak met de resultaten van klinische studies, die wel meer effectiviteit lieten zien wanneer er hogere doseringen gebruikt werden. In de klinische studies is deze concentratieafhankelijkheid alleen significant aangetoond indien er vloeibare kweken bij patiënten waren afgenomen, maar niet voor de CFU resultaten. Mogelijk is dit ook een van de redenen waarom wij dit effect niet in ons HFIM konden aantonen, aangezien we alleen CFU's gemeten hebben. Daarnaast zijn bij TB patiënten vele factoren van invloed op de respons op antibiotica, die in onze HFIM experimenten nu niet meegenomen zijn, zoals de aanwezigheid van meerdere metabole subpopulaties, intracellulaire bacteriën, verschillen in weefselpenetratie en verschillende immunologische factoren. Deze factoren kunnen ook bijdragen aan de gevonden verschillen tussen de uitkomsten in het HFIM en klinische studies.

Kortom, de resultaten van hogere doseringen rifamycines in een standaard HFIM kwamen niet volledig overeen met bestaande klinische data. Hierdoor is de plaats van het HFIM in deze vorm binnen het preklinisch onderzoek nog niet helemaal duidelijk, maar lijkt het HFIM het meest bruikbaar voor het maken van doseerschema's door de PK/PD parameter te achterhalen welke het meeste effect sorteert. Om de translationele waarde van het HFIM te verhogen zou het interessant zijn om andere factoren in het model te incorporeren zoals geïnfecteerde macrofagen, of verschillende metabole populaties.

Concluderend, hebben we door de hierboven beschreven onderzoeken meer inzicht gekregen in de diagnostiek, en potentiële nieuwe behandelingen van TB. Waarbij we de nieuwe QuantiFERON test hebben gevalideerd, maar deze helaas niet in staat bleek de behandelindicatie beter vast te stellen. Verder hebben we aangetoond dat BDL een veelbelovende nieuwe behandelcombinatie voor resistente TB is, evenals de moxifloxacin-linezolid combinatie. De toegevoegde waarde van verapamil als drug-potentiator bleek echter beperkt. Ons 18b TKK model lijkt een toegevoegde waarde te hebben binnen het preklinisch onderzoek, terwijl de resultaten van het HFIM in zijn huidige vorm in dat kader tegen vielen.

Chapter 7

Appendices

Chapter 7.1

Curriculum vitae

CURRICULUM VITAE

Elise Dagmar Pieterman was born on the 2nd of June in 1989 in Nijmegen. She received her gymnasium diploma in 2007 from the Sint-Jans Lyceum in 's-Hertogenbosch. In the same year she started with her medical training at the Erasmus Medical Centre in Rotterdam. Her graduation research focussed on (recurrent) thrombotic events in HIV patients, which she performed on the department of Medical Microbiology and Infectious Diseases of the Erasmus MC under supervision of dr. C. Rokx. In 2015 she obtained her Medical Doctor degree. In 2016 she started with her PhD research regarding improving the treatment of tuberculosis at the department of Medical Microbiology and Infectious Diseases of the Erasmus MC under supervision of prof. dr. A. Verbon, dr. J.E.M. de Steenwinkel and dr. H.I. Bax, of which the result can be read in this thesis. During her PhD research she attended from 2017 the Master Evidence Based Practice in Health Care at the University of Amsterdam, which she completed successfully in 2019. In 2020 she started as an internal medicine resident in the Franciscus Gasthuis & Vlietland, where she started in January 2021 with her medical specialisation in internal medicine under supervision of dr. Y.C. Schrama and dr. A.A.M. Zandbergen.

Chapter 7.2

List of publications

LIST OF PUBLICATIONS

Publications for this thesis

A multicentre verification study of the QuantiFERON®-TB Gold Plus assay
E.D. Pieterman, F. Liqui Lung, A. Verbon, H.I. Bax, C.W. Ang, J. Berkhout, G. Blaauw, A. Brandenburg, N.D. van Burgel, A. Claessen, K. van Dijk, M. Heron, M. Hooghiemstra, R. Leussenkamp-Hummelink, E. van Lochem, I.H.M. van Loo, B. Mulder, A. Ott, O. Pontesilli, A. Reuwer, P. Rombouts, V. Saegeman, M. Scholing, S. Vainio, J.E.M. de Steenwinkel
Tuberculosis (Edinb). 2018 Jan;108:136-142

