ptimizing TB treatment

Gerjo de Knegt

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Optimaliseren van de behandeling van tuberculose

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Voor papa (10-5-1958 – 26-06-2007)

Jouw stelling was altijd: *"Iedereen in Nederland kan een auto rijden, maar door hard te werken bepaal je zelf of dat een oude Opel Corsa is of een mooie nieuwe bak"*

Chapter 1

Introduction and outline of the thesis

Tuberculosis (TB) has become the single largest cause of death of any infectious disease [1]. Today, TB is also one of the top 10 causes of death worldwide. Over the past two hundred years it has killed more than a billion lives, more than any other infectious disease in history [1]. The World Health Organization (WHO) estimated that in 2015 TB killed 1.8 million people and that there were 10.4 million new TB cases, including one million children [2]. Globally, three people die as a consequence of TB every minute. To stop this insidious disease, the WHO launched the 'END TB STRATEGY' in May 2014. To realize the vision 'a world free of TB', targets were set including a 95% reduction in number of TB deaths and a 90% reduction in TB incidence rate in 2035 compared to 2015. Therefore, new treatment options are urgently needed to attain these targets. The WHO targets are even higher when one realizes that an estimated two to three billion people are latently infected with *Mycobacterium tuberculosis.* Of these latently infected people 5 to 15% will develop active TB during their life [2]. Anyone can get TB, but several factors can increase the risk of developing active disease. The main risk factors are health problems affecting the immune systems like HIV, but also smoking or substance abuse, living in poverty and diabetes are known risk factors [3].

Mycobacterium tuberculosis

The bacterium causing TB is called "*Mycobacterium tuberculosis*". This bacterium has been with humans for millennia. Descriptions of a TB-like disease can be found in the Vedic writings of India (~1500 BC) [4] and in the Old Testament of the Bible (~1300 – 400 BC) [5]. In Greek the disease was called "phthisis", meaning "wasting away". Referring to the symptomatic course of pulmonary TB, which was the basis for the common 19th century name "consumption". The actual tubercle bacillus was discovered by Robert Koch in 1882 [6]. It belongs to the genus of Mycobacteria which also includes *Mycobacterium leprae*, causing leprosy, and *Mycobacterium bovis,* causing TB in cattle. The genome of *Mycobacterium tuberculosis* is one of the biggest genomes among bacteria, is GC-rich (65%) and encodes about 4000 proteins [7]. *Mycobacterium tuberculosis* is classified as Gram-positive and has a thick cell wall built of polysaccharides, peptidoglycans and mycolic acid. This thick cell wall is impermeable and renders the bacteria acid-fast. This feature can be used to visualize the bacteria with a Ziehl-Neelsen staining for microscopy.

Pathogenesis

Mycobacteria causing pulmonary TB enter the body via inhaled aerosols, small droplets produced by coughing TB-patients. After entering the airways *Mycobacterium tuberculosis* can be phagocytosed by alveolar macrophages. After infection, a pro-inflammatory response is induced and mono nuclear cells and T-lymphocytes are attracted to build a granuloma. At this stage of the infection, the immune system of healthy individuals can control the pathogen but they remain latently infected. However, the host-pathogen

interaction is a delicate balance. On the one hand, *Mycobacterium tuberculosis* requires an immune response that is adequate to establish a granuloma, but not to strong that it lead to complete sterilization. On the other hand, inflammation over time should be sufficient to promote the eventual breakdown of some granulomas. These granulomas can maturate to different states; i.e. solid, necrotic and caseous. In the caseous state, *Mycobacterium tuberculosis* strongly increases in number before the granulomas burst open and the mycobacteria will be released into the airways and coughed out and the circle of infection is started.

The 'classic' clinical symptoms of TB are cough, severe weight loss, night sweats and fever, but also weakness, chestpain, loss of appetite and chills are common. When left untreated, 70% of smear-positive pulmonary TB-patients are expected to die within ten years after infection [8].

TB-drugs

Before the Second World War there was no drug available to treat TB. But in 1944 streptomycin was isolated from *Streptomyces griseus* by Schatz and Waksman and para aminosalicylic acid (PAS) was discovered and thus the first steps in TB treatment were made [9,10]. In 1952 isoniazid was discovered and by combining these drugs an effective regimen was possible. However, treatment duration was at least 18 months [11, 12]. In 1967 the second cornerstone drug rifampicin was first used for TB. Strikingly, this still forms the base for the current drug regimen [13].

The standard treatment recommended by the WHO for drug-susceptible TB consists of a 2-months intensive phase with at least isoniazid, rifampicin, pyrazinamide and ethambutol, followed by a 4-months continuation phase with at least isoniazid and rifampicin. This standard treatment, often referred to as the 2HRZE - 4HR regimen can only be used to treat drug susceptible mycobacteria and is associated with a 5 to 8% chance of relapse of infection [14, 15].

The mycobacterial population in a TB patient is considered not to be homogeneous [16]. It consists of mycobacteria in various metabolic states i.e. fast-replicating, slow-replicating and non-replicating mycobacteria, which can respond differently to antibiotic treatment. It is thought that isoniazid is responsible for the elimination of the fast-replicating mycobacteria in the first phase of treatment (2 months), whilst for elimination of the slow and non-replicating population other drugs are needed [17]. For a therapy to be successful, the combination of drugs must be active against both the replicating and the persistent subpopulation. Persistent bacteria are considered hard to eliminate; they are thought to be in a slow-metabolic state possibly due to environmental stress, such as low oxygen levels, lack of nutrients or low pH, which makes them less susceptible to killing by drugs [18]. What is more, they reside inside the thick-walled granulomas where they are almost unreachable for antibiotics.

Resistance

Drug-resistant TB poses a major threat to control of TB worldwide [19]. Soon after the introduction of streptomycin the first single-drug resistant mutants were discovered [20]. Nowadays, multidrug-resistant (MDR) *Mycobacterium tuberculosis* strains are frequently isolated. MDR is defined as bacteria that are resistant to at least the first-line drugs isoniazid and rifampicin. The WHO estimated that nearly half a million cases of MDR-TB have occurred in 2015 [2]. The treatment of MDR-TB is complex due to an extended treatment duration of at least 20 months and complexity of administration. Second-line treatment consists of injectables, fluoroquinolones, prothionamide/ethionamide, cyclyserine/terizidone, ethambutol and/or pyrazinamide. Nevertheless, approximately only 50% of the MDR-TB patients respond favourably to treatment [21]. Also, it is calculated that in Europe the treatment costs for a patient with a drug sensitive TB are €334 (41-1299), whereas the treatment of MDR-TB costs €23.272 (2112-77.354) per patient [22].

What is worse, however, that almost 10% of MDR-TB patients were found to have been infected with an extensively drug-resistant (XDR) strain [2]. XDR-TB is defined as a mycobacterial strain being resistant to both isoniazid and rifampicin, to a fluoroquinolone and to at least one of the three injectable second-line drugs amikacin, capreomycin or kanamycin [23]. Its costs are much higher than the costs of MDR-TB treatment, up to mean €93.962 (56.028-180.237) in Europe [22]. Also the mortality rate is higher compared to MDR-TB [24]. On top of this, four countries have recently reported strains that are resistant to all drugs tested, also known as totally drug-resistant TB (TDR-TB) [25-28].

A major problem with the treatment of drug-resistant TB are the tolerability and toxicity of the treatment regimens. Various severe side effects can occur which dramatically reduce the quality of life of patients. Side effects were described by patients with MDR-TB as: "as bad as or worse than the illness itself" and "I thought I was going to die because of the medicines…it was that bad" [29].

Given all these problems, it will be clear that new drugs and better drug combinations, also effective against drug-resistant *Mycobacterium tuberculosis* are urgently needed. Preferably, these new regimens are also affordable, and enabling shortening the treatment duration without severe side effects.

Optimize treatment with new TB drugs

The first new TB-drug since the discovery of rifampicin in 1967 was bedaquiline, a diarylquinoline [30]. Followed by the discovery of delamanid, a nitroimidazooxazole [31]. Both drugs received FDA approval and are currently used in phase 3 clinical trials to find their exact place in the TB treatment. Several other new promising drug candidates, such as PBTZ-169, sutezolid, SQ109 and pretomanid, are currently in development, but it might take quite some years before all preclinical studies are finished [32].

Before new compounds can be used on label for TB-patients they must be extensively tested in *in vitro* and *ex vivo* assays and *in vivo* animal models, followed by clinical development in phase I, phase II and Phase III clinical trials. This preclinical phase can take many years [33]. Therefore, two major TB-consortia, the European PreDiCT-TB consortium and their US counterpart, the "Critical Path to TB Drug Regimens" (CTPR), try to find a more rapid though reliable way of identifying the most potent combinations of new drugs and hasten their arrival in the clinic. Data from *in vitro* models, the hollow fiber infection model, *ex vivo* models, various animals models and data from human trials are combined using mathematical modelling techniques, to allow comprehensive understanding of the translational value of different preclinical models.

Optimize treatment by increasing the efficacy of current drugs

A different strategy in the battle against TB is finding ways to optimize the activity and efficacy of currently used TB-drugs. Increasing the drug concentrations at the site of infection by inhibiting efflux pumps can be an advantageous strategy to enhance the effect of the currently used TB-drugs.

Efflux pumps are protein complexes present in the cell membrane that remove drugs and toxins from inside the cell, thereby lowering the drug concentrations inside the cell. Some pumps are highly specific whereas others can recognize and transport a wide variety of structurally diverse compounds [34, 35]. There are five different groups of efflux pumps, and these can be found in both prokaryotes and eukaryotes. Administered TB-drugs encounter the first transporters in the intestines, liver and kidneys, and next at the site of the macrophages in which the bacteria can reside. After the macrophage membrane, the final barrier is encountered, which is formed by the thick *Mycobacterium tuberculosis* cell wall and the plasma membrane.

Mycobacterium tuberculosis harbours a huge number of transporters involved in the extrusion of exogenous compounds; the genes encoding for only the ABC-transporters occupy about 2.5% of the genome [36]. Efflux pumps can be inactivated by "efflux pump inhibitors" [37]. Different types of efflux pump inhibitors exhibit different modes of action. Some have a broad activity whilst others are specific to one class of efflux pumps [38]. Blocking or inactivation of efflux pumps may increase the TB-drug exposure at the site of infection, without elevating systemic concentrations of the drug associated with adverse effects. In addition, higher intrabacterial concentrations may increase the antimycobacterial effect and prevent selection of drug-resistant mutants [39].

Another new approach to achieve higher intrabacterial concentrations of TB-drugs is the addition of colistin. Colistin, a member of the polymyxins group, is active against *Mycobacterium tuberculosis* through interaction with the bacterial lipopolysaccharide and fatty acids of the bacterial outer membrane [40]. As a result, destabilization of the bacterial membrane occurs leading to enhanced membrane permeability permitting a higher influx

of other TB drugs [41]. The first results are promising, as colistin was found to potentiates the activity of isoniazid and amikacin against extracellular *Mycobacterium tuberculosis* [42].

Taken together, promising drugs and novel therapeutic strategies are currently evaluated at different (pre) clinical stages. Still, the predictive value of the various preclinical models needs to be further optimized.

In this thesis we focussed on optimizing TB treatment. On the one hand, by increasing the efficacy of currently used TB-drugs using efflux pump inhibitors. On the other hand by evaluating the bactericidal activity and sterilizing capacity of currently used TB-drugs in our mouse TB model, to optimize the predictive value of our mouse TB model.

Aim of this thesis

The overall aim of this thesis is to explore and study new therapeutic options to optimize TB treatment.

The specific objectives are:

- To assess the therapeutic potential of co-administration of efflux pump inhibitors, i.e. thioridazine or SILA-421 with the standard treatment regimen
- To study a moxifloxacin and linezolid combination as a novel treatment regimen for MDR-TB and potentiate this combination with efflux pump inhibitors and cell wall destabilizing drugs
- To characterize the effect of rifampicin on the transcription profile of a rifampicin-susceptible and a rifampicin-resistant *Mycobacterium tuberculosis* strain
- To study the added value of using a *Mycobacterium tuberculosis* strain of the clinical highly relevant Beijing genotype in our mouse TB model
- To compare Colony Forming Units counting, the Time to Positivity assay and the Molecular Bacterial Load assay, and explore whether the last assays can detect a mycobacterial subpopulation which is unable to grown on solid media

Outline of this thesis

Optimizing TB treatment:

In this thesis we describe different ways to optimize TB therapy, with a special focus on efflux pump inhibition. **Chapter 1** contains a general introduction and short outline of this thesis. In **Chapters 2 & 3** the antimycobacterial effects of the efflux pump inhibitor thioridazine and SILA-421 were explored. Both compounds were tested *in vitro* as single drug as well as in combination with isoniazid, rifampicin and pyrazinamide. Both compounds were also studied in our mouse TB model when added to the standard regimen of isoniazid, rifampicin and pyrazinamide. In **Chapter 4** we evaluated the activity of a moxifloxacin plus linezolid combination as a novel regimen for MDR-TB treatment. Fur-

thermore, we explored the effects of the potentiator drugs colistin, verapamil, SQ109 and timcodar on the activity of the moxifloxacin plus linezolid regimen. All compounds were *in vitro* tested as single drug, and the potentiator drugs were also studied in combination with the moxifloxacin and linezolid combination. In **Chapters 5 & 6** we described the validation of the "Dried Blood Spot" technique for moxifloxacin and linezolid for the use in pharmacokinetic and pharmacodynamics (PK/PD) studies in mice. We also defined the pharmacokinetic equivalent dose in mice for both drugs.

In **Chapter 7** we described the effect of rifampicin on the overall transcription profile of *Mycobacterium tuberculosis* and addressed in particular the differences between rifampicin-susceptible and rifampicin-resistant strains. We performed a genome-wide transcriptional profile analysis using microarray technology and compared the profiles of an isogenic pair of rifampicin-susceptible and rifampicin-resistant strains after exposure to rifampicin.

Optimizing our preclinical TB model:

In **Chapter 8** we described an evaluation of the suitability of our mouse TB model to determine therapeutic activity and sterilizing capacity of drug combinations. We assessed the bactericidal activity of a broad range of TB drugs and the sterilizing capacity of ten rifampicin containing and non-rifampicin containing treatment arms.

To further optimize the way we monitor the mycobacterial load during infection and treatment we compared colony forming unit (CFU) counting with time to culture positivity and the molecular bacterial load assay. CFU counting is a much used method to monitor drug activity and therapeutic efficacy in *in vivo* studies but may not be the most optimal method. Therefore, we explored in **Chapter 9** whether mycobacterial subpopulations that are unable to grow on solid media can be detected using the time to positivity assay or the molecular bacterial load assay. **Chapter 10** provides a summary of results of the studies included in this thesis and discusses the main findings. The discussion ends with the recommendations for future research. In **Chapter 11** the main findings are summarized in Dutch.

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Chapter 2

Enhancement of *in vitro* activity of tuberculosis drugs by addition of thioridazine is not reflected by improved *in vivo* therapeutic efficacy

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ABSTRACT

Objectives: Assessment of the activity of thioridazine towards *Mycobacterium tuberculosis*, *in vitro* and *in vivo* as a single drug and in combination with anti-tuberculosis (TB) drugs.

Methods: The *in vitro* activity of thioridazine as single drug or in combination with TB drugs was assessed in terms of MIC and by use of the time-kill kinetics assay. Various *Mycobacterium tuberculosis* strains among which the Beijing genotype strain BE-1585 were included.

In vivo, mice with TB induced by BE-1585 were treated with a TB drug regimen with thioridazine during 13 weeks. Therapeutic efficacy was assessed by the change in mycobacterial load in the lung, spleen and liver during treatment and 13 weeks post-treatment.

Results: *In vitro*, thioridazine showed a concentration-dependent and time-dependent bactericidal activity towards both actively-replicating and slowly-replicating *Mycobacterium tuberculosis*. Thioridazine at high concentrations could enhance the activity of isoniazid and rifampicin, and in case of isoniazid resulted in elimination of mycobacteria and prevention of isoniazid-resistant mutants. Thioridazine had no added value in combination with moxifloxacin or amikacin.

In mice with TB, thioridazine was poorly tolerated, limiting the maximum tolerated dose (MTD). The addition of thioridazine at the MTD to an isoniazid-rifampicin-pyrazinamide regimen for 13 weeks did not result in enhanced therapeutic efficacy.

Conclusions: Thioridazine is bactericidal towards *Mycobacterium tuberculosis in vitro*, irrespective the mycobacterial growth rate and results in enhanced activity of isoniazid or rifampicin. The *in vitro* activity of thioridazine in potentiating isoniazid and rifampicin is not reflected by improved therapeutic efficacy in a murine TB-model.

INTRODUCTION

Resistance to tuberculosis (TB) drugs is rapidly emerging worldwide with nearly half a million cases of multi-drug resistant TB (MDR-TB) recorded annually [1]. MDR-TB is caused by *Mycobacterium tuberculosis* strains that are resistant to the two most prominent drugs in the treatment of TB; rifampicin and isoniazid. Extensively drug-resistant tuberculosis (XDR-TB) is caused by *Mycobacterium tuberculosis* strains that are resistant to rifampicin, isoniazid to any of the fluoroquinolones and to any of the three second-line injectable drugs such as amikacin, capreomycin or kanamycin [1]. Due to the very poor treatment outcome of MDR-TB and XDR-TB [1], more effective treatment is urgently needed.

Acquired drug resistance in other bacterial species is generally mediated through horizontal transfer of mobile genetic elements [2]. However, in *Mycobacterium tuberculosis* acquired drug resistance is mainly caused by selection of bacteria with spontaneous mutations in chromosomal genes, during sub-optimal treatment [3]. In addition, accumulative evidence suggests an important role for mycobacterial efflux pumps in the extrusion of TB drugs and emergence of drug resistance [4, 5]. Analysis of the genome of *Mycobacterium tuberculosis* reveals that it has one of the largest numbers of putative efflux pumps among bacteria compared to its genome size [6]. These efflux pumps can extrude several compounds and chemicals out of the bacterial cell resulting in decreased intra-cellular drug concentrations [7]. It has also been demonstrated that antibiotics can serve as inducers by regulating the expression of efflux pumps at the level of gene transcription [8].

Phenothiazines are compounds that were previously used as anti-psychotic drugs. Additionally, phenothiazines also show a significant inhibitory effect on the growth of various clinical *Mycobacterium tuberculosis* isolates [9]. Although, the molecular mechanism of anti-mycobacterial activity of phenothiazines is still unclear, there are several hypotheses. Phenothiazines can interact with the DNA metabolism [10], or can disturb the Ca^{2+} -calmodulin signalling [11] and can cause alterations in the bacterial cell membrane [12]. Additionally, there is the alleged ability of phenothiazines to inhibit efflux pumps [5, 13]. One of the phenothiazines is thioridazine, which is active against intra- and extracellular, drug-susceptible and drug-resistant *Mycobacterium tuberculosis* and is concentrated by human macrophages [9, 14, 15].

We hypothesized that combining TB drugs with thioridazine results in increased intracellular drug concentrations and enhancement of the efficacy of the TB drugs.

In the current study, we conducted the *in vitro* concentration-dependent and time-dependent killing capacity (time-kill kinetics) of thioridazine towards a *Mycobacterium tuberculosis* strain of the Beijing genotype [16]. Furthermore, we investigated whether the addition of thioridazine to several TB drugs can enhance the time-kill kinetics of these TB drugs towards a Beijing genotype strain.

Finally, we investigated the therapeutic potential of co-administration of thioridazine to a regimen of isoniazid, rifampicin and pyrazinamide in a murine TB-model, induced by a Beijing genotype strain [17]. Outcome parameters are mycobacterial elimination in infected organs and the prevention of emergence of drug resistance and relapse of TB.

MATERIALS AND METHODS

Mycobacterium tuberculosis cultures

Mycobacterium tuberculosis strains H37Rv (ATCC 27294), H37Rv-H526Y, Beijing-1585 (BE-1585), BE-1585-H526Y and East-African/Indian-1627 (EAI-1627) were used. H37Rv-H526Y and BE-1585-H526Y were derived from their respective parental strains by exposure to 64 mg/L of rifampicin as described before [18]. Resistance was due to the His526Tyr mutation in codon 526 in the *rpoB* region. The MIC of rifampicin for the H37Rv strain is 0.5 mg/L, and for the BE-1585 strain and the EAI-1627 strain is 0.25 mg/L [16]. The MIC of rifampicin for both rifampicin-resistant strains is 256 mg/L.

Mycobacterium tuberculosis suspensions were cultured in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA), supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (OADC, Baltimore Biological Laboratories, Baltimore, 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 at 96 rpm at 37^0C. Cultures on solid media were grown on Middlebrook 7H10 agar (Difco), supplemented with 10% OADC and 0.5% glycerol for 28 days at 37°C with 5% CO_2.

TB drugs

Isoniazid (Hospital Pharmacy; Rotterdam, The Netherlands), rifampicin (Rifadin®, Aventis Pharma B.V, Hoevelaken, The Netherlands), amikacin (Hospira Benelux BVBA, Brussels, Belgium) moxifloxacin (Avelox®, Bayer Schering Pharma A.G, Berlin, Germany) and thioridazine (Sigma Chemical Co, St. Louis, MO, USA) were prepared according to the recommendations of the manufacturers.

Minimal inhibitory concentration (MIC)

The MIC of thioridazine for the *Mycobacterium tuberculosis* strains was determined in duplicate using the agar proportion method as described by the CLSI [19]. In short, colonies grown on solid media were suspended in broth using glass beads and vortexed during 4 minutes. The suspension was allowed to settle down for 30 minutes, after which the supernatant was taken and set to an optical density of McFarland standard 1. Using broth, a 1:10 dilution of this *Mycobacterium tuberculosis* suspension was plated onto solid media containing serial two-fold dilution concentrations of TB drug. After incubation the degree

of growth was assessed. The MIC was defined as the lowest concentration of TB drug that resulted in >99% growth inhibition.

In vitro time-kill kinetics

The concentration-dependent and time-dependent bactericidal activity of thioridazine as a single drug were determined as described previously [18]. In short, low-density cultures of actively replicating BE-1585 or high-density cultures of slowly replicating BE-1585 were exposed to two-fold increasing concentrations at 37^0C. On days 1, 3 and 6 samples (200 µl) were taken, provided that the mycobacterial suspension did not show visible aggregation, and were sub-cultured on drug-free solid media for CFU counts after 28 days of incubation at 37^0C with 5% CO_2.

The time-kill kinetics of the drug combinations in low-density cultures of actively replicating BE-1585 was determined for thioridazine in combination with isoniazid, rifampicin, moxifloxacin and amikacin. The concentrations of TB drugs selected for these experiments resulted in a maximum 10-fold change of *Mycobacterium tuberculosis* numbers after 6 days of single drug exposure compared to day 0. thioridazine concentrations ranging from ¼ x MIC to 4x the MIC were added to TB drugs at two-fold increasing concentrations. On days 3 and 6 samples (200 µl) were taken, provided that the mycobacterial suspension did not show visible aggregation, and were sub-cultured on drug-free and drug-containing solid media with 4-fold the critical concentrations of the drug [19], i.e. 0.8 mg/L isoniazid, 4 mg/L rifampicin, 8 mg/L moxifloxacin or 20 mg/L amikacin to select drug-resistant mutants if present. After 28 days of incubation at 37^0C with 5% CO_2 the number of CFU at the subculture plates was determined.

Synergistic activity of drugs was determined after 6 days of drug exposure and was defined as a $\geq$ 100-fold (Δlog 2) decrease in colony count obtained using the drug combination compared to the colony count obtained using the most active single agent, or when the drug combination resulted in complete mycobacterial elimination while the most active single agent did not.

Animals

Specified pathogen-free female BALB/c mice were obtained from Charles River (Les Oncins, France). At the day of infection, animals were 13-15 weeks old and weighed 20-25 gram. 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 protocol (117-09-04).

Infection

Mice were infected as described previously [17]. In short, mice under anaesthesia were infected by intratracheal instillation of a suspension containing 6.4 x 10^4 CFU of BE-1585, followed by proper inhalation.

Treatment with TB drugs

For thioridazine a dose-finding study was performed in BE-1585 infected mice with four different thioridazine dose-schedules added to a combination of isoniazid, rifampicin and pyrazinamide (HRZ) in human pharmacokinetic-equivalent doses [20]. The basic experimental scheme is shown in table 1. Briefly, treatment was started 2 weeks after infection and mice were randomly divided in five groups in which the thioridazine dosages slowly increased every 3 weeks from 3 up to 12 mg/kg/day in group 1, from 6 up to 18 mg/kg/day in group 2, from 9 up to 27 mg/kg/day in group 3 and from 12 up to 36 mg/kg/day in group 4. Group 5 were controls receiving HRZ only. thioridazine in a total volume of 200 µl Aqua Destillata (AD) was freshly prepared and daily administered intraperitoneally, HRZ in human pharmacokinetic-equivalent doses in a total volume of 300 µl was daily administered subcutaneously [20]. Treatment duration was 13 weeks.

Subsequently, the treatment schedule of thioridazine at the optimal dose obtained in the dose-finding study in combination with HRZ was administered to determine therapeutic efficacy.

Table 1. Basic experimental scheme of the dose finding study

Time (weeks) →	0-2	3-5	6-8	9-15
Treatment group ↓				
1	Infection	THZ 3 mg/kg + HRZ	THZ 6 mg/kg + HRZ	THZ 12 mg/kg + HRZ
2	Infection	THZ 6 mg/kg + HRZ	THZ 12 mg/kg + HRZ	THZ 18 mg/kg + HRZ
3	Infection	THZ 9 mg/kg + HRZ	THZ 18 mg/kg + HRZ	THZ 27 mg/kg + HRZ
4	Infection	THZ 12 mg/kg + HRZ	THZ 24 mg/kg + HRZ	THZ 36 mg/kg + HRZ
5	Infection	HRZ	HRZ	HRZ

THZ = thioridazine, HRZ = isoniazid + rifampicin + pyrazinamide

Determination of viable *Mycobacterium tuberculosis* counts in infected organs

At the start of therapy, after 3, 7 and 13 weeks therapy and 13 weeks therapy followed by 13 weeks relapse assessment mice (n=4 per time point) were sacrificed by CO_2 exposure. The lung, spleen and liver were removed aseptically and processed as described previously [20]. To prevent carry-over of TB drugs, therapy was stopped 72 hours before sacrificing the mice. In addition, tissue homogenate suspensions were centrifuged at 14,000xg for 10 min, and pellets were re-suspended in Phosphate Buffered Saline (PBS). From the tissue

homogenate the 10-fold serial dilutions were performed and samples of 200 µl were cultured on drug-free and drug-containing solid media with 4-fold the critical concentrations of the drug [19], i.e. 0.8 mg/L isoniazid or 4 mg/L rifampicin to select drug-resistant mutants if present.

Assessment of relapse of infection

The number of viable *Mycobacterium tuberculosis* counts in lung, spleen and liver of mice (n=4) was assessed 13 weeks after termination of TB treatment. Relapse was defined as *Mycobacterium tuberculosis*-positive organ cultures, while immediately after termination of treatment organs were *Mycobacterium tuberculosis* culture-negative.

RESULTS

In vitro MIC

The MICs of thioridazine for the five *Mycobacterium tuberculosis* strains tested are presented in table 2. The MIC values for the three susceptible strains as well as the two rifampicin-resistant strains proved to be similar, being 4-8 mg/L thioridazine.

Table 2. MIC values of thioridazine for three wild-type *Mycobacterium tuberculosis* strains (H37Rv, BE-1585 and EAI-1627) and two rifampicin-resistant *Mycobacterium tuberculosis* strains (H37Rv-H526Y and BE-1585-H526Y).

Strain	MIC (mg/L)
H37Rv	4
H37Rv-H526Y	8
BE-1585	8
BE-1585-H526Y	8
EAI-1627	8

In vitro time-kill kinetics

Time–kill kinetic assays were performed for thioridazine as single drug or in combination with isoniazid, rifampicin, moxifloxacin and amikacin for the BE-1585 strain. The activity of thioridazine against low-density cultures of actively replicating BE-1585 is presented in figure 1A. Thioridazine showed a strong concentration- and time-dependent activity in the concentration range tested (0.5 up to 128 mg/L). To achieve ≥99% mycobacterial killing, 16 mg/L thioridazine was needed. To achieve complete mycobacterial elimination 32 mg/L thioridazine was required.

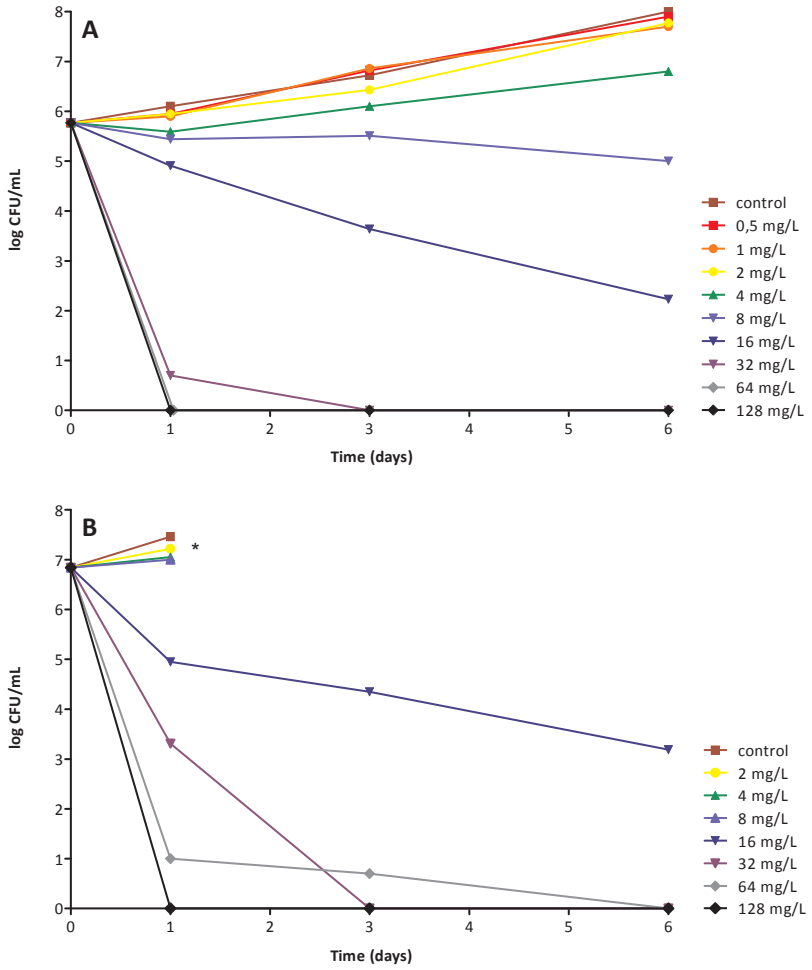

Figure 1. Concentration- and time-dependent bactericidal effect of thioridazine towards BE-1585. Low-density cultures of actively replicating *Mycobacterium tuberculosis* (starting at 5.9 x 10^5 CFU/mL) (**A**) and high-density cultures of slowly replicating *Mycobacterium tuberculosis* (starting at 6.9 x 10^6 CFU/mL) (**B**) were exposed to thioridazine at 2-fold increasing concentrations for 6 days at 37°C under shaking conditions. After 1, 3 and 6 days of drug exposure, samples were taken and quantitative cultures were performed.

* Due to aggregation of the mycobacteria in the culture, accurate CFU counts could not be performed.

Table 3. Effect of addition of thioridazine on the activity of isoniazid, rifampicin, moxifloxacin or amikacin towards BE-1585.

TB drugs tested (mg/L)	Log10 CFU/mL at day 0	Log10 CFU/mL at day 6	TB drug resistant mutants [1 per no. of bacteria]	**Change** in Log10 CFU/mL of the drug combination compared to the most active single agent
Control	5.7	7.3	-	
INH 0.063	5.6	5.4	[1:4.7 x 10^3]	
RIF 0.015	5.8	5.4	NF	
MXF 0.031	5.6	5.1	NF	
AMK 0.25	5.6	4.8	NF	
THZ 4	5.7	5.8	ND	
THZ 8	5.7	4.6	ND	
THZ 16	5.7	1.9	ND	
INH 0.063 + THZ 4	5.6	2.8	NF	-2.6*
INH 0.063 + THZ 8	5.6	0.0	NF	-4.0**
INH 0.063 + THZ 16	5.6	0.0	NF	-2.1**
RIF 0.015 + THZ 4	5.8	3.7	NF	-1.9
RIF 0.015 + THZ 8	5.8	3.4	NF	-1.6
RIF 0.015 + THZ 16	5.8	0.0	NF	-2.5**
MXF 0.031 + THZ 4	5.6	4.7	NF	-0.4
MXF 0.031 + THZ 8	5.6	4.1	NF	-0.7
MXF 0.031 + THZ 16	5.6	2.1	NF	+0.4
AMK 0.25 + THZ 4	5.6	4.1	NF	-0.7
AMK 0.25 + THZ 8	5.6	3.3	NF	-1.3
AMK 0.25 + THZ 16	5.6	1.3	NF	+0.1

* Synergy

** Complete elimination of mycobacteria

ND = Not Determined, NF = Not Found, THZ = thioridazine, INH = isoniazid, RIF = rifampicin, MXF = moxifloxacin,
AMK = amikacin

Low-density cultures of actively replicating BE-1585 were exposed to thioridazine for 6 days at 37°C under shaking conditions. At the time points indicated, samples were taken and quantitative cultures were performed on drug free and drug containing solid media.

The activity of thioridazine in high-density cultures of slowly replicating BE-1585 is presented in figure 1B. Concentrations of 2-8 mg/L thioridazine did not show any activity, resulting in mycobacterial aggregation, whereas 16 mg/L thioridazine resulted in >99% mycobacterial killing. At a concentration of 32 mg/L thioridazine, complete mycobacterial elimination was obtained after three days of exposure.

The results of the time-kill kinetics in low-density cultures of actively replicating BE-1585 after exposure to the drug combinations are presented in terms of Log10 CFU/mL in table 3. In the control BE-1585 cultures CFU counts increased from 5.7 (5.6 – 5.8) to 7.3 (7.0 – aggregation) within 6 days. Changes in CFU count of the BE-1585 cultures after 6 days of exposure to thioridazine as single agent were 5.7 (5.6-5.8) to 5.8 (5.1 – 6.5) for thioridazine 4 mg/L, 5.7 (5.6 – 5.8) to 4.6 (4.1 – 4.9) for thioridazine 8 mg/L and 5.7 (5.6 – 5.8) to 1.9 (1.3 – 2.3) for thioridazine 16 mg/L.

Addition of thioridazine at 4, 8 or 16 mg/L to isoniazid (0.063 mg/L) resulted in enhanced mycobacterial killing. A synergistic effect of the drug combination was obtained, when thioridazine 8 or 16 mg/L was added. Even complete elimination of mycobacteria was obtained and emergence of isoniazid resistance was prevented, while single drug exposure to isoniazid resulted in selection of isoniazid-resistant mutants (table 3). Addition of thioridazine to rifampicin (0.015 mg/L) also showed enhancement of rifampicin activity, but only with the highest concentration of thioridazine (16 mg/L) a clear synergistic activity was found. In contrast, addition of thioridazine to moxifloxacin (0.031 mg/L) or amikacin (0.25 mg/L) resulted in moderate improvement of the activity of moxifloxacin and amikacin, and synergistic activity was not obtained.

In vivo therapeutic efficacy

At two weeks after inoculation with the BE-1585 strain, mice had developed a TB infection including extra-pulmonary dissemination and treatment with thioridazine was initiated. Untreated control animals became moribund by three weeks after infection and were euthanized. The highest dose schedule of thioridazine in combination with HRZ (group 4) resulted in severe toxic events and finally all mice became moribund after 7 weeks of treatment. Of the mice from group 3, one mouse died after 4 weeks of treatment and the other mice became seriously ill and were euthanized. Mice from group 1 and 2 showed abnormal behaviour compared to the controls (group 5) but symptoms disappeared within 3 weeks. Based upon these results the thioridazine schedule of group 2 (from 6 to 18 mg/kg/day) was set as the maximum tolerated dosage (MTD) in our mouse model and used to assess the therapeutic efficacy (figure 2).

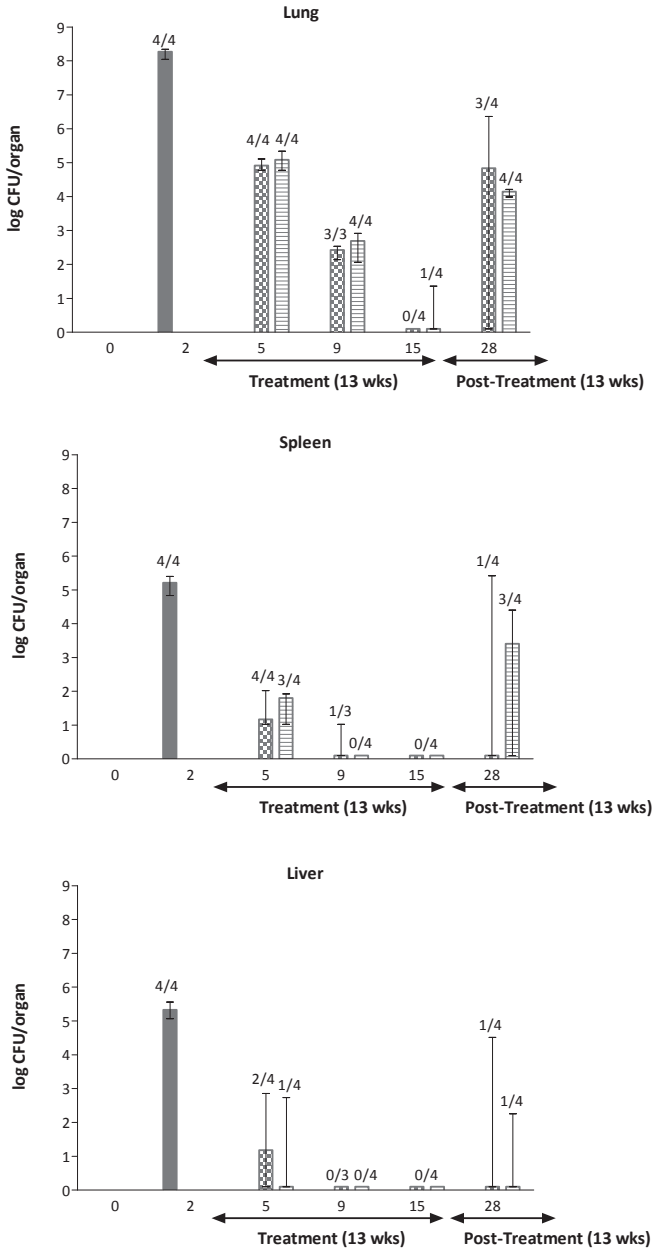

Figure 2. Therapeutic efficacy of treatment with HRZ or a combination of HRZ and thioridazine in mice with TB caused by BE-1585, started 2 weeks after inoculation and continued for 13 weeks. *Mycobacterium tuberculosis* load in infected organs in untreated mice (filled grey bars), in HRZ-treated mice (chequered bars) and in mice treated with HRZ + thioridazine (striped bars). Results are expressed as median ± range (error bars) of the colony forming units (CFU) per organ, n = 4 per time point. Numbers above bars are the numbers of culture-positive mice out of total mice.

To assess therapeutic efficacy the infected mice were treated for 13 weeks with HRZ or HRZ in combination with thioridazine (figure 2). After 13 weeks of treatment, lung, spleen and liver were culture-negative in mice receiving HRZ alone. The same results were obtained in mice receiving HRZ and thioridazine. Spleens and livers were all culture-negative although in one mouse some mycobacteria were found in the lung after 13 weeks of treatment. After a 13 weeks post treatment period in the HRZ treated group, relapse of infection was observed in the lungs of three out of four mice, and in the spleen and liver of one out of four mice (figure 2). All mice treated with HRZ plus thioridazine showed relapse of infection in the lungs, whereas three out of four mice were culture-positive in the spleen and in one out of four mice the liver was culture-positive.

DISCUSSION

Back in the 1960's the first cases were reported in which phenothiazines were used to treat TB-patient [21, 22]. In 1996 Amaral *et al.* postulated that thioridazine should be used in newly diagnosed TB-patients because it was shown that thioridazine inhibited the respiration of a clinical *Mycobacterium tuberculosis* isolate resistant to the first-line anti TB drugs [15]. Today, many years later thioridazine is still seldom used in the treatment of (MDR-) TB, with the exception of compassionate treatment in patients with XDR-TB [23]. The limited use of thioridazine might be explained by relatively few studies that evaluated its efficacy in preclinical models [24-27], by the variable response to thioridazine in these models, and by severe adverse effects that may occur with the use of thioridazine based on its use as a neuroleptic [28]. However, some promising results were obtained with respect to the activity of thioridazine against intracellular mycobacteria regardless the susceptibility status of the mycobacteria [14], and this encouraged further investigation of the use of thioridazine as a TB drug. Moreover, there is evidence that thioridazine is a potent inhibitor of efflux pumps, possibly causing accumulation of drugs inside phagocytic and bacterial cells and as such yielding a synergistic effect [5, 13]. The present study evaluates the activity of thioridazine, as single drug or in combination with TB drugs, towards actively replicating or slowly replicating mycobacteria of the Beijing genotype *in vitro*, and in murine TB caused by the same Beijing strain *in vivo*.

In the present study the *in vitro* activity of thioridazine in terms of MIC was demonstrated towards H37Rv, BE-1585 and EAI-1627 strains and two rifampicin-resistant isogenic mutants of the H37Rv and BE-1585. We found that thioridazine MIC values were similar and conclude that thioridazine is active against extracellular *Mycobacterium tuberculosis*, irrespective their susceptibility or resistance to rifampicin, suggesting that the mechanism of action of thioridazine is not influenced by rifampicin resistance.

Among the *in vitro* susceptibility assays currently used, the MIC assay provides end-point data that are obtained after 4 weeks of *Mycobacterium tuberculosis* exposure to TB drugs. With such an assay only inhibition of mycobacterial growth is detected. In the present study we also performed the time-kill kinetic assay which is more informative with respect to the concentration-dependent and time-dependent activity of the drug (figure 1A and 1B), and also determines mycobacterial killing. Using this assay, we conclude that thioridazine is effective in killing of extra-cellular BE-1585. As a remark, relatively high concentrations of thioridazine (≥ 16 mg/L) are needed to achieve mycobacterial killing. It is also shown that the bactericidal activity is irrespective of the replicating activity of the mycobacteria. The relevance of this observation lies in the fact that the killing capacity of thioridazine towards slowly replicating mycobacteria is important from a therapeutic point of view, as it is thought that deep-seated *Mycobacterium tuberculosis* in tissues during the dormant state probably exhibit low metabolic activity. It is expected that these low-active *Mycobacterium tuberculosis* are difficult to eliminate because most currently used TB drugs are primarily active against actively replicating *Mycobacterium tuberculosis*.