Assessment of the additional value of verapamil to a moxifloxacin and linezolid combination regimen in a murine tuberculosis model.
Elise D. Pieterman, Lindsey H.M. te Brake, Gerjo J. de Knegt, Aart van der Meijden, Jan-Willem C. Alffenaar, Hannelore I. Bax, Rob E. Aarnoutse, Jurriaan E.M. de Steenwinkel
Antimicrob Agents Chemother. 2018 Aug 27;62(9):e01354-18

Advanced quantification methods to improve the 18b dormancy model for assessing the activity of tuberculosis drugs *in vitro*
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Knowledge-based reconstruction for measurement of right ventricular volume on CMR images in a mixed population.
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Incidence and Risk Factors for Venous Thrombosis in Human Immunodeficiency Virus (HIV) Infection, Data from the Dutch Athena Cohort Study.
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Oral presentation ASH, 2016

Low-dose, automated TAVR guidance 3rd generation dual-source CT.
Saru RG, Dijkshoorn ML, Ouhlous M, **Pieterman ED**, Krestin GP, Nieman K.
Oral presentation RSNA, 2014

Quantification of left ventricular regional myocardial function using MRI feature tracking in healthy children - a dual-center study.
Eichhorn JG, Buss S, Foell A, Helling A, Robbers-Visser D, **Pieterman ED**, Bossers S, Lossnitzer D, Gorenflo M, Helbing WA.
Journal of Cardiovascular Magnetic Resonance, 2013

Chapter 7.3

PhD Portfolio

PHD PORTFOLIO

(inter)national conferences and courses

2016 Course on translational research and medicine development, Liverpool
2017 Workshop Photoshop and Illustrator CS6, Rotterdam
2017 Nederlandstalige tuberculose en diagnostiek dagen, Groningen
2018 Workshop Clinical pharmacology of TB drugs, Den Haag
2018 Graph Pad Prism course, Rotterdam
2018 Biomedical English Writing course, Rotterdam
2018 Research integrity, Rotterdam
2019 Master Evidence Based Practice, Amsterdam

Presentations

2017 QFT-plus validation study, Nederlandstalige tuberculose diagnostiek dagen, Groningen
2018 Efficacy assessment of bedaquiline, delamanid and linezolid as an MDR-TB regimen in a TB mouse-model, NCOH-AMR, Nijmegen
2018 The Hollow Fibre Infection Model to further fill our preclinical drug development pipeline; focus on rifampin dosing, Clinical pharmacology of TB drugs workshop, Den Haag
2019 Mycobacterium tuberculosis (in het bijzonder: uitleg en interpretatie IGRA), Serologiecursus, Amsterdam

Teaching

2016 Supervising research internship of infection and immunity master student of Erasmus University
2016 Supervising research internship of medicine master student of University of Leiden
2016 Supervising research internship of medicine master student of Erasmus University
2018 Vaardigheidsonderwijs Bachelor medicine of Erasmus University
2018 Summer course Master Infection and Immunity of Erasmus University

Chapter 7.4

Dankwoord

DANKWOORD

In dit laatste hoofdstuk van mijn proefschrift wil ik graag diegene bedanken die hier direct of indirect een belangrijke bijdrage aan geleverd hebben.

Allereerst, mijn promotor, Annelies Verbon. Ongeveer 5 jaar geleden spraken wij elkaar over mijn wens om onderzoek te doen bij de MMIZ naar aanleiding van mijn leuke tijd tijdens mijn masteronderzoek en oudste coschap. Jij vertelde me dat er verschillende opties waren en gelukkig mocht ik ook daadwerkelijk beginnen. Tijdens mijn onderzoeksfase was je vooral op de achtergrond betrokken en ik was altijd erg onder de indruk hoe jij in korte tijd alles snel wist te analyseren en tot de kern te brengen.

Mijn copromotoren, Jurriaan de Steenwinkel en Hannelore Bax. Ik wil jullie enorm bedanken voor de ontzettend fijne samenwerking in de afgelopen jaren. Ik heb heel veel op onderzoeksgebied en schrijfstijl van jullie geleerd. De besprekingen waren naast leerzaam ook erg gezellig, evenals de uitstapjes naar Groningen en Den Haag. Maar bovenal wil ik jullie bedanken voor jullie oprechte persoonlijke betrokkenheid ook buiten onderzoeksgebied, hoe jullie ondanks jullie drukke agenda's veel rekening hielden met mijn tijdslijnen die vaak net wat te krap door mij bedacht waren en dat ik vrijwel anytime voor vragen of problemen bij jullie terecht kon. Jurriaan, jij weet zelfs de meest vervelende boodschap nog in iets grappigs te verpakken. Daarbij heb je enorm veel creatieve ideeën en het was leuk en leerzaam om daarover van gedachten te wisselen. Hannelore, het was ontzettend fijn om binnen het labonderzoek ook een echt klinische dokter betrokken te hebben. Je hebt hele scherpe analyses en weet moeilijke dingen op een heldere manier te verwoorden. Daarbij heb jij mij tijdens mijn oudste coschap echt enthousiast gemaakt voor de infectiologie en kijk er ook erg naar uit om straks tijdens de opleiding in het EMC weer samen te werken.