In the present study also combinations of several common TB drugs and thioridazine were tested in low-density cultures of actively replicating BE-1585. In these studies the concentrations of TB drugs were selected that resulted in a maximum of 10-fold change in CFU counts during 6 days of single drug exposure compared to day 0. The value of addition of thioridazine to the TB drugs was most pronounced for isoniazid. With the three concentrations of thioridazine tested in combination with isoniazid, a substantial increased killing of *Mycobacterium tuberculosis* was achieved and synergistic activity was obtained. Thioridazine 8 and 16 mg/L even resulted in complete elimination of the mycobacteria. Furthermore, whereas isoniazid-resistant mutants occurred after exposure of *Mycobacterium tuberculosis* to isoniazid as single drug, the addition of thioridazine at all three concentrations prevented selection of isoniazid-resistant mutants. This result expands upon a prior study of Dutta *et al.* in which they presented in an acute murine model that treatment with isoniazid + thioridazine reduced the frequency of isoniazid-resistant mutants by 7-fold [24]. Machado *et al*. showed *in vitro* that isoniazid-resistance can be reduced by inhibiting certain mycobacterial efflux pumps by exposure to thioridazine [29]. The potential of thioridazine in this respect is of high importance in view of the worldwide emerging isoniazid resistance.

The enhanced activity of TB drugs following the addition of thioridazine was also observed in the combination of thioridazine-rifampicin. This suggests that the activity of thioridazine targeting the cell wall may result in an increased cell wall permeability or inhibition of efflux pumps, enabling an increase in intrabacterial concentrations of rifampicin [30]. The synergy found of thioridazine and rifampicin is in concordance with a study of Viveiros *et al.* in which they also demonstrated an enhanced activity of rifampicin following addition of thioridazine [31]. However, they did not find enhanced activity of isoniazid after

addition of thioridazine [31], which might be explained by the use of poly-drug resistant *Mycobacterium tuberculosis* strains or the much lower concentrations of isoniazid applied in their experiments.

The *in vitro* observations of the present study were realized with thioridazine concentrations that are clinically not achievable. However, it is known that accumulation of phenothiazines can occur within alveolar macrophages [32], and this might justify the application of thioridazine in potentiating the activity of TB drugs in the treatment of TB in a murine model.

Despite the promising results found *in vitro* in the time-kill kinetics following the addition of thioridazine to isoniazid or rifampicin, enhancement of therapeutic efficacy of the HRZ regimen following co-administration with thioridazine was not found in the treatment of TB caused by BE-1585 in mice. Due to severe toxic events following thioridazine administration, the maximum tolerable dosing schedule of thioridazine escalated the dose up to 18 mg/kg/day. We did not perform a pharmacokinetic study, but thioridazine was clearly absorbed by our mice, considering the dose-limiting adverse effects that we observed. Furthermore, the dose of 18 mg/kg/day is close to the 25 mg/kg/day dose that yields exposures similar to those in humans after a daily dose of 25 mg, as shown by Dutta *et al.* in the same mice [24]. Dutta *et al*. also showed that thioridazine in this dose range concentrates up to 30-fold in murine lung tissue relative to serum in this dose range. Therefore, we conclude that addition of thioridazine at the MTD has no additional value in the therapeutic efficacy of HRZ treatment in our murine model. These results are in concordance with a study of Dutta *et al.* in which they also showed in their chronic infection murine model that 2 months treatment with isoniazid + thioridazine or rifampicin + thioridazine does not yield to any additive effect on CFU load in the lung. In their acute model they showed some additive effect of thioridazine in combination with isoniazid, but very limited [24]. In 2013, Dutta *et al.* also demonstrated relatively high mortality when using a range of thioridazine dosages in their guinea pig TB model, and no significant decrease in mycobacterial load was found when thioridazine as single agent was administered for one month with dosages ranging from 2.5 to 80 mg/kg [25].

In contrast to our observations, van Soolingen *et al.* showed in 2010 enhanced therapeutic efficacy in a mouse TB model when thioridazine (32 and 70 mg/kg doses) was added to a HRZ treatment [27]. After 60 days of treatment the *Mycobacterium tuberculosis* load in the lung was significantly lower in the group receiving HRZ + thioridazine compared to the group receiving HRZ only. In spite of the statistical significance achieved, the decrease in *Mycobacterium tuberculosis* load was very limited and lungs still remained culture positive. Also in 2007 Martins *et al.* found a decrease in *Mycobacterium tuberculosis* load in the lung in their murine TB model when mice were treated for 300 days with thioridazine as single agent (0.5 mg/day) [26]. However, no decrease of *Mycobacterium tuberculosis* load in the spleen and liver were observed. Therefore extra-pulmonary organs could remain

a source of *Mycobacterium tuberculosis* causing re-infection of the lung. This can be an explanation for the *in vivo* results observed in the present study, where relapse occurs after 13 weeks therapy followed by a 13 weeks post-treatment period in both the group receiving HRZ + thioridazine as well as in the control group receiving only HRZ.

Based on the data from the present study and earlier studies by Martins *et al.*, Dutta *et al.* and from van Soolingen *et al.* we can conclude that there is a lack of conclusive evidence that proves that thioridazine will be the drug to beat MDR-TB or XDR-TB. However, we provide further evidence that an efflux pump inhibitor indeed can be of added value. Therefore development of less toxic efflux pump inhibitors is urgently needed.

Summarizing, we conclude that *in vitro* thioridazine in itself is bactericidal towards a Beijing genotype *Mycobacterium tuberculosis* strain, irrespective the growth rate of the mycobacteria. Furthermore, the addition of thioridazine to isoniazid or rifampicin results in enhanced activity of both drugs, which is most pronounced for isoniazid. Moreover, the addition of thioridazine prevents emergence of isoniazid resistance. The translational value of the *in vitro* observations to the *in vivo* therapeutic efficacy in the murine TB model caused by the same Beijing genotype strain is disappointing. Due to toxicity only limited dosages of thioridazine are allowed. At the maximum tolerated dose thioridazine has no added value to the therapeutic activity of HRZ in mice.

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Chapter 3

SILA-421 activity *in vitro* against a rifampicin-susceptible and rifampicin-resistant *Mycobacterium tuberculosis,* and *in vivo* in a murine TB model

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ABSTRACT

Due to the emergence of multidrug-resistant tuberculosis (TB) and extensively drug-resistant TB, there is an urgent need for new TB drugs or compounds that improve the efficacy of currently used drugs. In this study we performed time-kill kinetics of SILA-421 as single drug and in combination with isoniazid, rifampicin, moxifloxacin or amikacin against *Mycobacterium tuberculosis.* We also assessed therapeutic efficacy *in vivo* in our mouse TB model. Further *in vitro* analysis was performed with a rifampicin-susceptible and rifampicin-resistant strains of *Mycobacterium tuberculosis.*

When used as single drug, SILA-421 showed *in vitro* a concentration-dependent and time-dependent bactericidal activity. SILA-421 also enhanced the activity of isoniazid and rifampicin, in case of isoniazid resulted in synergy. The emergence of isoniazid-resistance after exposure to isoniazid can be prevented by the addition SILA-421. SILA-421 had no additional value in combination with moxifloxacin or amikacin. Furthermore, SILA-421 enhanced the activity of rifampicin towards a rifampicin-resistant strain, and resulted in complete elimination of rifampicin-resistant mycobacteria. Unfortunately, in mice with TB, induced by a Beijing genotype strain, the addition of SILA-421 to an isoniazid-rifampicin-pyrazinamide regimen for 13 weeks did not result in enhanced therapeutic efficacy.

INTRODUCTION

Tuberculosis (TB) is one of the major life-threatening infectious diseases [1]. Most currently used TB-drugs being developed more than 50 years ago and due to an increasing amount of multi-drug resistant TB (MDR-TB) [1], we must conclude that there is an urge to develop new treatment regimens. Despite the fact that several novel compounds with anti TB activity are in different phases of development, there continues to be a need for new TB-drugs or compounds that enhance the therapeutic efficacy of existing TB-drugs [2].

It has been demonstrated that compounds that inhibit efflux pumps can enhance the intracellular mycobacterial killing themselves and/or may potentiate the effect of other TB-drugs [3]. It has been previously demonstrated that thioridazine used as TB-drug/efflux pump inhibitor (EPI) has *in vitro* activity. Thioridazine shows a synergistic effect when combined with isoniazid and rifampicin [3-7]. However, when used in a murine TB-model, thioridazine shows toxic effects and enhanced therapeutic efficacy of the standard TB regimen was not observed [8]. The development of novel EPIs is needed.

The potency of an organosilicon (SILA) compound, SILA-421, to inhibit efflux pumps in cancer cells and *Escherichia coli* has been demonstrated [9, 10]. In addition, SILA-421 showed *in vitro* activity against *Mycobacterium tuberculosis*[11], and *ex vivo* it enhances intracellular killing of an extensively drug resistant (XDR) TB isolate inside human macrophages [12].

We hypothesized that combining SILA-421 with TB-drugs of different classes, namely isoniazid, rifampicin, moxifloxacin and amikacin could result in enhancement of activity of these TB-drugs *in vitro*, as well as improvement of therapeutic efficacy in our mouse TB model.

We studied the time-kill kinetics of SILA-421 towards a *Mycobacterium tuberculosis* strain of the Beijing genotype, and investigated if the addition of SILA-421 to several TB drugs would lead to synergy between compounds. We also assessed the time-kill kinetics of SILA-421 in combination with rifampicin towards an isogenic mutant strain of the Beijing strain which is resistant to rifampicin. Subsequently, we evaluated the effect of the combination of SILA-421 with the standard therapy regimen in a TB model in mice infected with *Mycobacterium tuberculosis* Beijing genotype strain. Outcome parameters are mycobacterial elimination in infected organs, the prevention of emergence of drug resistance and relapse of TB.

MATERIALS AND METHODS

Materials

SILA-421 (1,3-dimethyl-1,3-bis(4-fluorophenyl)-1,3-bis{3-1(4-butyl-piperazinyl)-propyl}-disiloxan tetrahydrochlorid), a patented compound was provided by Dr G. Hajos (Institute

for Biomolecular Chemistry, Budapest, Hungary) and prof. L. Amaral (Travel Medicine of the Center de Malaria e Doencas Tropicais, Institute of Hygiene and Tropical Medicine, Universidade Nova de Lisboa, Lisbon, Portugal) and was dissolved in water.

Isoniazid (Hospital Pharmacy; Rotterdam, The Netherlands), rifampicin (Rifadin®, Aventis Pharma B.V, Hoevelaken, The Netherlands), pyrazinamide (Sigma Chemical Co, St. Louis, MO, USA), amikacin (Hospira Benelux BVBA, Brussels, Belgium) and moxifloxacin (Avelox®, Bayer Schering Pharma A.G, Berlin, Germany) were used according to the recommendations of the manufacturers.

Mycobacterium tuberculosis suspensions for the *in vitro* assays were cultured in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA), supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (OADC, Baltimore Biological Laboratories, Baltimore, MD, USA), 0.5% glycerol (Scharlau Chemie S.A, Sentmenat, Spain) and 0.02% Tween 20 (Sigma Chemical Co, St. Louis, MO, USA), under shaking conditions at 96 rpm at 37^0C. All cultures on solid media were grown on Middlebrook 7H10 agar (Difco), supplemented with 10% OADC and 0.5% glycerol for 28 days at 37°C with 5% CO_2.

Bacterial strains

The *Mycobacterium tuberculosis* genotype strain Beijing VN 2002-1585 (BE-1585) which is susceptible to isoniazid (MIC 0.125 mg/L), rifampicin (MIC 0.25 mg/L), moxifloxacin (MIC 0.25 mg/L) and amikacin (MIC 2 mg/L) and the progeny strain BE-1585-H526Y were used[1 3]. The BE-1585-H526Y is resistant to rifampicin (MIC 512 mg/L) due to a mutation in the *rpoB* gene and was derived from the BE-1585 by exposure to 64 mg/L of rifampicin as described before [13, 14].

Minimal inhibitory concentration assay (MIC)

The solid media MIC of SILA-421 for the Beijing strains were determined using the agar proportion method as derived from the standards of the CLSI [15].

In vitro time-kill kinetics

The concentration- and time-dependent bactericidal activity of SILA-421 as single drug or in combination with TB-drugs were determined in liquid media as described previously [16]. In short, low-density cultures of actively replicating (exponential-phase) BE-1585 were exposed to two-fold increasing concentrations of SILA-421 at 37^0C and 96 rpm. On day 1, 3 and 6 samples (200 µL) were taken, provided that the mycobacterial suspension did not show visible aggregation. Samples were centrifuged at 14.000xg to avoid drug carry-over and were sub-cultured on drug-free solid media for CFU counts after 28 days of incubation at 37^0C with 5% CO_2. To assess the selection of drug-resistant *Mycobacterium tuberculosis*, samples were sub-cultured on drug-containing solid media. The drug concentration in

the subculture plates were 4-fold the critical concentrations, i.e. 0.8 mg/L isoniazid, 4 mg/L rifampicin, 8 mg/L moxifloxacin or 20 mg/L amikacin.

The time-kill kinetics of the drug combinations in low-density *Mycobacterium tuberculosis* cultures of actively replicating BE-1585 were determined for SILA-421 in combination with isoniazid, rifampicin, moxifloxacin or amikacin. The concentrations of TB-drugs selected for these experiments were chosen based on the results obtained as single exposure where the mycobacterial load on day 6 deviated not more than 1 log compared to day 0. SILA-421 concentrations of 1, 2 and 4 mg/L were added to the TB-drugs.

The time-kill kinetics of actively replicating rifampicin-resistant BE-1585-H526Y were determined for SILA-421 (2 and 4 mg/L) in combination with rifampicin (64, 128 and 256 mg/L).

The two endpoints of this experiment were drug synergy and the prevention of the emergence of TB-drug resistance. Synergistic activity of a drug combination was determined after 6 days of exposure and was defined as $\geq$ 100-fold (Δlog 2) decrease in mycobacterial load in the combination compared to the mycobacterial load in the most active single agent, or when the drug combination resulted in complete mycobacterial elimination while the most active single agent did not [17]. Lower limit of quantification (LLQ) was 5 CFU (Log 0.7).

Infection Model

Mice were infected as described previously [18]. In short, mice under anaesthesia were infected by intra-tracheal instillation of a suspension (40 µL) containing 3.8 x 10^4 CFU of BE-1585, followed by proper inhalation, to ensure the formation of a bilateral TB infection.

Animals

Specified pathogen-free female BALB/c mice were obtained from Charles River (Les Oncins, France). At the day of infection, 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 (117-12-12).

Treatment with TB-drugs

A tolerability study in infected mice was performed using dosages of 5 and 10 mg/kg/day SILA-421 in combination with isoniazid, rifampicin and pyrazinamide (HRZ) in human pharmacokinetic-equivalent doses, 25, 10 and 150 mg/kg/day respectively[19, 20], for 3 weeks.

In the therapeutic efficacy study in BE-1585 infected mice, treatment was started 2 weeks after infection and mice were randomly divided in two groups. The first group

received HRZ in human pharmacokinetic-equivalent doses 5 days/week, the second group received HRZ and 10 mg/kg SILA-421, 5 days/week. HRZ was administered orally in a total volume of 0.3 mL, SILA-421 was administered intra-peritoneally in a volume of 0.3 mL. Treatment was continued for 13 weeks, followed by a 13 weeks post-treatment period to check for relapse of TB infection.

Determination of viable *Mycobacterium tuberculosis* counts in infected organs

To assess the therapeutic efficacy and prevention of relapse TB infection, mice were sacrificed by CO_2 exposure at several time points: at the start of therapy (n=3), after 8 (n=4) and 13 weeks therapy (n=4) and at week 26 (13 weeks after termination of therapy) (n=12). To prevent carry-over of TB-drugs on subculture plates, therapy was stopped 72 hours before sacrificing the mice. The lungs and spleens were removed aseptically and homogenized in the M-tubes using the gentleMACS Octo Dissociator (Miltenyi Biotec BV, Leiden, the Netherlands) in 2 mL Phosphate Buffered Saline (PBS). Tissue homogenates were centrifuged at 14,000xg for 10 min, again to prevent carry–over of TB-drugs. Pellets were re-suspended in PBS. From each tissue homogenate 10-fold serial dilutions were performed and samples of 200 µL were cultured on drug-free solid media.

Assessment of relapse of TB infection

The number of viable *Mycobacterium tuberculosis* counts in lung and spleen of mice (n=12) was assessed 13 weeks after termination of TB treatment. Relapse was defined as *Mycobacterium tuberculosis*-positive cultures of infected organs, while immediately after termination of treatment organs were *Mycobacterium tuberculosis* culture-negative.

RESULTS

In vitro activity, MIC

The MICs of SILA-421 for BE-1585 and BE-1585-H526Y were similar, being 8 mg/L SILA-421.

In vitro time-kill kinetics towards BE-1585

In figure 1, the activity of SILA-421 as single drug exposure against BE-1585 is presented. SILA-421 showed a strong concentration- and time-dependent activity in the concentration range tested (0.5 – 64 mg/L). After 6 days of exposure to 8 mg/L SILA-421, all *Mycobacterium tuberculosis* cultures became sterile, whereas 16-64 mg/L resulted in complete *Mycobacterium tuberculosis* elimination already after 1 day of exposure.

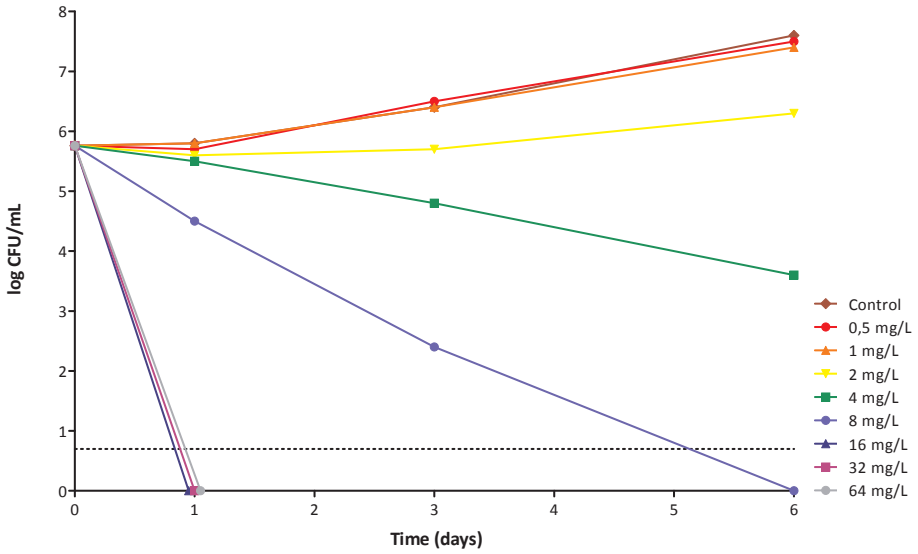

Figure 1. Concentration- and time-dependent bactericidal effect of SILA-421 against *Mycobacterium tuberculosis* BE-1585. Cultures of actively replicating *Mycobacterium tuberculosis* (starting at 5.8 x 10^5 CFU/mL) were exposed to SILA-421 at 2-fold increasing concentrations for 6 days at 37°C under shaking conditions. After 1, 3 and 6 days of drug exposure, samples were taken and quantitative cultures were performed. The dotted horizontal line is the lower limit of quantification of log CFU/ml.

The activity of SILA-421 in combination with the TB-drugs against BE-1585 is shown in table 1. In the unexposed *Mycobacterium tuberculosis* cultures, CFU counts increased from (median + range) 5.8 (5.6 – 5.9) to 7.2 (7.1 – aggregation) within 6 days. Changes in CFU count of the *Mycobacterium tuberculosis* cultures after 6 days SILA-421 single drug exposure were (median + range) 5.8 (5.6 – 5.8) to 7.4 (7.2 – 7.5), to 6.8 (6.5- 7.0) and to 4.2 (3.9 – 4.4) for 1, 2 and 4 mg/L SILA-421 respectively.

Table 1. Effect of SILA-421 on the bactericidal activity of isoniazid, rifampicin, moxifloxacin or amikacin against BE-1585.

TB-drugs tested (mg/L)	Log10 CFU/mL at day 0 LLQ = 0.7	Log 10CFU/mL at day 6 LLQ = 0.7	TB-drug resistant mutants	Δ Log10 CFU/ mL between drug combination and most active single drug at day 6
Control	5.8 (5.6 - 5.9)	7.2 (7.1 – aggregation)	-	
INH 0.063	5.8	5.1	[1:6.2 x 10^3]	
RIF 0.015	5.6	5.3	0	
MXF 0.031	5.8	4.9	0	
AMK 0.25	5.9	4.7	0	
SILA 1	5.8 (5.6 - 5.9)	7.4 (7.2 – 7.5)	ND	
SILA 2	5.8 (5.6 - 5.9)	6.8 (6.5 – 7.0)	ND	
SILA 4	5.8 (5.6 - 5.9)	4.2 (3.9 – 4.4)	ND	
INH 0.063 + SILA 1	5.8	5.0	[1:5.0 x 10^3]	-0.1
INH 0.063 + SILA 2	5.8	3.6	[1:5.4 x 10^2]	-1.5
INH 0.063 + SILA 4	5.8	2.0	0	-2.4*
RIF 0.015 + SILA 1	5.6	5.1	0	-0.2
RIF 0.015 + SILA 2	5.6	4.6	0	-0.7
RIF 0.015 + SILA 4	5.6	2.5	0	-1.7
MXF 0.031 + SILA 1	5.8	5.2	0	+0.3
MXF 0.031 + SILA 2	5.8	4.7	0	-0,2
MXF 0.031 + SILA 4	5.8	3.6	0	-0.3
AMK 0.25 + SILA 1	5.9	6.7	0	+2.0
AMK 0.25 + SILA 2	5.9	5.8	0	+1.1
AMK 0.25 + SILA 4	5.9	4.0	0	-0.1

Cultures of BE-1585 were exposed to SILA-421 for 6 days at 37°C under shaking conditions At day 6, samples were taken and quantitative cultures were performed on drug free and drug containing solid media.

Δ Log10 CFU/mL is determined as: Log10 CFU/mL of a combination minus the Log10 CFU/mL of the most active single drug of the combination.

Resistant mutants are presented as the mutation rate, determined as: the amount of resistant mutants divided by the total amount of bacteria cultured on day 6.

* Synergy, ND = Not determined, LLQ = Lower Limit of Quantification, INH = isoniazid, RIF = rifampicin, MXF = moxifloxacin, AMK = amikacin

Addition of 2 mg/L SILA-421 to isoniazid 0.063 mg/L resulted in enhanced mycobacterial killing and a synergistic effect was obtained when 4 mg/L SILA-421 was added to isoniazid 0.063 mg/L. When the BE-1585 was exposed to isoniazid as single drug, isoniazid resistant mutants were obtained from drug-containing plates, whereas the combination of 4 mg/L SILA-421 with 0.063 mg/L isoniazid resulted in prevention of emergence of resistance. Furthermore, addition of 2 and 4 mg/L SILA-421 to rifampicin (0.015 mg/L) resulted in enhanced mycobacterial killing. No effect was observed when SILA-421 was added to moxifloxacin (0.031), however, when 1 and 2 mg/L SILA-421 were added to amikacin

(0.25 mg/L) an antagonistic effect (Δlog 2 increase in mycobacterial load) between the compounds was observed.

In vitro, time-kill kinetics towards BE-1585-H526Y

The activity of SILA-421 in combination with 128 and 256 mg/L rifampicin against the rifampicin resistant isogenic BE-1585-H526Y, is presented in table 2 and figure 2. The combination of rifampicin 64 mg/L and SILA-421 is presented in table 1 of the supplementary

Table 2. Effect of SILA-421 on the bactericidal activity of rifampicin against BE-1585-H526Y

TB-drugs tested (mg/L)	Log10 CFU/mL at day 0 LLQ = 0.7	Log 10CFU/mL at day 6 LLQ = 0.7	Δ Log10 CFU/mL between drug combination and most active single drug at day 6
Control	5.6	7.1	
RIF 128	5.6	4.2	
RIF 256	5.6	3.4	
SILA 2	5.6	5.8	
SILA 4	5.6	3.6	
RIF 128 + SILA 2	5.6	3.1	-1.1
RIF 128 + SILA 4	5.6	1.5	-2.1*
RIF 256 + SILA 2	5.6	3.3	-0.1
RIF 256 + SILA 4	5.6	<LLQ	-3.4*

Cultures of BE-1585-H526Y (rifampicin-resistant) were exposed to SILA-421 and RIF for 6 days at 37°C under shaking conditions. At day 6, samples were taken and quantitative cultures were performed on solid media.
Δ Log10 CFU/mL is determined as: Log10 CFU/mL of a combination minus the Log10 CFU/mL of the most active single drug of the combination.
* Synergy, LLQ = Lower Limit of Quantification, RIF = rifampicin

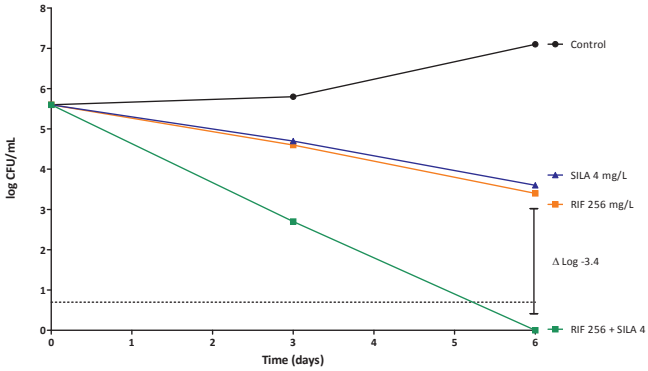

Figure 2. Concentration- and time-dependent bactericidal effect of SILA-421 against *Mycobacterium tuberculosis* BE-1585-H526Y (rifampicin-resistant). Cultures of actively replicating *Mycobacterium tuberculosis* (starting at 5.6 x 10^5 CFU/mL) were exposed to 4 mg/L SILA-421 and to 256 mg/L rifampicin as single drug or in combination for 6 days at 37°C under shaking conditions. After 3 and 6 days of drug exposure, samples were taken and quantitative cultures were performed. The dotted horizontal line is the lower limit of quantification of log CFU/ml.

data. The addition of SILA-421 to rifampicin 64 mg/L resulted in enhanced mycobacterial killing, where the addition of 4 mg/L SILA-421 to 128 and 256 mg/L rifampicin resulted in enhanced mycobacterial killing and a synergistic effect was obtained with both rifampicin concentrations. Even complete elimination of rifampicin-resistant *Mycobacterium tuberculosis* could be achieved after exposure to a combination of SILA-421 with rifampicin.

In vivo therapeutic efficacy

Zalatnai *et al.*, showed that mice could tolerate 10 mg/kg SILA-409 for 34 days, SILA-409 and SILA-421 are closely related compounds and very similar to each other [10, 21]. Based on this study we started a small experiment to assess a maximum tolerated dose (MTD), in order to evaluate the novel combination of SILA-421 and a standard TB regimen in our mouse TB model. We observed that our mice when treated with SILA-421 (5 mg/kg or 10 mg/kg) in combination with HRZ did not show toxic side effects, although some mice receiving 10 mg/kg appeared agitated the first 2 weeks of treatment. Based upon these observations on the behavior and survival of the mice we used 10 mg/kg SILA-421 for further experiments.

At two weeks after inoculation with the BE-1585 strain, mice had developed a TB infection including extra-pulmonary dissemination. Untreated control animals became moribund by three weeks after infection and were euthanized. To assess the therapeutic efficacy of the combination SILA-421 (10 mg/kg) and HRZ, mice were treated for 13 weeks (figure 3). After 13 weeks of treatment all lungs were culture-positive in the group of mice receiving HRZ only, whereas in the group of mice receiving HRZ + SILA-421 lungs of 2 out of 4 mice were culture-positive with very small numbers of *Mycobacterium tuberculosis*, showing no significant difference at this time point. After 13 weeks post-treatment all mice showed relapse/regrowth of *Mycobacterium tuberculosis*. From the spleen (data not shown), *Mycobacterium tuberculosis* was never cultured after 13 weeks treatment in both groups of mice. At 13-weeks post-treatment, 10 out of 12 mice in the HRZ only group were culture-positive, whereas 11 out of 12 mice in the HRZ+SILA-421 group were culture-positive.

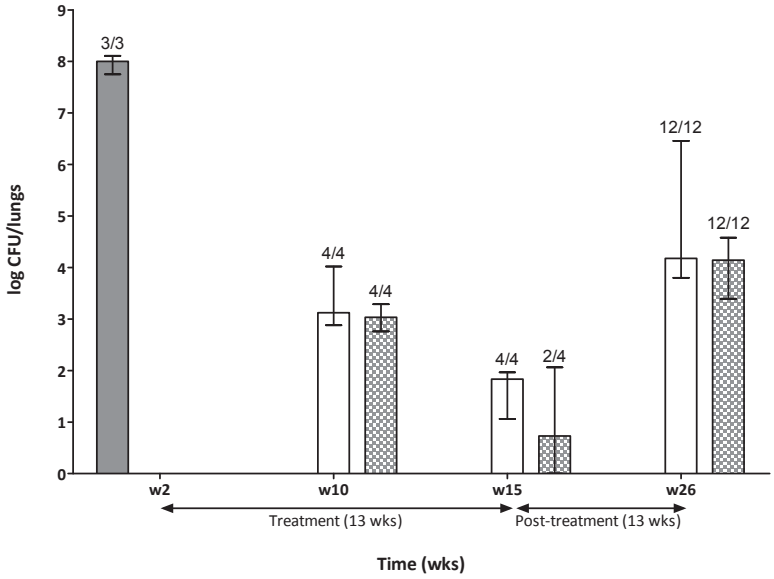

Figure 3. Mycobacterial load in infected lungs after treatment with HRZ (open bars) or a combination of HRZ and SILA-421 (chequered bars) of mice with TB caused by BE-1585. Treatment was started 2 weeks after infection (grey bars). Results are expressed as median ± range (error bars) of the colony forming units (CFU) per total lung. Numbers above bars are the numbers of culture-positive mice out of total number mice.

DISCUSSION

The aim of this study was to evaluate whether SILA-421 had activity in itself and additionally could improve the activity and efficacy of currently used TB-drugs. By using time-kill kinetics, we were able to show the time- and/or concentration-dependent activity of SILA-421 as single drug, as well as in combination with isoniazid, rifampicin, moxifloxacin or amikacin. We found for SILA-421 as single drug exposure a time- and concentration-dependent effect on *Mycobacterium tuberculosis* of the Beijing genotype. An important observation was that SILA-421 is bactericidal, after 6 days exposure to 8 mg/L SILA-421 *Mycobacterium tuberculosis* cultures were negative, whereas by increasing the concentration to 16 mg/L complete mycobacterial elimination after just 1 day of exposure was achieved. SILA-421 at 4 mg/L resulted in inhibition of mycobacterial growth after 6 days of exposure. This observation expands upon prior studies of Martins *et al.* and Simons *et al.*[11, 12], in which they present MIC values of SILA-421 in a range of 2 to 16 mg/L. Resistance of *Mycobacterium tuberculosis* against SILA-421 was not assessed, because no clinical breakpoints are available for SILA-421 to define resistance. Further research is necessary to investigate whether mycobacteria can be selected which are able to grow in the presence

of high concentrations SILA-421 and characterize them for example with whole genome sequencing.

To our knowledge this is the first study providing evidence that SILA-421 can potentiate the activity of isoniazid *in vitro*. Furthermore, the emergence of isoniazid-resistance after exposure to isoniazid as single drug can be prevented by the addition SILA-421. When SILA-421 is combined with rifampicin we also observe substantial increased killing of *Mycobacterium tuberculosis*. Both these findings are important results in view of the urgent need of new drugs, that enhance the therapeutic efficacy of existing TB-drugs for the treatment of drug susceptible TB and prevention of MDR-TB. Given the fact that similar results are obtained when thioridazine was used as a compound to enhance the effect of TB-drugs [8], our results support the hypothesis that both isoniazid and rifampicin can be enhanced by adding an EPI such as SILA-421 [9, 10] used in the present study. Also the antagonistic effect of SILA-421 on amikacin is interesting. A possible explanation for this observation can be the binding of SILA-421 to amikacin which may result in diminished binding of amikacin to the 30S ribosome, or limited influx of amikacin into the cell. Normally, amikacin binds to the 30S ribosome and as such interferes with the protein synthesis [22], however, the mechanism resulting in antagonism between amikacin and SILA-421 is unclear.

We could not demonstrate enhancement of the therapeutic efficacy of the HRZ regimen when combined with SILA-421 in the treatment of TB in mice. After 13 weeks of HRZ treatment all mice were *Mycobacterium tuberculosis* culture positive whereas 2 out of 4 mice were *Mycobacterium tuberculosis* culture negative after HRZ + SILA-421 combination treatment. However, all mice exhibited regrowth/relapse of TB infection after a 13 weeks post-treatment period. Possibly the relatively short treatment period of 13 weeks only is too ambitious for achieving superior therapeutic efficacy. Further research is needed to investigate the additional value of SILA-421 in long-lasting treatment periods. A limitation of the present study is the relatively low number of animals, which does not allow us to investigate the efficacy of more treatment schedules or do a proper study to determine the MTD. Another limitation is that we could not perform a pharmacokinetic study, due to the lack of a proper bio-analytical methods. In addition, a bio-analytical detection method such as a HPLC-MS should be developed and a pharmacokinetic study should be performed to assess the actual plasma and tissue concentrations of SILA-421 that are achieved after a dose of 10 mg/kg in mice, and to compare the (active, i.e. protein-unbound) *in vivo* concentrations to those studied in our in *vitro* time-kill kinetics experiments. In 2009, Martins *et al.* already demonstrated that SILA-421 at a concentration of 0.1 mg/L is able to kill intracellular *Mycobacterium tuberculosis*[12]. However, the translational value of this *in vitro* observation to the *in vivo* TB infection is difficult as data on the availability of SILA-421 in mice at the site of infection is currently not available.

Our *in vitro* time-kill kinetics assays and *in vivo* therapeutic efficacy experiments with SILA-421 largely focused on drug susceptible TB strains, but we also assessed the time-kill kinetics of SILA-421 in combination with rifampicin against the rifampicin-resistant BE-1585-H526Y strain. In a previous study, we performed a genome-wide transcriptional profile analysis with micro-array, and showed that rifampicin-resistant *Mycobacterium tuberculosis* exposed to 256 mg/L still exhibited a response. Several genes involved in efflux and transport appeared up-regulated in the rifampicin-resistant mutants compared to the rifampicin-susceptible strain [14]. We therefore hypothesized that by inhibiting the efflux of rifampicin following addition of SILA-421, we might be able to kill rifampicin-resistant *Mycobacterium tuberculosis*. In the present study we show that exposure of a rifampicin-resistant *Mycobacterium tuberculosis* isolate to SILA-421 and high concentrations of rifampicin results in synergy and complete elimination of the culture is achieved. With the currently used rifampicin doses, an active (i.e. protein-unbound) rifampicin concentration of 256 mg/L seems clinically not achievable. Nevertheless, de Steenwinkel *et al.* showed in the same mouse TB model that high doses of rifampicin up to 160 mg/kg/day in combination with isoniazid and pyrazinamide during a 2 months treatment period were tolerated, and the application of these high rifampicin dosage schedules allowed reduction of the treatment period from 6 months to 2 months, without relapse of infection [20]. Boeree *et al.* showed in TB patients that rifampicin doses up to 35 mg/kg were well tolerated [23]. These combined results in TB infected mice and TB patients open new possibilities for studies investigating the capacity of high doses of rifampicin in combination with an EPI such as SILA-421 in the treatment of MDR-TB. To support clinical studies in this respect, a mouse MDR-TB model should be developed and the therapeutic efficacy of combinations of SILA-421, or other EPIs and higher doses of rifampicin should be further evaluated.

Summarizing, we demonstrated *in vitro* that SILA-421 in itself has bactericidal activity towards rifampicin-susceptible and rifampicin-resistant *Mycobacterium tuberculosis* strains of the Beijing genotype. We show that SILA-421 enhanced the mycobacterial killing capacity of isoniazid and rifampicin, even in the rifampicin-resistant strain. Unfortunately, enhanced therapeutic efficacy following addition of SILA-421 to the currently used HRZ treatment schedule in our mouse TB model was not demonstrated.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY DATA

Table S1. Effect of SILA-421 on the bactericidal activity of RIF against BE-1585-H526Y.

TB-drugs tested (mg/L)	Log10 CFU/mL at day 0 LLQ = 0.7	Log 10CFU/mL at day 6 LLQ = 0.7	Δ Log10 CFU/mL between drug combination and most active single drug at day 6
Control	5.6	6.8	
RIF 64	5.6	5.0	
SILA 2	5.6	5.8	
SILA 4	5.6	3.6	
RIF 64 + SILA 2	5.6	3.5	-1.5
RIF 64 + SILA 4	5.6	2.2	-1.4

Cultures of BE-1585-H526Y (RIF-resistant) were exposed to SILA-421 and RIF for 6 days at 37°C under shaking conditions. At day 6, samples were taken and quantitative cultures were performed on solid media.
Δ Log10 CFU/mL is determined as: Log10 CFU/mL of a combination minus the Log10 CFU/mL of the most active single drug of the combination.
* Synergy, RIF = rifampicin

Chapter 4

Activity of moxifloxacin and linezolid against *Mycobacterium tuberculosis* in combination with potentiator drugs verapamil, timcodar, colistin and SQ109

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ABSTRACT

The current treatment of tuberculosis (TB) is complicated by the emergence of multi-drug resistant TB (MDR-TB). As a result, there is an urgent need for new powerful TB regimens and novel strategies. In this study we aim to potentiate a moxifloxacin – linezolid backbone as treatment for MRD-TB with efflux pump inhibitors verapamil and timcodar as well as with drugs that act on the mycobacterial cell wall stability like colistin and SQ109.

Using a time kill kinetics assay the activity of moxifloxacin, linezolid, verapamil, timcodar, colistin and SQ109 as single drugs against *Mycobacterium tuberculosis* was evaluated. In addition the activity of the moxifloxacin + linezolid backbone in combination with one of the potentiator drugs was assessed.

As little as 0.125 mg/L moxifloxacin achieved 99% killing of *Mycobacterium tuberculosis* after 6 days of exposure. Linezolid showed moderate killing but 99% killing was not achieved. Verapamil, timcodar and colistin only resulted in killing with the highest concentrations tested but 99% killing was not achieved. SQ109 resulted in complete elimination after 1 day of exposure to 256 mg/L and in 99% elimination after 6 days of exposure to 1 mg/L SQ109. Furthermore, we found that colistin added to the backbone resulted in increased elimination. Verapamil, timcodar and SQ109 showed no added value to the moxifloxacin + linezolid backbone. Our finding that colistin potentiates the activity of the moxifloxacin + linezolid backbone against *Mycobacterium tuberculosis* suggests its potential role in further studies on the applicability of a moxifloxacin + linezolid treatment of MDR-TB.

INTRODUCTION

Tuberculosis (TB) is the leading cause of death due to an infectious disease worldwide [1, 2]. Treatment of this dreadful disease is complicated by the emergence of multi-drug resistant TB (MDR-TB), occurring in about half a million new TB cases annually [2]. The current treatment of MDR-TB is long and complex, with several drugs to be used in combination, resulting in patient non-compliance, inadequate response in 50% of patients and emergence of further drug resistance [2]. Therefore, every effort should be made to improve MDR-TB treatment.

Moxifloxacin, one of the most potent fluoroquinolones, is the cornerstone of MDR-TB treatment in many countries, together with levofloxacin in others. In addition linezolid, one of the oxazolidinones, shows excellent efficacy but also toxicity in MDR-TB treatment [3]. The potential of oxazolidinones is underlined by the ongoing development of structural analogues to linezolid as TB drugs [4, 5].

As to adjuvant strategies in MDR-TB treatment, accumulating evidence shows that there is a significant role for mycobacterial efflux pumps in the extrusion of TB drugs and emergence of resistance towards different TB-drugs [6], including fluoroquinolones and oxazolidinones [7]. Inactivation of these mycobacterial efflux pumps with efflux pump inhibitors (EPI) may increase the intrabacterial concentration of TB-drugs, thereby increasing efficacy while avoiding high systemic exposures associated with adverse effects. This concept may even prevent or reverse resistance [8, 9]. Recently, a combination of moxifloxacin, linezolid and the EPI thioridazine resulted in a favourable response in patients with extensively-drug resistant TB (XDR-TB), suggesting the potential of EPIs as treatment adjuncts for the treatment of *Mycobacterium tuberculosis* infections [10].

Verapamil is a calcium channel antagonist, recognized to inhibit bacterial efflux pumps and has been shown to accelerate mycobacterial killing in mice infected with *Mycobacterium tuberculosis* and it decreased relapse rates with shortened treatment regimens [11-13]. It also reduced the mycobacterial drug tolerance induced in the intracellular department in macrophages as well as in zebrafish granuloma-like lesions [14].

Timcodar has also been described as a bacterial efflux pump inhibitor [15]. Timcodar, used as a single agent, has *in vitro* a moderate effect against *Mycobacterium tuberculosis* in liquid cultures [16]. However, this effect is more pronounced in a *Mycobacterium tuberculosis*-infected macrophage assay [16]. Additionally, timcodar added to rifampicin consistently improved the clearance of *Mycobacterium tuberculosis* in infected mice [16].

A different strategy to increase intrabacterial drug concentrations and improve the effect of TB drugs is to target the stability of the mycobacterial cell wall. Colistin is an old antimicrobial and part of the polymyxin family acting primarily on the cell wall by causing changes in the permeability of the plasma membrane of gram-negative bacteria [17]. Recently, it has been shown that colistin potentiates the activity of isoniazid and amikacin

against *Mycobacterium tuberculosisinvitro* and also prevented the emergence of isoniazid resistance [18].

SQ109 is a TB drug candidate that was identified by creating more potent analogues of ethambutol, but it demonstrated a different mode of action compared to ethambutol [19]. SQ109 decreases the incorporation of mycolic acids into the mycobacterial cell wall, thereby targeting the mycobacterial cell wall [20]. SQ109 is also an EPI at least to human efflux pumps [21]. It demonstrated synergistic interaction with isoniazid and rifampicin as well as with moxifloxacin [20].

In this study we focus on combining moxifloxacin and linezolid as a proposed fluoroquinolone-oxazolidinone backbone for the treatment of MDR-TB. We aimed to further potentiate the anti-mycobacterial effect of this backbone by using potentiator drugs verapamil and timcodar as EPIs, and by colistin and SQ109, drugs that target the stability of the mycobacterial cell wall.

MATERIALS AND METHODS

Materials

Moxifloxacin, linezolid and verapamil were all purchased from Sigma Chemical Co, St. Louis, MO, USA, and were dissolved in water. Colistin sulphate (Hospital Pharmacy; Rotterdam, The Netherlands) was dissolved in water, Timcodar (Vertex Pharmaceuticals Incorporated, Boston, MA, USA) was dissolved in DMSO. SQ109 (Sequella Inc., Rockville, MD, USA) was dissolved in water.