Graag wil ik de overige leden van de leescommissie, prof. dr. W. Bitter, prof. dr. A. Geluk en prof. dr. J.H. Richardus, hartelijk danken dat jullie in deze drukke COVID periode tijd hebben vrijgemaakt voor het lezen en beoordelen van mijn proefschrift. Ook wil ik graag prof. dr. R. van Crevel, dr. O.W. Akkerman en dr. M. Bakker bedanken voor het plaatsnemen in de grote commissie.

Casper Rokx, jij hebt aan het begin gestaan van mijn onderzoekswerk binnen de MMIZ. Voor mijn masteronderzoek kreeg ik van jou de mogelijkheid om onderzoek te doen naar trombose en HIV. Dank voor deze mooie kans, maar zeker ook voor je begeleiding des tijds ten aanzien van loopbaankeuzes en het vinden van een plek voor mijn oudste coschap. Ik heb het klinisch onderzoek des tijds als enorm leuk en leerzaam ervaren en is ook een van de redenen geweest om later een master klinische epidemiologie te volgen. Inmiddels ben je al even staflid in het EMC dus ik kom je tijdens de opleiding vast weer tegen en wie weet zit er ooit weer een samenwerking in op onderzoeksgebied.

Daarnaast wil ik graag de TB groep bedanken. Allereerst Aart, heel erg bedankt voor al je hulp op het lab, maar ook in de laatste fase met het maken van grafieken en het oplossen

van problemen die ik vanuit huis niet goed kon regelen. Behalve dat ik altijd alles aan je kon vragen, was het ook altijd erg gezellig om even het weekend door te nemen en de vorderingen qua hardlooptrainingen te horen. Corné ook jij hebt mij enorm geholpen met het aanleren van labvaardigheden, helaas is ons MBL project nooit echt een succes geworden, maar dat lag in elk geval niet aan de inzet. Gerjo, je bent natuurlijk al even weg bij de MMIZ, maar je hebt me tijdens het opstarten van mijn onderzoek met veel van de procedures wegwijs gemaakt en het was leuk je laatst in je nieuwe rol in het AMC tegen te komen, helaas is dat koffietje er door COVID niet meer van gekomen, maar wie weet in de toekomst. Heleen, dank voor je hulp bij de muizenstudies. Marjan, dank voor de fijne tijd in 'jouw' lab. Je hebt me veel over labwerk geleerd en stond altijd klaar om te helpen. Sanne, bedankt voor jouw hulp bij ons hollow fiber project. Van jou heb ik echt geleerd hoe belangrijk het is op het lab om precies te werken, zaken te standaardiseren en duidelijk procedures te beschrijven. Dit heeft er zeker voor gezorgd dat de voorbereiding voor veel projecten goed was, waardoor de experimenten ook beter verliepen.

Maarten, inmiddels zelf al bijna phd student af, maar je hebt mij tijdens je masteronderzoek deed ontzettend goed geholpen met de 18b studie. Je hebt een hele leuke creatieve kijk op dingen, waardoor je met veel mooie nieuwe onderzoeksideeën kwam. Daarnaast was je ook een erg gezellige collega en bureaubuurman. Gelukkig lijken onze phd projecten toch iets beter te verlopen dan ons avocadoplant project..

Rixt, wat bofte ik toen ik naast jou terecht kwam toen ik begon met mijn phd traject. Jij was ook net begonnen en dat schepte meteen een band. Ik had altijd zin om na het weekend weer even bij te kletsen over alles, waarbij soms de halve ochtend alweer voorbij was voor we echt aan het werk gingen. Ik heb je erg gemist toen je opeens naar Australië ging, maar het was leuk om te horen hoe het leven daar was en hoe erg je daarvan genoten hebt. Ik ben blij dat we elkaar nog steeds spreken en dat ik af en toe een update over mijn pannekoekenplant baby krijg.