Mycobacterium tuberculosis suspensions for the *in vitro* assays were cultured in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA), supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (OADC, Baltimore Biological Laboratories, Baltimore, MD, USA), 0.5% glycerol (Scharlau Chemie S.A, Sentmenat, Spain) and 0.02% Tween 20 (Sigma Chemical Co, St. Louis, MO, USA), under shaking conditions at 96 rpm at 37^0C. All cultures on solid media were grown on Middlebrook 7H10 agar (Difco), supplemented with 10% OADC and 0.5% glycerol for 28 days at 37°C with 5% CO_2.

Bacterial strain

The *Mycobacterium tuberculosis* genotype strain Beijing VN 2002-1585 (BE-1585) with a MIC of 0.25 mg/L moxifloxacin and 1 mg/L of linezolid was used [22, 23]. MICs were determined using CLSI standards [24].

In vitro time-kill kinetics

The activities of moxifloxacin, linezolid, verapamil, timcodar, colistin and SQ109 were first determined as single compounds in liquid media, as described previously [25]. In short,

cultures of actively replicating (exponential-phase) BE-1585 were exposed to increasing concentrations of the drugs at 37^0C and 96 rpm. Concentrations of moxifloxacin and linezolid tested were based on the maximum concentration of unbound drugs which can be achieved in patients (fC_{max}). Ranges were 4x to 1/256x the fC_{max}. Samples (200 µL) were taken on day 1, 2, 3 and 6, provided that the mycobacterial suspension did not show visible aggregation. Samples were centrifuged at 14.000xg, washed with PBS to avoid drug carry-over and sub-cultured on drug-free solid media. After 28 days of incubation at 37^0C with 5% CO_2 the number of CFU was counted. To assess selection of moxifloxacin-resistant or linezolid-resistant bacteria, samples were sub-cultured on drug-containing solid media. The drug concentrations in the subculture plates were 4-fold the critical concentrations, i.e. 2 mg/L of moxifloxacin or 8 mg/L of linezolid as reported by the CLSI [24].

Next, the time-kill kinetics of drug combinations with exponential-phase BE-1585 were determined for both moxifloxacin and linezolid as single drugs in combination with the potentiator drugs verapamil, timcodar, colistin or SQ109 and for the moxifloxacin + linezolid backbone in combination with the potentiator drugs. A concentration of moxifloxacin of 0.031 mg/L (1/64 x fC_{max}) and a linezolid concentration of 0.069 mg/L (1/16 x fC_{max}) were selected based on results in the single drug experiments. These concentrations resulted in a mycobacterial load on day 6 that deviated no more than 1.5 log compared to day 0. Similarly, the concentrations of potentiator drugs selected for these experiments resulted a limited change in a mycobacterial load on day 6 compared to day 0 in the single drug experiments. Verapamil concentrations of 16, 64 and 256 mg/L; timcodar concentrations of 4, 16 and 64 mg/L; colistin concentrations of 16, 64 and 256 mg/L and SQ109 concentrations of 0.063, 0.25 and 1 mg/L were added to the moxifloxacin + linezolid backbone.

All time-kill kinetic assays were performed in duplicate. The lower limit of quantification (LLQ) was 5 CFU (Log 0.7). Emergence of resistance to moxifloxacin or linezolid was tested by sub-culturing of samples on drug-containing solid media.

Endpoints for assessment of anti-TB drug combinations

The two endpoints of this experiment were drug synergy and the prevention of the emergence of drug resistance. Synergistic activity of a 2 or 3-drug combination was determined after 6 days of exposure and was defined as $\geq$ 100-fold (Δlog 2) decrease in mycobacterial load in the 2 or 3-drug combination compared to the mycobacterial load achieved with the potentiator drug as single agent and moxifloxacin or linezolid as single agent and in case of a 3-drug combination the backbone of moxifloxacin and linezolid. The definition of synergy was also met when a 2 or 3-drug combination achieved mycobacterial elimination which was not achieved during exposure to moxifloxacin or linezolid as single agents or the backbone of moxifloxacin and linezolid [8, 18, 26].

RESULTS

Single-drug exposure of moxifloxacin and linezolid

The bactericidal activity of moxifloxacin as single-drug exposure is illustrated in figure 1A. Moxifloxacin showed a concentration dependent killing against BE-1585. A concentration of 0.125 mg/L moxifloxacin was needed to achieve 99% killing. However, there seemed to be an optimum concentration (0.5 mg/L) beyond which an increase in concentration does not result in more efficient killing of *Mycobacterium tuberculosis*. A concentration of 8 mg/L of moxifloxacin even resulted in a 1.5 Log less effective killing of *Mycobacterium tuberculosis* after 6 days of exposure. The concentration of the fC_{max} (2 mg/L) resulted in an almost 3.5 Log decrease compared to the control after 6 day of exposure.

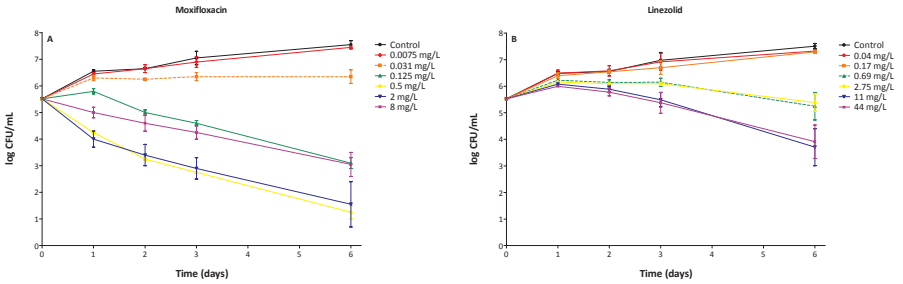

Figure 1. Bactericidal activity of moxifloxacin and linezolid as single drug against BE-1585. Cultures were exposed to 2-fold increasing drug concentrations based on the fC_{max} for 6 days. On days 1, 2, 3 and 6, samples were collected for cfu counting. Data are presented as median with ranges (n=2). Dashed line represents the drug concentration used in the combination experiments.

In figure 1B the bactericidal activity of linezolid as single-drug exposure is illustrated. Linezolid showed a moderate activity against *Mycobacterium tuberculosis*, as the concentration of the fC_{max} (11 mg/L) resulted in a 1.5 Log decrease after 6 days of exposure. With the concentrations tested, 99% killing was not achieved with linezolid.

Both moxifloxacin as well as linezolid resistant *Mycobacterium tuberculosis* were not found in these experiments.

Single-drug exposure of the potentiator drugs verapamil, timcodar, colistin and SQ109

The activity of verapamil (2A), timcodar (2B), colistin (2C) and SQ109 (2D) as single-drug exposures is presented in figure 2. Exposure to verapamil or colistin only resulted in killing of *Mycobacterium tuberculosis* with the highest concentrations tested, 99% killing of *Mycobacterium tuberculosis* was not achieved. Timcodar showed almost no bactericidal

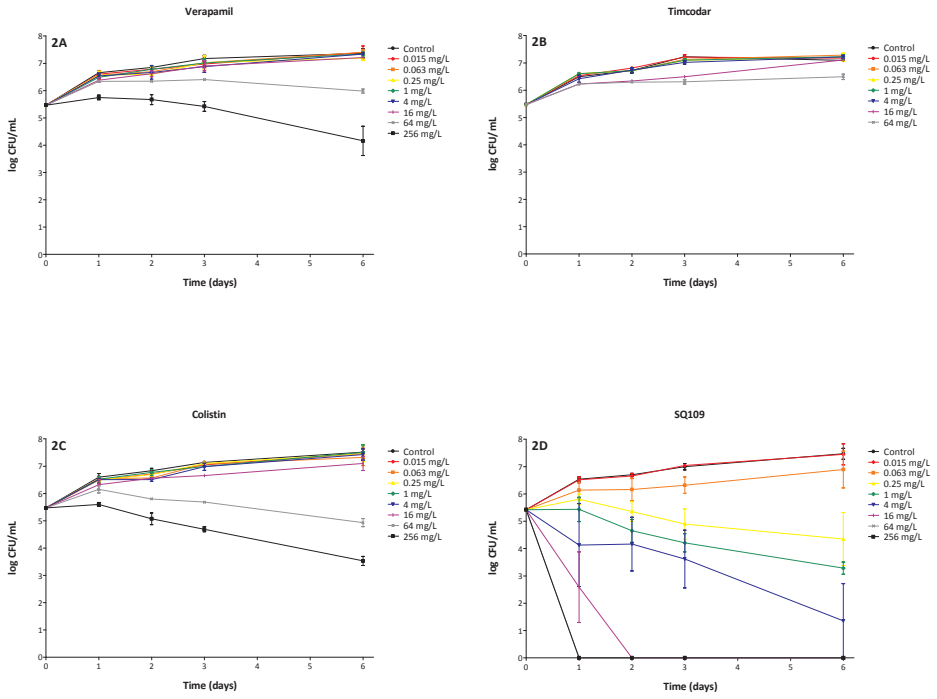

Figure 2. Bactericidal activity of the 4 potentiator drugs (verapamil, timcodar, colistin and SQ109) against BE-1585. Cultures were exposed to 2-fold increasing drug concentrations for 6 days. On days 1, 2, 3 and 6, samples were collected for cfu counting. Data are presented as median with ranges (n=2).

activity. Due to precipitation of timcodar in the broth, 64 mg/L was the highest concentration possible to test. SQ109 showed a clear concentration-dependent anti-mycobacterial activity. Exposure to 64 mg/L SQ109 resulted in 99% killing within one day and 256 mg/L resulted even in complete elimination of *Mycobacterium tuberculosis*. After 6 days of exposure, 1mg/L of SQ109 was needed to achieve 99% killing.

Combined exposure of the moxifloxacin or linezolid with the potentiator drugs

Data obtained combining either moxifloxacin or linezolid with the potentiator drugs is presented in the supplementary data, table S1. Addition of verapamil or timcodar to moxifloxacin or linezolid does not result in an improved or decreased activity of moxifloxacin of linezolid. The addition of colistin to moxifloxacin or linezolid resulted in some improved activity of both moxifloxacin as well as linezolid, however, the results does not met the

synergy criteria. The results of the addition of SQ109 to both moxifloxacin and linezolid are diverse, in the lowest concentration SQ109 the combination is more active against *Mycobacterium tuberculosis* compared to moxifloxacin and linezolid as single drug. Whereas by increasing the SQ109 concentration this effect is no longer observed in combination with moxifloxacin, furthermore, in combination with linezolid these higher concentrations of SQ109 resulted in a decreased activity of the combination compared to the most active single drug.

Combined exposure of the moxifloxacin + linezolid backbone with the potentiator drugs

As shown in table 1, addition of verapamil to the backbone showed no improved or decreased bactericidal activity. Addition of timcodar to the backbone resulted in limited improvement of the bactericidal activity. However, synergy, defined as ≥ 100-fold (Δlog 2) decrease in mycobacterial load in the 3-drug combination compared to the mycobacterial load achieved with the potentiator drug as single agent or the backbone of moxifloxacin and linezolid, was not achieved. Similarly, addition of colistin to the backbone resulted in increased mycobacterial killing; addition of 256 mg/L colistin showed a 2.6 Log difference compared to the moxifloxacin + linezolid backbone. However, only a 1.4 Log difference was observed when the 3-drug combination was compared to colistin as single drug. As a result, the 3 drug combination did not reach the synergy criteria. The combination of SQ109 with the backbone did not improve bactericidal activity either and it even resulted

Table 1. The anti-mycobacterial effect of the moxifloxacin + linezolid backbone in combination with a potentiator drug. Abbreviations: MXF: moxifloxacin, LZD: linezolid, VP: verapamil, TIM: timcodar, CST: colistin

TB-drugs tested (mg/L)	Log10 CFU/mL at day 0 LLQ = 0,7	Log10 CFU/mL at day 6 LLQ = 0,7	ΔLog10 CFU/mL between drug combination and most active single drug/ combination at day 6
Control	5.4 (5.1 - 5.6)	7.1 (7.0 - 7.5)	-
MXF+LZD	5.5 (5.5 - 5.6)	5.1 (5.0 - 5.2)	-
VP 16		7.3 (7.2 - 7.4)	-
VP 64		6.0 (5.9 - 6.0)	-
VP 256		4.0 (3.9 - 4.1)	-
MXF + LZD + VP 16		5.4 (5.3 - 5.5)	0.3
MXF + LZD + VP 64		5.2 (5.2 - 5.2)	0.1
MXF + LZD + VP 256		4.1 (4.0 - 4.2)	0.1
MXF+LZD	5.2 (5.1 - 5.3)	5.5 (5.1 - 5.8)	-
TIM 4		7.4 (7.2 - 7.5)	-
TIM 16		6.4 (6.4 - 6.4)	-
TIM 64		5.6 (5.1 - 6.1)	-

Table 1. The anti-mycobacterial effect of the moxifloxacin + linezolid backbone in combination with a potentiator drug. Abbreviations: MXF: moxifloxacin, LZD: linezolid, VP: verapamil, TIM: timcodar, CST: colistin (continued)

TB-drugs tested (mg/L)	Log10 CFU/mL at day 0 LLQ = 0,7	Log10 CFU/mL at day 6 LLQ = 0,7	ΔLog10 CFU/mL between drug combination and most active single drug/ combination at day 6
MXF + LZD + TIM 4		5.1 (5.0 - 5.2)	-0.4
MXF + LZD + TIM 16		5.1 (4.9 - 5.2)	-0.4
MXF + LZD + TIM 64		4.9 (4.6 - 5.2)	-0.6
MXF+LZD	5.2 (5.1 - 5.3)	5.5 (5.1 - 5.8)	-
CST 16		7.3 (7.2 - 7.4)	-
CST 64		5.2 (4.8 - 5.6)	-
CST 256		4.3 (3.8 - 4.8)	-
MXF + LZD + CST 16		4.8 (5.4 - 4.1)	-0.7
MXF + LZD + CST 64		4.3 (4.0 - 4.6)	-0.9
MXF + LZD + CST 256		2.9 (2.2 - 3.6)	-1.4
MXF+LZD	5.5 (5.5 - 5.6)	5.1 (5.0 - 5.2)	-
SQ109 0.063		7.3 (7.3 - 7.3)	-
SQ109 0.25		3.6 (3.5 - 3.7)	-
SQ109 1		2.3 (2.1 - 2.4)	-
MXF + LZD + SQ109 0.063		4.7 (4.5 - 4.8)	0.4
MXF + LZD + SQ109 0.25		4.4 (4.1 - 4.7)	0.8
MXF + LZD + SQ109 1		3.0 (2.8 - 3.1)	0.7

in decreased activity. Addition of 0.25 mg/L SQ109 resulted in a 0.8 Log higher mycobacterial load compared to 0.25 mg/L SQ109 as single drug after 6 days of exposure. Neither moxifloxacin nor linezolid resistant *Mycobacterium tuberculosis* were not found in these experiments.

DISCUSSION

To treat MDR-TB, options are limited with isoniazid and rifampicin no longer available [2]. Therefore other, less effective, more toxic and more costly second-line TB drugs are combined, leading to a prolonged treatment time and successful treatment in only 50% of patients [2, 27].

Fluoroquinolones and especially moxifloxacin have proven to be highly effective in the treatment of susceptible TB (although they did not achieve treatment shortening [28]), and they are of great value in the treatment of MDR-TB [29]. In the present study we show using time-kill kinetics that 99% killing of *Mycobacterium tuberculosis* can be achieved with a moxifloxacin concentration below the fC_{max}, thereby confirming its potential. However,

using moxifloxacin we repeatedly found that increasing the concentration leads to an optimum effect reached at 0.5 mg/L, whereas higher concentrations up to 64 mg/L where less bactericidal, but by increasing concentrations up to 128 and 256 mg/L complete elimination can be achieved *in vitro* (data no shown). This phenomenon was first described by Eagle *et al.* in 1948 for penicillin against various types of bacteria and is called the paradoxical effect, also known as the "Eagle effect" [30]. This paradoxical effect is also described for several fluoroquinoles [31], and later by Drlica *et al.* using ciprofloxacin against *Mycobacterium bovis* in 1996 [30, 32]. This paradoxical effect is possibly explained by the observation that at high drug concentrations, synthesis of RNA and proteins, both of which are necessary for killing is inhibited [33, 34].

Linezolid is currently a group 5 anti-TB drug, i.e. not recommended for routine use in the treatment of MDR-TB. However, *in vitro* and *in vivo* studies with linezolid have shown good activity against *Mycobacterium tuberculosis,* and the current study showed moderate activity against this mycobacterium. Also off-label, clinical use as compassionate drug has shown that when linezolid is incorporated in the treatment of MDR-TB patients it might improve outcome [10, 35-38].

We aimed to combine moxifloxacin and linezolid with a companion drug to increase their efficacy. It appeared that the EPIs verapamil and timcodar did not improve the *in vitro* activity of moxifloxacin as single drug or the combination of moxifloxacin and linezolid. These results contrast with data presented by Adams *et al.* who showed a reduced macrophage induced tolerance of moxifloxacin when verapamil was added [14]. This effect was also observed when isoniazid, rifampicin and bedaquiline were combined with verapamil. A reduced tolerance of linezolid when combined with verapamil was not observed, which is in line with results in the current study. Gupta *et al.* showed using a murine TB model, acceleration of bacterial elimination when verapamil was added to the standard regimen of isoniazid, rifampicin and pyrazinamide [12]. Addition of verapamil also resulted in significantly decreased relapse rates. Furthermore, verapamil increased the bactericidal efficacy of sub inhibitory doses of bedaquiline in TB infected BALB/c mice [13]. In a different study Gupta *et al.* showed that the MIC of clofazamine and bedaquiline against *Mycobacterium tuberculosis* decreases by 8-fold when verapamil was added. However, verapamil did not contribute to a MIC reduction of moxifloxacin [11], which is comparable with the data presented in this study.

Our data of timcodar are approximately similar to that of Grossman *et al.*, who also found no additive effect of timcodar in combination with moxifloxacin and linezolid when used in broth cultures [16]. In an acute, high-dose TB infection model they found a statistically significant reduction of the mycobacterial load when mice where treated with a combination of bedaquiline and timcodar compared to bedaquiline as single drug. However, this effect was not observed when moxifloxacin and sutezolid where combined with timcodar [16].

A possible explanation for the lack of improvement of the activity of the moxifloxacin + linezolid backbone when verapamil or timcodar were added *in vitro,* is that verapamil and timcodar do not inhibit the mycobacterial transporters that are accountable for the transport of moxifloxacin or linezolid through the bacterial cell. However, in macrophages and mice, verapamil can reduce the transmembrane potential and thereby inhibit eukaryotic transporters [13, 14], possibly leading to an increased moxifloxacin or linezolid concentration inside the macrophages. Therefore an *in vivo* study using a murine TB model studying the role of verapamil in combination with the moxifloxacin + linezolid backbone is warranted.

The most striking result of this study was the effect of colistin on the moxifloxacin + linezolid backbone. As single drug, colistin displayed limited activity, as also shown by Bax *et al*. [18], but it enhanced the activity of both moxifloxacin and linezolid as single drug as well as the activity of the moxifloxacin + linezolid backbone. Although synergy criteria were not met, with the 3 concentrations tested we showed 1.4-2.6 log CFU/mL increased killing when colistin was added to the moxifloxacin + linezolid backbone. A drawback of the effect observed with colistin is that only high concentrations of colistin can potentiate the moxifloxacin + linezolid backbone, i.e. concentrations systemically not achievable in patients. However, administration of colistin via inhalation deserves attention as several (pre-)clinical studies have shown dramatically increased colistin concentrations in pulmonary epithelial lining fluid and in sputum [39, 40].

Also, the observation that SQ109 in combination with moxifloxacin plus linezolid does not increase the efficacy of the backbone is very interesting, it even limited the activity of the backbone. The same is observed in the combination of linezolid with SQ109, the higher the concentration of SQ109, the less active the combination is. A possible explanation could be that SQ109 exerts its effect against actively replicating *Mycobacterium tuberculosis,* whereas linezolid as a protein-synthesis inhibitor might growth arrest the bacteria and thereby preclude the effect of SQ109. As single drug we showed that SQ109 is very active against *Mycobacterium tuberculosis*, with concentrations far below the C_{max} observed in TB-patients treated with SQ109 [41]. Heinrich *et al.* recently showed that SQ109 is safe to use for at least 14 days in TB patients, but it was not active as single drug or in combination with rifampicin in these patients over the first 14 days of treatment [41]. In addition, in a clinical study recruitment of patients was stopped in the SQ109 containing treatment arms because the pre-specified efficacy thresholds were not met [42]. Both the SQ109 containing arms also contained rifampicin and one also moxifloxacin, indicating that further research is needed to study the role of SQ109 as anti-TB drug.

In summary, we showed the strong bactericidal activity of moxifloxacin and SQ109 as single drugs against *Mycobacterium tuberculosis* as well as the ability of colistin to potentiate the moxifloxacin + linezolid combination.

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Table S1. The anti-mycobacterial effect of moxifloxacin or linezolid in combination with a potentiator drug.

TB drugs tested (mg/L)	Log10 CFU/mL at day 0 LLQ = 0,7	Log10 CFU/mL at day 6 LLQ = 0,7	ΔLog10 CFU/mL between drug combination and most active drug at day 6
Control	5.4 (5.2 - 5.5)	7.5 (7.4 - 7.6)	-
MXF 0.031		6.7 (6.7 - 6.7)	-
LZD 0.69		5.6 (5.4 - 5.7)	-
VP 16		7.4 (7.3 - 7.4)	-
VP 64		6.7 (6.6 - 6.7)	-
VP 256		4.3 (4.2 - 4.3)	-
MXF + VP 16		6.5 (6.5 - 6.5)	-0.2
MXF + VP 64		5.9 (5.6 - 6.1)	-0.6
MXF + VP 256		4.6 (4.5 - 4.6)	0.3
LZD + VP 16		5.8 (5.6 - 5.9)	0.2
LZD + VP 64		5.6 (5.5 - 5.6)	0.0
LZD + VP 256		4.8 (4.5 - 5.0)	0.5
Control	5.4 (5.2 - 5.5)	7.5 (7.4 - 7.6)	-
MXF 0.031		6.7 (6.7 - 6.7)	-
LZD 0.69		5.6 (5.4 - 5.7)	-
TIM 4		7.5 (7.4 - 7.6)	-
TIM 16		7.6 (7.4 - 7.7)	-
TIM 64		7.5 (7.4 - 7.5)	-
MXF + TIM 4		7.0 (6.8 - 7.1)	0.3
MXF + TIM 16		6.5 (6.4 - 6.6)	-0.2
MXF + TIM 64		6.3 (6.1 - 6.4)	-0.4
LZD + TIM 4		5.7 (5.4 - 5.9)	0.1
LZD + TIM 16		5.6 (5.4 - 5.8)	0.0
LZD + TIM 64		5.5 (5.3 - 5.6)	-0.1
Control	5.2 (5.2 - 5.2)	7.4 (7.2 - 7.5)	-
MXF 0.031		6.1 (5.8 - 6.4)	-
LZD 0.69		5.5 (5.4 - 5.5)	-
CST 16		6.9 (6.8 - 7.0)	-
CST 64		5.2 (4.7 - 5.6)	-
CST 256		4.0 (3.5 - 4.5)	-
MXF + CST 16		5.7 (5.4 - 5.9)	-0.4
MXF + CST 64		4.3 (4.1 - 4.4)	-0.9
MXF + CST 256		3.8 (3.4 - 4.1)	-0.2
LZD + CST 16		5.0 (5.0 - 5.0)	-0.5
LZD + CST 64		4.4 (4.3 - 4.4)	-0.8
LZD + CST 256		4.2 (4.1 - 4.2)	0.2

Control	5.2 (5.2 - 5.2)	7.4 (7.2 - 7.5)	-
MXF 0.031		6.1 (5.8 - 6.4)	-
LZD 0.69		5.5 (5.4 - 5.5)	-
SQ109 0.063		6.6 (6.4 - 6.8)	-
SQ109 0.25		3.1 (3.1 - 3.1)	-
SQ109 1		2.3 (2.2 - 2.3)	-
MXF + SQ109 0.063		5.0 (4.1 - 5.8)	-1.1
MXF + SQ109 0.25		3.0 (2.9 - 3.0)	-0.1
MXF + SQ109 1		2.7 (2.4 - 2.9)	0.4
LZD + SQ109 0.063		4.5 (4.3 - 4.7)	-1.0
LZD + SQ109 0.25		4.3 (4.2 - 4.3)	1.2
LZD + SQ109 1		3.9 (3.7 - 4.0)	1.6

Abbreviations: MXF: moxifloxacin, LZD: linezolid, VP: verapamil, TIM: timcodar, CST: colistin

Chapter 5

Dried Blood Spot Analysis and Human Pharmacokinetic Equivalent Dose of Moxifloxacin in Mice

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Submitted

ABSTRACT

The use of dried blood spot (DBS) analysis for the quantification of drug concentrations can reduce the number of animals used in pre-clinical drug development. In order to evaluate the use of DBS analysis we conducted a pharmacokinetic study in healthy mice to compare moxifloxacin concentrations in plasma with murine blood spots. We also determined the pharmacokinetic parameters to define a human equivalent dose of moxifloxacin. A Passing-Bablok plot showed a small positive bias for DBS versus plasma measurements and Bland-Altman analysis showed a small range around the mean DBS/plasma moxifloxacin concentration ratio. This indicates that DBS concentrations can predict plasma concentrations with acceptable precision. Analyses of pharmacokinetic parameters showed that a 200mg/kg dose of moxifloxacin in mice would approximate a human equivalent exposure, based on AUC values. These results suggests that DBS analysis can be used to predict moxifloxacin concentrations in plasma in pre-clinical murine models.

INTRODUCTION

Despite the recent failure of moxifloxacin to effectively shorten treatment duration of drug susceptible tuberculosis (TB) in recent trials [1], it remains a strong companion drug within novel TB drug combinations for susceptible TB [2]. In addition, it is still of great value for multidrug-resistant TB (MDR-TB) treatment. MDR-TB requires a prolonged treatment time, drug treatment is expensive and yields successful response in only 50% of MDR-TB patients [3]. Preclinical studies presented promising results of moxifloxacin as part of novel regimens against drug resistant *Mycobacterium tuberculosis* [4, 5], and (as a result of such preclinical studies) moxifloxacin is being evaluated as part of a universal regimen for susceptible and MDR-TB [6].

The use of moxifloxacin in pre-clinical studies as well as its subsequent evaluation in novel treatment regimens in TB patients requires a clear understanding of its dose-response relationships. Pharmacokinetic confirmation and optimum dose finding in preclinical models may prevent suboptimal exposures in efficacy studies and avoid inaccurate comparisons between regimen potencies [7].

In these preclinical studies, moxifloxacin is ideally measured in small sample volumes allowing repeated sampling within one animal. These small blood samples can be collected via the tail vein with limited animal discomfort and can possibly replace blood collection via cardiac puncture. This would allow for a significant decrease in the number of animals used for pre-clinical pharmacokinetic studies, which is of ethical importance. Also more than one blood sample can be taken from the animals via the tail vein, this will increase the accuracy in PK studies. Dried blood spots (DBS) analysis in combination with high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis may permit these novel sampling strategies, which could possibly replace conventional sampling.

The objectives of our study were to evaluate the use of DBS analysis for measuring moxifloxacin concentrations in murine blood spots, as well as to identify the human equivalent dose (HED) of moxifloxacin.

MATERIALS AND METHODS

Animals

Specified pathogen-free female BALB/c mice were obtained from Charles River (Les Oncins, France). At the day of the experiments, animals were 13-15 weeks old and weighed 20-25 gram. 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

A single-dose pharmacokinetic study was performed in healthy mice given oral doses of moxifloxacin (Sigma-Aldrich, Zwijndrecht, the Netherlands) dissolved in sterile water (50, 100, 200 and 400 mg/kg). Mice were sacrificed by CO_2 exposure and blood samples, approximately 700µl were obtained via cardiac punction (n=2 mice/dose/timepoint) at 0.25, 0.50, 0.75, 1, 1.5, 3, 5 and 10 h after dosing. Samples were collected in microcentrifuge tubes with EDTA. To compare plasma analysis with DBS 40µl of blood was spotted onto Whatman FTA/DMPK-C cards (Whatman, Kent, UK). Cards were allowed to dry for 3 hours. Remaining blood was centrifuged at 10.000xg for 5 min to obtain plasma. Cards and plasma were frozen at -80°C.

Analysis of plasma and DBS moxifloxacin concentrations

Moxifloxacin concentrations in plasma were analyzed with a LC-MS/MS assay, validated for human plasma, at the University Medical Center Groningen, the Netherlands [8].

Cross-validation of the assay for human plasma to murine plasma was performed by comparing responses of fivefold measurements of three 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%, the within-run CV amounted to 4.0-6.2% and the LLOQ was 0.05 mg/L.

Protein-unbound (free, active) concentrations of moxifloxacin were measured in all samples and were obtained following ultrafiltration of plasma, to separate unbound from bound moxifloxacin, using Centrifree YM-T 30 kDa ultrafiltration tubes (Merck-Millipore, Amsterdam, the Netherlands). Clear ultrafiltrate was analyzed via LC-MS/MS.

To quantify DBS samples, a validated extraction method was used [9, 10]. Extracts were analyzed using the same LC-MS/MS analysis method as for plasma. Cross-validation of the assay for human to murine DBS samples was performed by comparing responses of fivefold measurements of three QC moxifloxacin DBS samples (concentrations 0.5, 2.5 and 5 mg/L) in human blood with those in murine blood (40 µL blood spots) after analysis of all samples with the LC-MS/MS assay for human DBS samples. Accuracy of measurement of murine DBS samples with the assay for human DBS samples was between 123-125%, the within-run CV amounted to 2.0-3.6% and the LLOQ was 0.05 mg/L. The positive bias of 23-25% may be explained by differences in hematocrit concentrations in humans and mice, i.e circa 35% versus measured hematocrit of 55% (95% CI 44-67%), respectively. DBS sampling is known to be hampered by variation in hematocrit, resulting in differences in blood viscosity values and sample volumes, yielding a proportional analytical bias. Therefore, we corrected all murine DBS concentrations with a factor 0.82 (1/mean bias).

Agreement analysis of moxifloxacin concentrations in plasma and DBS

The level of agreement between the DBS method and the plasma method was analyzed by comparing the moxifloxacin concentrations in DBS samples with the concentrations in plasma using Passing-Bablok regression and Bland-Altman analysis with Analyze-it version 2.20 (Analyze-it Software, Ltd.) [11]. Only DBS concentrations were included that were below the highest QC that was cross-validated, namely 5 mg/L.

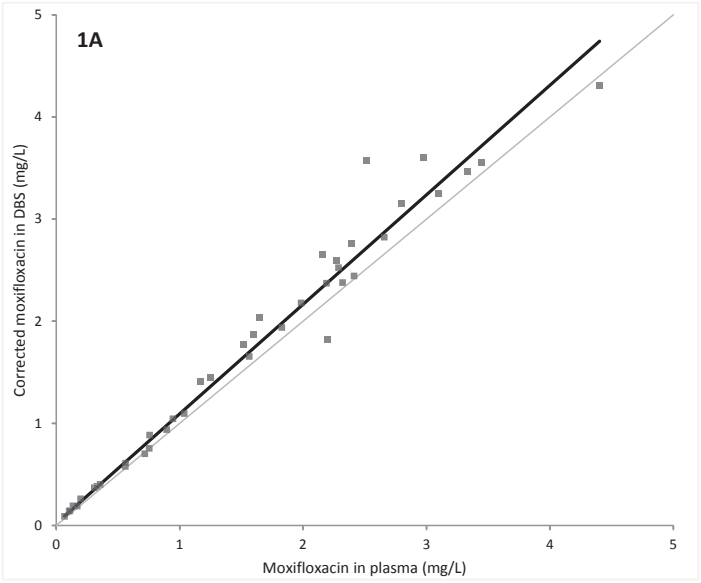
Pharmacokinetic evaluation

PK parameters were assessed using standard noncompartmental methods in Phoenix WinNonlin version 6.4 (Pharsight Corporation), as described previously [12]. To determine exposure equivalence with human moxifloxacin exposures, areas under the plasma concentration-time curve up to infinity ($AUC_{0-\infty}$) and peak (C_{max}) values after a single dose in mice were compared to clinically relevant AUC_{0-t} and C_{max} steady state moxifloxacin exposures in humans, considering that $AUC_{0-\infty}$ after a single dose is equivalent to AUC_{0-t} in a dosing interval t at steady-state.

RESULTS

Comparison of moxifloxacin concentrations in plasma and DBS

The level of agreement between moxifloxacin measured in murine plasma and with DBS was determined using Passing-Bablok regression and Bland-Altman analysis and is shown in figure 1. The Passing-Bablok analysis (1A) showed a high level of similarity between both methods with an intercept of 0.02155 (95% CI -0.01765 – 0.04669) mg/L and a slope of 1.07 (95% CI 1.03 – 1.14), indicating an average 7% positive proportional bias for DBS versus plasma measurements of moxifloxacin in mice.



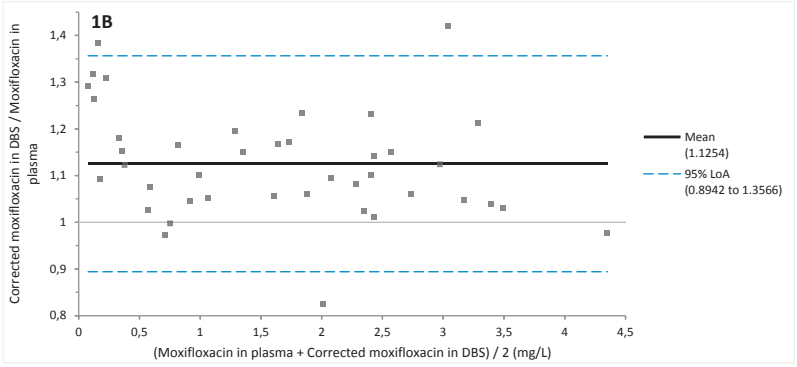


Figure 1. Assessment of the level of agreement between moxifloxacin concentrations measured in plasma and in DBS using Passing-Bablok regression (A) and Bland-Altman analysis (B).In the scatter plot with Passing-Bablok fit (A), moxifloxacin concentrations in DBS are plotted against their concentration in plasma. The line of identity is presented in solid grey and in black the Passing-Bablok regression line, y=0.02155+1.072x. Bland-Altman analysis plots (B) the moxifloxacin DBS/plasma concentration ratio versus the average concentration in DBS and plasma. The line representing the mean ratio is solid, while the lines depicting the 95% Limits of Agreement (LoA), containing 95% of the data points, are dashed.

Bland-Altman analysis (1B) showed a mean DBS/plasma concentration ratio of 1.13 with a 95% CI of 1.09 – 1.16, with a lower limit of agreement (LoA) of 0.89 (95% CI 0.83-0.96) and an upper LoA of 1.36 (95% CI 1.29-1.42), which means that 95% of the data points is within 0.89 and 1.36 concentration ratios.

Pharmacokinetic evaluation

Figure 2 displays the concentration-time profiles as measured in plasma and table 1 the main pharmacokinetic parameters of single doses of moxifloxacin in uninfected mice. After oral administration of moxifloxacin in mice the $AUC_{0-\infty}$ values were 4.5, 11, 24 and 76 mg/L/h for 50, 100, 200 and 400 mg/kg, respectively. Pharmacokinetics appeared to be dose linear with increasing concentrations, except for the increase from the 200 to 400 mg/kg dose, where multiplying the dose by two resulted in a threefold increase in exposure. The C_{max} values were 2.8, 6.7, 12 and 18 mg/L for 50, 100, 200 and 400 mg/kg, respectively. The mean percentage unbound moxifloxacin in all 64 samples was 55% (42–76%) and seems comparable, but somewhat higher compared to the unbound fraction in humans (39-50%) [13, 14].

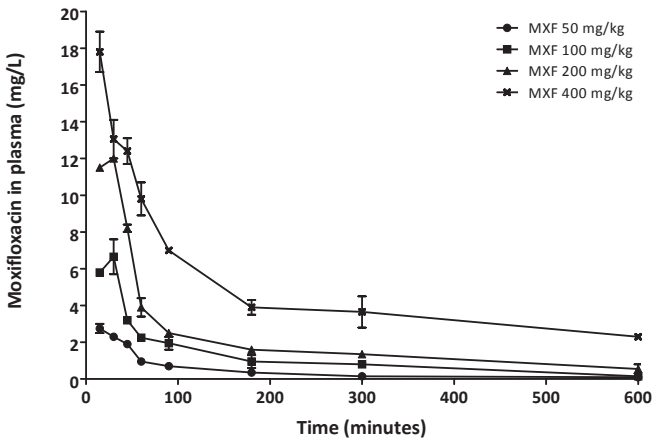


Figure 2. Moxifloxacin profiles in plasma of healthy BALB/c mice following single oral doses of 50 mg/kg (circles), 100 mg/kg (squares), 200 mg/kg (triangles) and 400 mg/kg (crosses) of moxifloxacin.
Plasma concentrations are plotted as median with ranges of two mice per dosage and time point.

Table 1. Pharmacokinetic parameters of moxifloxacin in plasma following single dose of 50, 100, 200 and 400 mg/kg in BALB/c mice.

Dose (mg/kg)	$AUC_{0-\infty}$ (mg/L.h)	C_{max} (mg/L)	Cl/F (L/h)	Vd/F (L)	T½ (h)
50	4.5	2.8	0.25	1.2	3.2
100	11	6.7	0.19	0.67	2.4
200	24	12	0.19	1.1	4.1
400	76	18	0.12	1.5	8.8

PK parameters are based on the average concentration of two sacrificed mice per dosage and time point (n=64)

DISCUSSION

Moxifloxacin is a promising building block in novel TB regimens for drug susceptible and resistant TB and remains an essential drug in the battle against MDR-TB. Therefore a clear understanding of its pharmacokinetics and pharmacodynamics in preclinical models is pivotal. In the present study we validate the use of DBS sampling by comparing concentrations in whole blood with concentrations in plasma, obtained from uninfected female BALB/c mice. When comparing DBS with plasma, the Passing-Bablok plot showed a positive bias with an acceptable level, demonstrating that data obtained with both methods are in concordance. Bland-Altman analysis showed a lower (0.89) and upper (1.36) 95% LoA indicating that DBS values can predict plasma concentrations with acceptable precision. Vu *et al.* found significantly higher moxifloxacin concentrations in DBS compared to plasma samples from actual TB-patients [10]. This discrepancy between DBS and plasma concentrations is higher than found in the current study in mice. This might be explained by interspecies differences in the distribution of moxifloxacin between plasma and blood cells caused by a difference in binding capacity to plasma proteins and blood cells [15].

Based on these results we conclude that DBS can be used in preclinical drug development models to assess moxifloxacin pharmacokinetics. Multiple blood sampling via the tail vein is possible up to 20% of the circulatory blood volume in 24h, this allow up to four samples from a single animal [16]. In addition in PK/PD studies, mice can be used for a PK study and remain in the study for PD assessment. This significantly decrease the number of mice necessary as well as the costs of the experiments. A limitation of the study is the validated range of concentrations (0.5 – 5 mg/L) when cross-validating human DBS with murine DBS samples, in some samples the moxifloxacin concentrations in blood were higher than the upper limit and were excluded in the comparison.

Before performing efficacy studies with moxifloxacin in mice it is of great importance to use a HED. In the present study, the pharmacokinetics of moxifloxacin were studied to determine the HED in mice that can be used for further drug efficacy testing in TB infected mice. The AUC relative to the minimal inhibitory concentration has been suggested as the

best parameter to predict *in vivo* efficacy against *Mycobacterium tuberculosis*[17]. Based on the protein binding which seems slightly lower in mice compared to humans and the $AUC_{0-\infty}$ calculated in the current study, a single dose of 200 mg/kg moxifloxacin in mice would approximate a human equivalent free drug exposure (AUC_{0-24} 24.8 mg*h/L / *f*AUC_{0-24} 17.3 mg*h/L [18]), which can be used in efficacy studies. This suggested 200 mg/kg dose complements the data from Poissy *et al.* who found AUC values close to those found in humans when a similar dose was used as well as the highest decline in CFU with this dose [19].

The C_{max} of moxifloxacin in humans after the standard dose of 400mg is 2.5 - 3.3 mg/L [18, 20]. The C_{max} in mice obtained with the 100, 200 and 400 mg/kg dose was higher, where 50 mg/kg resulted in a C_{max} of 2.8 mg/L which is in the C_{max} range found in TB-patients [18, 20]. However, the suggested 200 mg/kg dose yields an increased C_{max} compared to those in humans, therefore an additional study must be performed to determine whether a fractionated dose over the day will minimize the C_{max} and maintain the AUC. The median half-life of moxifloxacin in mice is 3.65 h compared to 8.3 – 15.6 in humans [13, 20].

Our results are in contrast with $AUC_{0-\infty}$ and C_{max} data presented by Rosenthal *et al.* who found higher $AUC_{0-\infty}$ and C_{max} values in mice after a single dose of moxifloxacin (100, 200 and 400 mg/kg), except for the $AUC_{0-\infty}$ after a 400 mg/kg dose which is higher in the current study [21].

In summary, we showed that DBS can be used to perform further moxifloxacin studies in murine models to study pharmacokinetics and pharmacokinetic-pharmacodynamic relationships. Furthermore, we identified that 200 mg/kg moxifloxacin is an appropriate dose to perform efficacy studies in a murine TB model.

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Chapter 6

Human pharmacokinetic equivalent dose and dried blood spot analyses of linezolid in BALB/c mice

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ABSTRACT

Linezolid is a promising agent for future multidrug-resistant TB (MDR-TB) regimens, to be evaluated in the TB mouse model. To improve clinical predictability from animal efficacy studies, it is important to test a linezolid dose that is pharmacokinetically equivalent to the dose used in MDR-TB patients. Furthermore, pharmacokinetic analyses in mice would benefit from measurement of analytes in small blood volumes using the 'dried blood spot' (DBS) technique, because it allows for the assessment of drug concentrations over time within a single mouse. This study aimed to identify the human pharmacokinetic equivalent dose of linezolid, as well as to evaluate DBS analysis for measuring linezolid concentrations in murine blood spots. We identified 50 mg/kg oral linezolid ($AUC_{0-\infty}$ 78 mg/L*h) in BALB/c mice as the pharmacokinetic equivalent of a dose between 300 and 600 mg in humans. We recommend this dose for future MDR-TB regimen efficacy testing. Nonlinear kinetics of linezolid were observed, which complicated deriving an exact human pharmacokinetic equivalent dose. Concentrations in murine blood spots were slightly lower than in plasma: Passing-Bablok regression demonstrated a significant proportional bias with a slope of 0.81 (95% CI 0.79-0.84). To compensate for the differences in murine plasma and blood, we propose a conversion factor of 1.2 to predict plasma concentrations from DBS. Overall, we feel that the applied measurement of drug concentrations in murine DBS may significantly reduce the number of animals needed for future PK-PD assessments.