Hassna, ook wij begonnen ongeveer tegelijkertijd aan ons onderzoek, helaas ging jij alleen al een stuk eerder weg. Vond het altijd erg gezellig met je en leuk dat we nu waarschijnlijk toch ongeveer tegelijk ons proefschrift gaan verdedigen.

Carla, bedankt voor het voorbereiden van de honderden liters platen en broth..

Verder wil ik graag alle andere medewerkers van de MMIZ bedanken. In de afgelopen 6 jaar die ik hier gewerkt heb, heb ik een ontzettend fijne, leerzame en gezellige tijd gehad zowel als oudste coassistent, masteronderzoekstudent en als phd student.

Verder wil ik de arts-assistenten van de interne geneeskunde in het Franciscus bedanken voor de flexibiliteit met de roosters, waarbij ik erg veel (avond)diensten heb kunnen doen, waardoor ik genoeg tijd heb gehad om mijn proefschrift af te ronden. Daarbij was er niet meer zoveel tijd (en mogelijkheid door COVID) om privé leuke dingen te doen, maar was het gelukkig op werk wel altijd erg gezellig.

Tiarah, het was altijd heerlijk om tussen de experimenten door weer even samen te lunchen ergens tussen het EMC en het oogziekenhuis in en ben heel blij dat we nu niet alleen een stuk dichter bij elkaar wonen maar nu ook nog in hetzelfde ziekenhuis werken!

Pauline, dank voor onze vriendschap van de afgelopen 18 Jaar. Het is altijd een welkome afwisseling om het over heel andere zaken dan medische dingen te hebben, maar tegelijkertijd was je ook altijd erg betrokken bij hoe mijn onderzoek vorderde.

Annelie, Charlotte, Dana, Lara, Lesley, Lise, Marieke, Solange en Warnyta, dank voor alle keren dat we pas om half 9 gingen eten omdat ik nog uit Rotterdam moest komen, jullie blijvende interesse in de totstandkoming van dit proefschrift en de fantastische studententijd die ik met jullie gehad heb. Annelie in het bijzonder ook dank voor jou omdat zonder jouw logeerkamer de logistiek ten aanzien van mijn hollow fiber experimenten een stuk problematischer was geweest en de daarbij behorende etentjes maakten deze onderzoeksavonden ook meteen een stuk gezelliger.

Verder wil ik ook graag mijn schoonouders, Willem en Ineke bedanken voor jullie interesse in mijn onderzoek, de vele artikelen over tuberculose die ik van Willem doorgestuurd kreeg en onze gesprekken over onderzoek en wetenschap in het algemeen. Daarbij zijn jullie natuurlijk een belangrijke steun geweest bij de zorg voor Philip in het afgelopen drukke jaar.

Mijn broertjes, Kay en Roel en nu ook mijn paranimfen. Het was heel gezellig om de afgelopen jaren samen met jullie te studeren in het EMC, lunchen en alle hoogte en dieptepunten van onze studie te kunnen delen.

Mijn zusje Myrthe, dank voor alle logeerpartijen als ik weer eens tot laat experimenten moest doen en niet meer met de trein naar Amsterdam kon. Jammer dat er nu je werkt geen tijd meer is voor lekkere lunchmomentjes bij Vermeyden, maar dat moeten we gewoon wat vaker in het weekend gaan plannen.

Papa en mama, dankzij jullie heb ik een heerlijke jeugd gehad, wat natuurlijk de belangrijkste basis is geweest voor mijn verdere ontwikkeling later en dus ook voor dit boekje. Mama, het was heel leuk om het met jou het over mijn onderzoek te hebben en hoe jij door mijn onderzoek weer nieuwe ideeën kreeg voor de oncologie. Papa, het was natuurlijk enorm gezellig om met jou in hetzelfde ziekenhuis te werken ook al kwamen we elkaar vrijwel nooit spontaan tegen. Ik mis onze regelmatige lunches. Daarbij ben ik jullie enorm dankbaar voor jullie goede zorg voor Philip in het afgelopen jaar, waardoor ik altijd met een fijn gevoel naar mijn werk kan.

Lieve Philip, jij hebt natuurlijk geen echte bijdrage aan dit boekje geleverd, maar wel mijn leven nog een stuk mooier gemaakt dan het al was.

Bart, jij bent onmisbaar geweest in dit hele traject. Je hebt me altijd gestimuleerd om alles te doen wat ik wil, ook al zorgde dat ervoor dat ik de afgelopen jaren erg veel aan het werk en studeren was. Jij zorgde ervoor dat dit mogelijk was en alles thuis soepel verliep. Ik kan me niemand beters naast mij wensen.