INTRODUCTION

Globally, an estimated 480,000 tuberculosis (TB) patients suffer from multi-drug resistant TB (MDR-TB), a level that has changed little in recent years [1]. Only 50% of MDR-TB patients were successfully treated in 2014 [1], indicating that MDR-TB treatment remains far from adequate. The oxazolidinone linezolid, a group 5 TB drug in the WHO treatment guideline, has shown good activity against drug resistant strains in preclinical studies [2, 3], with growing evidence for its efficacy in MDR-TB and XDR-TB patients [4, 5].

Before the contribution of linezolid to novel MDR-TB regimens can be investigated in patients, this first has to be tested in appropriate preclinical models, such as the TB mouse model. Pharmacokinetic data are indispensable in these studies, as linezolid exposure is considered predictive for treatment outcome and adverse effects such as hematologic toxicity and peripheral neuropathy [6-8]. The linezolid pharmacokinetic-pharmacodynamic (PK-PD) parameters of interest are the (protein-unbound) area under the concentration versus time curve (AUC) to minimum inhibitory concentration (MIC) ratio, and time above MIC [7, 9].

To improve clinical predictability from animal efficacy studies, it is important to test a linezolid dose that is pharmacokinetically equivalent to the dose used in MDR-TB patients. Furthermore, pharmacokinetics analyses in mice would benefit from measurement of analytes in small blood volumes using the 'dried blood spot' (DBS) technique [10], because it allows for the assessment of drug concentrations over time within a single mouse. This could significantly reduce the number of animals needed for pharmacokinetic assessment, as conventionally one animal is euthanized for collection of a single blood sample to ensure sufficient quantities of analytical sample.

To optimize linezolid efficacy testing in mice, the aim of this study was to identify the linezolid dose resulting in exposures equivalent to those found in humans, as well as to evaluate DBS analysis for measuring linezolid concentrations in murine blood spots.

METHODS

Animals

Specified-pathogen-free female BALB/c mice were obtained from Charles River (Les Oncins, France). Experimental protocols adhered to the rules specified in the Dutch Animal Experimentation Act (1977) and the published Guidelines on the Protection of Experimental Animals by the Council of the EC (1986). The Institutional Animal Care and Use Committee of the Erasmus MC Rotterdam approved the protocol [117-12-14, EMC2888].

Experimental set-up

Pharmacokinetic studies were performed in 64 mice after oral administration of single doses of linezolid (Sigma-Aldrich, Zwijndrecht, the Netherlands). Mice were treated with a linezolid dosage range (12.5, 25, 50 and 100 mg/kg dissolved in PBS) and samples were taken at 0.25, 0.5, 0.75, 1, 1.5, 3, 5 and 10 h after the dose. Two animals were euthanized for each dosage and sampling time point. Blood was collected via cardiac puncture, after which DBS samples were obtained by pipetting 40 µl of blood onto Whatman DMPK-C cards (Whatman, Kent, UK). The remaining blood was centrifuged to obtain plasma, which was stored at -80 °C until further analysis.

Analysis of plasma and DBS linezolid concentrations

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 [11]. 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. Cross-validation 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 five-fold) 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%. Protein-unbound concentrations of linezolid were measured in all samples and were obtained following ultrafiltration of plasma, to separate bound from unbound linezolid, using Centrifree YM-T 30 kDa ultrafiltration tubes (Merck-Millipore, Amsterdam, the Netherlands). Clear ultrafiltrate was analysed via LC-MS/MS.

To quantify murine DBS samples a validated method for human samples was used [12]. The DBS assay accuracy was within 98.7-106.3%, the overall precision (CV) ranged from 5.3-17.2%, and the LLOQ was 0.05 mg/L. Cross-validation between human and murine DBS was performed by comparing responses of three QC linezolid concentrations (0.5, 15 and 30 mg/L in five-fold) in human DBS with those in mouse DBS (40 µL blood spots). The accuracy of murine relative to human DBS measurements was between 111-140% (mean bias 123%), with a within-run CV of 2.1-2.7%. The bias may be explained by measured differences in hematocrit concentrations in human and mice, 35% versus 55% (95% CI 44-67%), respectively. DBS sampling is hampered by variation in hematocrit due to differences in blood viscosity values, yielding a proportional analytical bias. Therefore, we corrected all murine DBS concentrations with a factor 0.82 (1/mean bias).

Pharmacokinetic evaluation

Pharmacokinetic parameters were assessed using standard noncompartmental methods in Phoenix WinNonlin version 6.4 (Pharsight Corporation) [13]. To determine the human

pharmacokinetic equivalent dose, $AUC_{0-\infty}$ single dose values in mice were compared to clinically relevant AUC_{0-t} linezolid exposures in humans at steady state.

Agreement analysis of plasma and DBS linezolid concentrations

The level of agreement between the DBS and the plasma method was analysed by comparing linezolid concentrations in DBS samples with those in plasma using Passing-Bablok regression and Bland-Altman analysis with Analyze-it version 2.20 (Analyze-it Software, Ltd.) [14]. Only DBS concentrations were included that were below the highest QC cross-validated (<30 mg/L).

RESULTS

Pharmacokinetic evaluation

Plasma concentration-time profiles and pharmacokinetic parameters of linezolid dosages are shown in Figure 1 and Table 1, respectively. Pharmacokinetics appeared to be nonlinear, as increases in exposure were not proportional to increases in dosage. The highest dose of 100 mg/kg was an exception to this, probably because limited solubility resulted in deviant absorption characteristics. The mean percentage unbound linezolid across all samples was 57 ± 9% (mean ± sd), which seems comparable, though somewhat lower, than the free fraction found in humans (70%) [9].

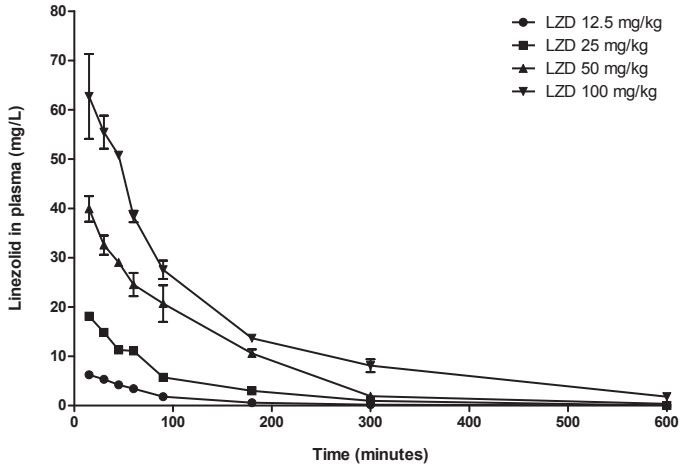


Figure 1. Linezolid plasma concentration-time profiles following single oral doses of 12.5 mg/kg (diamonds), 25 mg/kg (crosses), 50 mg/kg (circles) and 100 mg/kg (triangles) in BALB/c mice.
Plasma concentrations are plotted as mean and minimum-maximum of two mice per dosage and time point (n=64).

Table 1. Pharmacokinetic parameters of linezolid in plasma following single oral doses of 12.5 mg/kg, 25 mg/kg, 50 mg/kg and 100 mg/kg in BALB/c mice.

Dose (mg/kg)	$AUC_{0-\infty}$ (mg/l*h)	C_{max} (mg/l)	Cl/F (l/h)	Vd/F (l)	$t_{½}$ (h)
12.5	8.3	6.3	0.033	0.054	1.1
25	28	18	0.020	0.038	1.3
50	78	40	0.014	0.030	1.4
100[a]	141	63	0.016	0.054	2.4

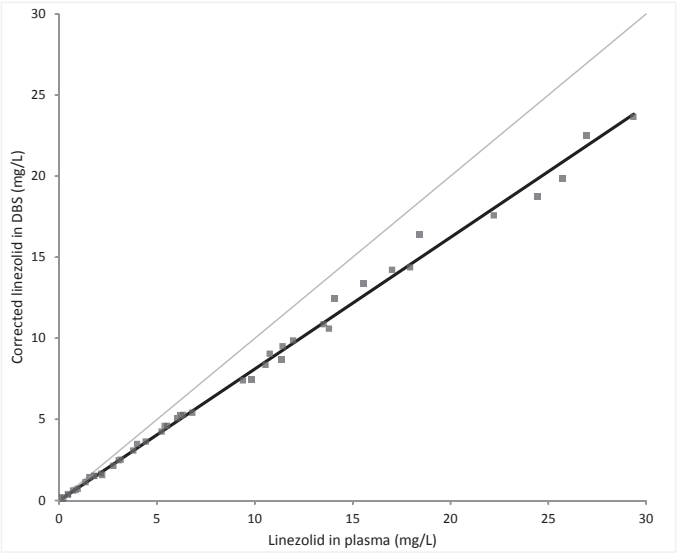
Pharmacokinetic parameters are based on the mean concentration of two sacrificed mice (n=64)

[a] Dosage was at the limit of solubility, homogeneity of suspension was strived for by vortexing just prior to administration

In literature, median AUC_{0-12h} in TB patients taking 300 mg twice daily was 58 mg/L*h [15]. For 600 mg twice daily, AUC_{0-12h} amounted to 140-146 mg/L*h [15, 16]. Single oral doses of 375 and 625 mg in healthy adults resulted in an $AUC_{0-\infty}$ of 66 and 102 mg/L*h, respectively [17]. Considering these exposures and the relatively comparable protein binding in humans, 50 mg/kg in BALB/c mice ($AUC_{0-\infty}$ of 78 mg/L*h) would approximate free linezolid exposures of a dose between 300 and 600 mg in humans.

Agreement between plasma and DBS concentrations

Figure 2 displays the assessment of the level of agreement between measurement of linezolid in murine plasma and DBS samples (n=46 data points <30 mg/L). A significant *proportional* bias was observed in the Passing-Bablok analysis with a slope of 0.81 (95% CI 0.79-0.84) (Figure 2A). The Bland-Altman analysis showed that the lower (0.71 (95% CI 0.69-0.73)) and upper (0.90 (95% CI 0.87-0.92)) 95% Limits of Agreement (LoA) were relatively narrow around the mean DBS/plasma linezolid concentration ratio of 0.80 (95% CI 0.79-0.82)) (Figure 2B). From a linear regression analysis with plasma concentration as dependent and DBS concentrations as independent variable, we derived a conversion factor of 1.23 to predict murine plasma concentrations from DBS levels (R^2 = 0.995, p<0.001).



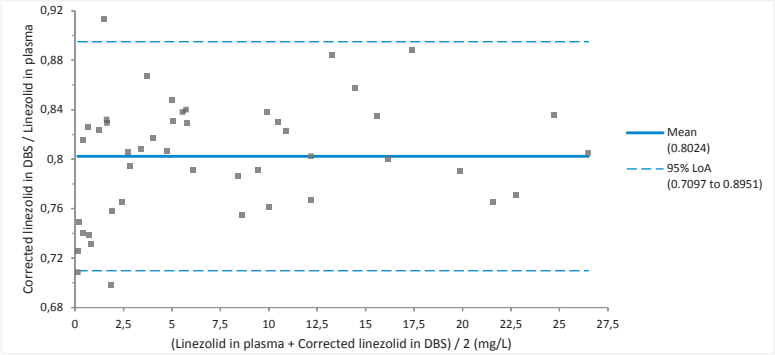


Figure 2. Assessment of the level of agreement between linezolid concentrations measured in plasma and in DBS using Passing-Bablok regression (A) and Bland-Altman analysis (B).

In the scatter plot with Passing-Bablok fit, linezolid concentrations in DBS are plotted against their concentration in plasma (A). The line of identity is presented in solid grey and the Passing-Bablok regression line is dashed. The regression line has a slope of 0.81 (95% CI 0.79-0.84) and an intercept of -0.015 (95% CI -0.064-0.041). Bland-Altman analysis plots the linezolid DBS/plasma concentration ratio versus the average concentration in DBS and plasma (B). The line representing the mean ratio is solid, while the lines depicting the 95% Limits of Agreement (LoA), containing 95% of the data points, are dashed. The mean ratio is 0.90 (95% CI 0.79-0.82).

DISCUSSION

In this study, we found that a linezolid dose of 50 mg/kg resulted in an $AUC_{0-\infty}$ of 78 mg/L*h in BALB/c mice, which approximates free linezolid exposures of a dose between 300 and 600 mg in humans. We recommend this dose for future MDR-TB regimen efficacy testing. In literature there has been an ongoing debate on the optimal linezolid dose or concentrations associated with treatment efficacy in humans. Initial treatment of MDR-TB with 600 mg of linezolid twice daily has been limited by adverse effects [15, 18]. Lower doses mostly seemed to retain effectiveness and limit toxicity [7, 15, 18]. Therefore, linezolid doses ≥100 mg/kg tested in mice previously, probably by far exceed clinically useable doses and exposures [19, 20].

Nonlinear kinetics of linezolid were observed. Clearance decreased 30% after a 2-fold increase in dose, which complicated deriving an exact human pharmacokinetic equivalent dose. Nonlinearity has been demonstrated in mice previously at lower oral linezolid doses (0.625-40 mg/kg) [21], but not after intraperitoneal or subcutaneous administration [22, 23]. This underlines the importance of route of administration as well as pharmacokinetic confirmation of drug exposures in drug efficacy studies. Nonlinearity has also been shown before in humans, but to different extends [9, 15, 17, 24]. In a population pharmacokinetic analysis linezolid nonlinearity could not be explained by enzyme kinetics and was thought to be related to mitochondrial function [24].

With respect to the analysis of linezolid in murine DBS, we showed that concentrations in blood are slightly lower than in plasma, as we found a significant proportional bias with a slope of 0.81 (95% CI 0.79-0.84). To compensate for the differences in murine plasma and blood, we propose a conversion factor of 1.2 to predict plasma concentrations from DBS levels. Bland-Altman analysis showed that the lower and upper 95% LoA are relatively narrow around the average linezolid DBS/plasma concentration ratio, namely 0.71-0.90, meaning that the DBS/plasma ratio can be estimated with rather good precision.

Current findings are in contrast with the higher linezolid levels found in human DBS compared to human plasma previously [12], which may be explained by interspecies differences in (binding to) blood components. Furthermore, less spread is observed in mice plasma/DBS ratios compared to human ratios [12], probably due to the larger heterogeneity in human blood/hematocrit composition. To our knowledge, no studies measuring linezolid (or any other TB drug) in murine DBS have been reported, even though the general feasibility of serial sampling in mice with the DBS technique has been published before [25, 26]. Compound specific validation in DBS matrix is indispensible, as factors such as blood/plasma partition ratios and the impact of hematocrit may be very drug specific, and can complicate the comparison with plasma samples [27].

Overall, this study provides very practical considerations and techniques for linezolid drug efficacy studies in mice, with linezolid as part of a future MDR-TB regimen. Further-

more, the applied measurement of drug concentrations in murine DBS may significantly reduce the number of animals needed for these future PK-PD assessments.

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Chapter 7

Rifampicin-induced transcriptome response in rifampicin-resistant *Mycobacterium tuberculosis*

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ABSTRACT

Tuberculosis (TB) is still a major life-threatening infectious disease, within which especially the rise of multidrug resistant TB (MDR-TB) is currently worrying. This study focuses on mechanisms of development of rifampicin resistance, since rifampicin seems to play an important role in the development of MDR-TB.

To provide further insight in rifampicin resistance, we performed a genome-wide transcriptional profile analysis for *Mycobacterium tuberculosis* using microarray technology and qRT-PCR analysis. We exposed a rifampicin-susceptible H37Rv wild type (H37Rv-WT) and a rifampicin-resistant progeny H37Rv strain with a H526Y mutation in the *rpoB* gene (H37Rv-H526Y) to several concentrations of rifampicin, to define the effect of rifampicin on the transcription profile.

Our study showed that there are resistance-dependant differences in response between both *Mycobacterium tuberculosis* strains. Gene clusters associated with efflux, transport and virulence were altered in the rifampicin-resistant H37Rv mutant compared to the rifampicin-susceptible H37Rv-WT strain after exposure to rifampicin.

We conclude that the small gene cluster Rv0559c-Rv0560c in the H37Rv-H526Y strain was remarkably up-regulated in the microarray analysis and qRT-PCR results and appeared to be dependent on rifampicin concentration and time of exposure. Therefore this study suggests that Rv0559c and Rv0560c play a pivotal role in rifampicin resistance of *Mycobacterium tuberculosis*. Further investigation of Rv0559c and Rv0560c is needed to reveal function and mechanism of both genes that were triggered upon rifampicin exposure.

INTRODUCTION

Tuberculosis (TB) is one of the major life-threatening infectious diseases. In 2011, with 8.8 million new cases reported, TB was responsible for the death of 1.5 million people, equal to 4100 deaths a day [1]. Due to a considerable increasing rate of isoniazid and rifampicin resistance among strains of *Mycobacterium tuberculosis* and the poor treatment outcome of multidrug resistant (MDR)-TB treatment [1], more insight in the development of MDR-TB is needed. The World Health Organization estimated that worldwide 3.3% of all new TB cases in 2009 were MDR-TB [2] and over 85% of rifampicin-resistant clinical isolates are also resistant to isoniazid [3]. Our study focused on rifampicin-induced transcriptome responses in rifampicin-susceptible and rifampicin-resistant *Mycobacterium tuberculosis* strains, since rifampicin seems to play a driving role in the development of MDR-TB.

Resistance can either be intrinsic or acquired via genotypic changes. *Mycobacterium tuberculosis* is intrinsically resistant to a wide ranges of antimicrobial compounds due to the unusually low permeability of the bacterial outer membrane [4]. Additionally, *Mycobacterium tuberculosis* can become resistant to rifampicin via various routes. Genotypic resistance towards rifampicin is caused for almost 95% by target alteration due to non-synonymous single nucleotide polymorphisms (nsSNP) [5]. The nsSNP, which primarily occurs in an 81 base pair region in the *rpoB* gene, known as the rifampicin resistance-determining region (RRDR), results in several changes in the β-subunit of RNA polymerase. Due to these changes, rifampicin is less competent to bind to the β-subunit of RNA polymerase [5, 6], resulting in *Mycobacterium tuberculosis* mutants that are less susceptible or resistant to rifampicin. Recently, Siu *et al.* showed two new mutations in the *rpoB* gene outside the RRDR that also resulted in rifampicin resistance [7]. Another form of genotypic resistance is for example porin loss by mutations in genes encoding porin proteins leading to decreased cell wall permeability [8]. Resistance can also be caused by efflux pump activity. Efflux pump-induced drug extrusion will result in suboptimal intracellular drug concentrations [9-11]. The activity of efflux pumps genes and transporter genes can be actively induced by rifampicin at the level of gene expression [12].

To define the effect of rifampicin on the transcription profile, we performed a genome-wide transcriptional profile analysis for *Mycobacterium tuberculosis* exposed to rifampicin using microarray technology. We compared the transcription profiles of an isogenic pair of rifampicin-susceptible and rifampicin-resistant *Mycobacterium tuberculosis* strains, to provide further insight in the influence rifampicin resistance on overall gene expression upon rifampicin exposure.

MATERIALS AND METHODS

Bacterial culture and drug exposure

The two *Mycobacterium tuberculosis* strains used were: a H37Rv wild type (H37Rv-WT) strain and a progeny H37Rv strain with a H526Y mutation in the *rpoB* gene (H37Rv-H526Y). The minimum inhibitory concentration (MIC) for rifampicin (Rifadin®, Aventis Pharma B.V, Hoevelaken, the Netherlands) was determined to be 0.25 mg/L for the H37Rv-WT strain and 512 mg/L for the H37Rv-H526Y strain, using CLSI guidelines.

Mycobacterium tuberculosis suspensions were taken from a frozen stock, thawed and cultured in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA), supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (OADC, Baltimore Biological Laboratories, Baltimore, MD, USA), 0.5% glycerol (Scharlau Chemie S.A, Sentmenat, Spain) and 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA), under shaking conditions at 96 rpm at 37°C. After 6 days of growth *Mycobacterium tuberculosis* cultures were exposed to 0.063 mg/L and 0.25 mg/L rifampicin for the H37Rv-WT, and 128 mg/L and 512 mg/L rifampicin for the H37Rv-H526Y. These rifampicin concentrations represent ¼x the MIC and 1x the MIC of rifampicin for both strains, in the controls solvent was added.

Mycobacterium tuberculosis was cultured to late log-phase, prior to addition of rifampicin. Samples were collected 30 minutes and 12 hours after exposure to rifampicin, or solvent.

RNA extraction

RNA was isolated using a FastPrep-24 with a FastRNA® Pro Blue Kit (MP Biomedicals, Irvine, CA, USA). To remove residual DNA, RNA samples were treated with RNase-free DNase (Ambion, Austin, TX, USA) and were purified using the RNeasy MiniElute Clean-up Kit (Qiagen Benelux, Venlo, the Netherlands), all according to the manufacturers protocol. The amount of RNA was measured using the NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA) and the integrity was investigated with the 2100 BioAnalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) using a RNA Nano 6000 kit (Agilent Technologies) yielding RIN-values $\geq$ 8.9.

Labeling protocol

Per test sample, 5 µg total RNA together with 1 µg random octamers (Biolegio, Nijmegen, the Netherlands) in 4.5 µl was heated to 65°C for 10 min to denature the RNA and was allowed to cool in an ice-water bath for 10 min. This 4.5 µl was supplemented to 10 µl with a first strand mastermix. The mastermix contained final concentrations of 50 mM Tris-Cl (pH 8.3), 3 mM $MgCl_2$, 75 mM KCl, 200 mM Raffinose (Sigma-Aldrich), 0.015% Triton X-100, 30 ng Actinomycin-D (Sigma-Aldrich), 0.01M DTT, 0.5 mM dGAC, 0.35 mM dUTP, 0.15 mM dUTP-Cy3 (test) or dUTP-Cy5 (common reference) (GE Healthcare, Hoevelaken,

the Netherlands) and 200U SuperScript-II (Life Technologies, Bleiswijk, the Netherlands). The mixture was incubated for 2 min at 25°C, 120 min at 42°C and 15 min at 70°C. Finally, 1.5 µl of 2.5M NaOH was added to hydrolyze the remaining RNA upon heating for 10 min at 70°C. 8.5 µl 2M MOPS was added for neutralization and the labeled cDNA was purified with the E.Z.N.A. MicroElute RNA Clean-up Kit (Omega Biotek, Norcross, GA, USA). Dye incorporation and cDNA yield was measured on the NanoDrop ND-1000 yielding 2-2.5 µg per sample and a FOI >8 pmol/µg. The common reference was made by an equimolar pool of the test samples (5 µg per sample) and subsequently labeled as the test samples with Cy5 incorporation.

Microarray hybridization and scanning

Each hybridization mixture was made up from 500 ng test (Cy3) and 500 ng common reference (Cy5) material. Hybridization mixtures were prepared as described in the Agilent Two-Color Microarray-Based Gene Expression Analysis guide version 5.5 (G4140-90050, Agilent technologies) without the inclusion of the RNA fragmentation mixture. *Mycobacterium tuberculosis* whole genome arrays were supplied by the Bacterial Microarray Group at St. George's Hospital, University of London. Hybridization samples were loaded onto 8x15k *Mycobacterium tuberculosis* microarrays (Design ID: 027543, Agilent Technologies) and hybridized for 17 hours at 65°C. Afterwards the slides were washed and scanned (20 bit, 5 µm resolution) in an ozone-free room with the Agilent G2505C scanner as described in the Agilent G4140-90050 guide. Data were extracted with Feature Extraction (v10.7.3.1, Agilent Technologies) with the GE2_107_Sep09 protocol for two-color Agilent microarrays.

Microarray data processing

The raw data from all arrays were subjected to multiple quality control checks, i.e. visual inspection of the microarray scans, testing against criteria for foreground and background signals, testing for consistent performance of the labeling dyes, checking for spatial effects through pseudo-color plots, and inspection of pre- and post-normalized data with box plots, ratio-intensity (RI) plots and PCA plots. These checks revealed significant position effects on a slide. Subsequently, within-array normalization was performed using LOESS. Between-array normalization was run on the Cy3-channel by summarizing the intensity values of the probes in a probe set using the robust multi-array average (RMA) algorithm. The study (basically a three-factorial design) was subjected to 2 types of contrast analyses using mixed linear models with coefficients for slide-position and sample isolation day (both random) and each experimental group (fixed), to determine differential gene expression between ¼x MIC, 1x the MIC of rifampicin and non-exposure, per genotype and time-point and also to determine differential gene expression between the H37Rv-H526Y and H37Rv-WT per MIC treatment and time-point [13, 14]. For hypothesis testing, a Fs test was used and correction of the resulting P-values for false discoveries was done according

to Storey and Tibshirani [15, 16]. All data handling was performed using R-2.11.1 (http://www.R-project.org) and Bioconductor (www.bioconductor.org) software. The array design is available in BμG@Sbase (Accession No. A-BUGS-41; http://bugs.sgul.ac.uk/A-BUGS-41) and also ArrayExpress (Accession No. A-BUGS-41). Fully annotated microarray data have been deposited in BμG@Sbase (accession number E-BUGS-139; http://bugs.sgul.ac.uk/E-BUGS-139) and also ArrayExpress (accession number E-BUGS-139).

qRT-PCR analysis

Reverse transcriptase was performed with 50 ng of total RNA of each sample using a Reverse Transcriptase Core kit (Eurogentec, Seraing, Belgium). Primers were designed using Primer-BLAST (NCBI) and primer sequences are shown in Table 3. Quantitative Real-Time PCR (qRT-PCR) was performed with a 7500 Fast Real-Time PCR System (Applied Biosystems, Bleiswijk, the Netherlands) using a MESA FAST qRT-PCR MasterMix Plus for SYBR® Assay Low ROX (Eurogentec). The relative fold induction level of each transcript was obtained by ΔΔCt analysis and was performed on duplicate biological samples that were assayed in duplicate. For quantitative results *sigA* was used for normalization.

RESULTS

Mycobacterium tuberculosis growth

H37Rv-WT and H37Rv-H526Y at a density of 7.4 x 10^5 [4.3 – 9.9 x 10^5] CFU/mL were cultured and after 6 days had increased up to 3.1 x 10^7 [2.1 – 4.0 x 10^7] CFU/mL. Both strains showed identical growth patterns. At day 6, rifampicin was added and quadruple cultures were exposed to either solvent or rifampicin concentrations at ¼x the MIC or 1x the MIC. After 30 minutes and 12 hours exposure the control curves of both strains reached 3.9 x 10^7 [3.0 – 5.0 x10^7] CFU/mL and 2.5 x 10^7 [1.9 – 2.8 x 10^7] CFU/mL respectively. Exposure to ¼x the MIC of rifampicin led in both strains to similar CFU counts being 3.0 x 10^7 [2.1 – 4.1 x 10^7] after 30 minutes and 2.1 x 10^7 [1.5 – 3.1 x 10^7] after 12 hours exposure. Exposure to 1x the MIC of rifampicin led in both strains to CFU counts of 3.1 x 10^7 [2.8 – 3.4 x 10^7] after 30 minutes and 2.3 x 10^7 [1.2 – 3.1 x 10^7] after 12 hours exposure.

Microarray analysis

After microarray analysis, all genes that responded to rifampicin (FDR p-values ≤ 0.05), at each time point were categorized and the associated distribution between functional categories is displayed in Table 1. Of the total number of up-regulated genes upon rifampicin exposure, 4 genes were up-regulated in both strains and 2 genes were down-regulated in both strains upon rifampicin exposure of the total number of down-regulated genes.

Table 1. Numbers of genes that responded to rifampicin in H37Rv-WT and H37Rv-H526Y, according to their functional class

		H37Rv-WT							
		¼x MIC RIF (0.06 mg/L)*				1x MIC RIF (0.25 mg/L)*			
	Total	30 min		12 h		30 min		12 h	
Functional class	nr. on array	Up	Down	Up	Down	Up	Down	Up	Down
1: Virulence, detoxification and adaption	216	0	0	0	1	0	0	5	5
2: Lipid metabolism	270	0	0	0	0	0	0	10	10
3: Information pathways	241	0	0	0	0	0	0	5	5
4: Cell-wall and cell processes	762	0	0	0	0	0	0	24	24
5: Stable RNAs	0	0	0	0	0	0	0	0	0
6: Insertion sequences and phages	101	0	0	0	0	0	0	1	6
7: PE and PPE proteins	165	0	0	0	0	0	0	5	8
8: Intermediary metabolism and respiration	920	0	0	0	0	0	0	28	30
9: Proteins of unknown function	13	0	0	0	0	0	0	1	1
10: Regulatory proteins	194	0	0	0	0	0	0	3	10
11: Conserved hypothetical proteins	1005	0	0	0	0	0	0	22	50
Total	3887	0	0	0	1	0	0	104	149

		H37Rv-H526Y							
		¼x MIC RIF (128 mg/L)*				1x MIC RIF (512 mg/L)*			
	Total	30 min		12 h		30 min		12 h	
Functional class	nr. on array	Up	Down	Up	Down	Up	Down	Up	Down
1: Virulence, detoxification and adaption	216	0	0	0	0	1	1	8	4
2: Lipid metabolism	270	0	0	0	3	0	2	5	4
3: Information pathways	241	0	0	0	0	0	0	4	1
4: Cell-wall and cell processes	762	7	0	8	0	9	1	13	7
5: Stable RNAs	0	0	0	0	0	0	0	0	0
6: Insertion sequences and phages	101	0	0	0	0	0	0	0	0
7: PE and PPE proteins	165	0	0	0	1	0	0	2	3
8: Intermediary metabolism and respiration	920	1	1	1	1	2	0	14	10
9: Proteins of unknown function	13	0	0	0	0	0	0	0	0
10: Regulatory proteins	194	1	0	2	0	2	0	16	0
11: Conserved hypothetical proteins	1005	1	0	2	1	4	1	13	10
Total	3887	10	1	13	6	18	5	75	39

* Expression after 30 minutes and 12 hours of exposure to ¼x the MIC and 1x the MIC of rifampicin, determined with microarray analysis.

In H37Rv-WT no genes were induced after 30 minutes exposure to ¼x the MIC and 1x the MIC of rifampicin. After 12 hours exposure to ¼x the MIC of rifampicin, Rv1397c (*vapC10*) encoding a possible toxin was found down-regulated, a 12 hour exposure to 1x the MIC of rifampicin resulted in 104 up-regulated genes and 149 down-regulated genes. After 12 hours exposure to 1x the MIC of rifampicin *vapC10* was also down-regulated which was a little bit stronger compared to exposure to ¼x the MIC.

The H37Rv-H526Y showed more response upon rifampicin exposure compared to the H37Rv-WT. Exposure to ¼x the MIC of rifampicin resulted in 10 up-regulated genes and 1 down-regulated gene after 30 minutes, and 13 up-regulated genes and 6 down-regulated genes after 12 hours. Exposure to 1x the MIC of rifampicin resulted in 18 up-regulated and 5 down-regulated genes after 30 minutes and 75 up-regulated and 39 down-regulated genes after 12 hours. Under all conditions in the H37Rv-H526Y strain nine genes were found up-regulated.

Twenty genes were selected that showed in at least 2 conditions differential expression ≥ 4 (14 genes) or $\leq ¼$ (6 genes) in H37Rv-H526Y and not in H37Rv-WT upon exposure to rifampicin (Table 2). From the 14 up-regulated genes, 8 appeared to be involved in cell wall and cell processes (Rv0450c, Rv0451c, Rv0559c, Rv0676c, Rv0677c, Rv1216c, Rv1217c and Rv1218c), 2 encode proteins involved in intermediary metabolism and respiration (Rv0560c and Rv0711), 1 encode regulatory proteins (Rv0452), 1 is associated with virulence, detoxification and adaption (Rv0251c), and 1 encodes a conserved hypothetical protein (Rv0678). Of the 6 down-regulated genes, 3 encode proteins that are involved in lipid metabolism (Rv0098, Rv0099 and Rv0101), 1 belongs to the PPE family proteins (Rv0096), 1 encodes a protein involved in intermediary metabolism and respiration (Rv0097), and 1 encodes a conserved hypothetical protein (Rv0100).

Table 2. Fold change of 20 differentially-expressed genes in H37Rv-H526Y

					¼x MIC RIF (128 mg/L)*		1x MIC RIF (512 mg/L) [qPCR]*		
Condition	**Rv number**	**Gene**	**Funct. Cat.**	**Description**	**30 min**	**12 h**	**30 min**	**12 h**	
Up-regulation	Rv0251c	hsp	1	HEAT SHOCK PROTEIN HSP (HEAT-STRESS-INDUCED RIBOSOME-BINDING PROTEIN A)	1,0	2,7	1,5	10,9	[3.9]
	Rv0450c	mmpL4	4	PROBABLE CONSERVED TRANSMEMBRANE TRANSPORT PROTEIN MMPL4	1,8	3,3	4,7	6,8	[1.1]
	Rv0451c	mmpS4	4	PROBABLE CONSERVED MEMBRANE PROTEIN MMPS4	2,1	3,8	4,4	5,3	[1.7]
	Rv0452	Rv0452	10	POSSIBLE TRANSCRIPTIONAL REGULATORY PROTEIN	2,9	5,1	6,4	5,0	[1.3]
	Rv0559c	Rv0559c	4	POSSIBLE CONSERVED SECRETED PROTEIN	9,3	13,3	18,9	18,1	[40.0]
	Rv0560c	Rv0560c	8	POSSIBLE BENZOQUINONE METHYLTRANSFERASE (METHYLASE)	52,9	98,3	72,7	137,1	[242.6]
	Rv0676c	mmpL5	4	PROBABLE CONSERVED TRANSMEMBRANE TRANSPORT PROTEIN MMPL5	22,6	11,6	34,6	26,0	[6.4]
	Rv0677c	mmpS5	4	POSSIBLE CONSERVED MEMBRANE PROTEIN MMPS5	14,5	8,0	34,7	18,3	[8.5]
	Rv0678	Rv0678	11	CONSERVED HYPOTHETICAL PROTEIN	16,0	6,9	31,5	25,2	[9.1]
	Rv0711	atsA	8	POSSIBLE ARYLSULFATASE ATSA (ARYL-SULFATE SULPHOHYDROLASE) (ARYLSULPHATASE)	-1,1	1,2	1,8	5,5	[3.7]
	Rv1216c	Rv1216c	4	PROBABLE CONSERVED INTEGRAL MEMBRANE PROTEIN	2,1	5,7	4,2	8,4	[1.5]
	Rv1217c	Rv1217c	4	PROBABLE TETRONASIN-TRANSPORT INTEGRAL MEMBRANE PROTEIN ABC TRANSPORTER	3,0	4,6	7,2	8,7	[1.9]
	Rv1218c	Rv1218c	4	PROBABLE TETRONASIN-TRANSPORT ATP-BINDING PROTEIN ABC TRANSPORTER	2,7	4,0	8,5	7,2	[1.6]
	Rv1219c	Rv1219c	10	PROBABLE TRANSCRIPTIONAL REGULATORY PROTEIN	1,7	4,4	1,9	6,4	[1.4]

Table 2. Fold change of 20 differentially-expressed genes in H37Rv-H526Y (continued)

					¼x MIC RIF (128 mg/L)*		1x MIC RIF (512 mg/L) [qPCR]*		
Condition	**Rv number**	**Gene**	**Funct. Cat.**	**Description**	**30 min**	**12 h**	**30 min**	**12 h**	
Down-regulation	Rv0096	PPE1	7	PPE FAMILY PROTEIN	1,0	-6,0	1,4	-9,9	[-2.1]
	Rv0097	Rv0097	8	POSSIBLE OXIDOREDUCTASE	1,0	-6,1	1,4	-9,0	[-2.6]
	Rv0098	fcoT	2	PROBABLE FATTY ACYL CoA THIOESTERASE TYPE III FCOT	1,0	-6,3	1,1	-12,7	[-2.6]
	Rv0099	fadD10	2	POSSIBLE FATTY-ACID-CoA LIGASE FADD10 (FATTY-ACID-CoA SYNTHETASE) (FATTY-ACID-CoA SYNTHASE)	-1,0	-6,6	1,1	-10,5	[-1.3]
	Rv0100	Rv0100	11	CONSERVED HYPOTHETICAL PROTEIN	1,0	-3,9	-1,0	-6,0	[-1.5]
	Rv0101	nrp	2	PROBABLE PEPTIDE SYNTHETASE NRP (PEPTIDE SYNTHASE)	1,2	-3,2	1,0	-5,0	[-1.1]

* Fold change after 30 minutes and 12 hours of exposure to ¼x the MIC and 1x the MIC of rifampicin determined with microarray analysis. qRT-PCR results obtained after 12 hours exposure to 1x the MIC of rifampicin are shown between brackets.

Table 3. Primers used for the qRT-PCR

Gene	Annotation	Forward primer	Reverse primer
Rv0096	*PPE1*	CAGCAGCTGGCAGGGAACCG	AGCTCTGCTGGGGTGGGCAT
Rv0097		TCCGTCTCCGCGGGAGTTCA	ATGCCAGAACGCGCCGGTTT
Rv0098	*fcoT*	ACACCGACTTGACGCCCTGC	TGGCGCTGTACTGTGCGTCG
Rv0099	*faD10*	GATGGCATCGGCCCTACCGC	CCGGTGTTCACCCAGCCGTC
Rv0100		CGCCGTCTGCGACGTGTTGTA	GGCCAATCGGGACGGCAGTT
Rv0101	*nrp*	ATTCCTGGCCGCTCTGCACG	GCACCGTATGCCGCACCAGT
Rv0251c	*hsp*	GCTGCGCCTGAGTTTCGGCT	TCATTCCGCCGCTCGTTCCG
Rv0450c	*MmpL4*	GTCGCTGCTCATCGCCAAGGG	CGCCATCCATCGCCGCACTA
Rv0451c	*MmpS4*	CACGGTAGCACCGCTCCATCG	GATCCGCGGCTTCTTCGGCTC
Rv0452		AACGGCTGTCGCAGGTCGTG	AGCTGCTCGCATACCGTGCC
Rv0559c		CGCCCACATGCTCAGGGCAG	GGCGTTGACGGCGATGCGTA
Rv0560c		GTGTCCCCGTTCGGCCAGTG	GGAGGGAGTCCGACCGCCAT
Rv0676c	*MmpL5*	CCAACGGCACACCGAGGGTC	CAGGACCGGGAGTCGGCGTA
Rv0677c	*MmpS5*	GCCATCGACGGTGATTCGGCA	GACCTCGACGCCAAGCCACAG
Rv0678		TCCGGGCAATGGCCGAACTG	GTCCTCTCCGGTTCGCTGGC
Rv0711	*atsA*	GCCCGACAAGCCGTGGTTCA	TGTCGGGTGGCACGATCCCT
Rv1216c		GCACGCGCTCAGCACCATCA	AGCACCACGATTGGCCCCAC
Rv1217c		CGAACGTCACGCCGTCGGAA	CGCTGTACCTGCTCGGCTCG
Rv1218c		TCGCACAGCGCTTCGGTCTC	GCGCGCACCTACTCCAAGGG
Rv1219c		CGGCGCGTACTCCTTCGTCC	AACGACCCGACCACCTGGCT
Rv2703	*sigA**	GTCGGAGGCCCTGCGTCAAG	AGGCCAGCCTCGATCCGCTT

* *SigA* was used for normalization

qRT-PCR confirmation

Aliquots of the RNA from rifampicin-treated and control samples were also used for qRT-PCR analysis. The samples obtained after 12 hours 1x the MIC of rifampicin exposure, were compared with the 12 hours control samples; *sigA* was used for normalization. Results of the ΔΔCt analysis are in agreement with the microarray data. The direction of change was in concurrence by both qRT-PCR and microarray analysis for all samples. However, the fold change determined by qRT-PCR is different from that determined by the microarray analysis as shown in Table 2 and Figure 1. Another ΔΔCt analysis of independent RNA samples showed that the direction of change was in agreement by both qRT-PCR and microarray analysis for 70% of the samples. However, in general gene-expression levels are lower compared to the microarray analysis and to the expression levels obtained from the first qRT-PCR experiment.

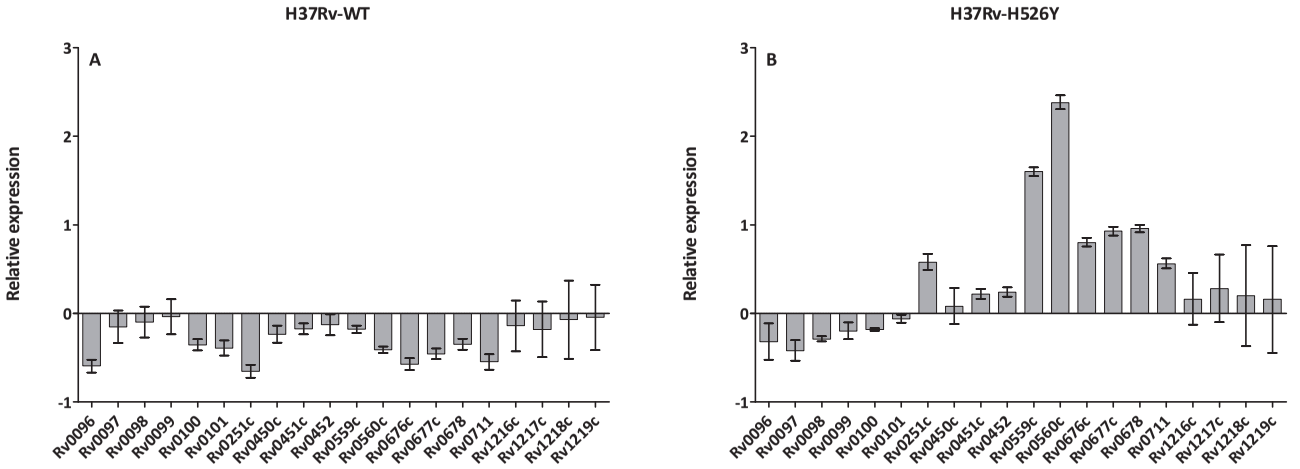

Figure 1. Differentially expressed genes assessed by quantitative Real-Time PCR. The relative gene expression of H37Rv-WT (A) and H37Rv-H526Y (B) assessed with qRT-PCR and normalised to *sigA* after 12 hours of exposure to 1x the MIC of rifampicin.

Of the 20 genes that were up-regulated in at least two conditions in the microarray analysis, only Rv0559c and Rv0560c were found strongly and constantly up-regulated in both qRT-PCR experiments in H37Rv-H526Y compared with H37Rv-WT.

DISCUSSION

We have investigated differences in rifampicin-induced gene responses between rifampicin-susceptible and rifampicin-resistant *Mycobacterium tuberculosis* strains using microarray analysis and qRT-PCR. At ¼x the MIC of rifampicin exposure more genes are differentially expressed in H37Rv-H526Y than H37Rv-WT, whereas at 1x the MIC of rifampicin and long exposure this is reversed. This shows that the rifampicin-susceptible and rifampicin-resistant *Mycobacterium tuberculosis* strain have an altered response upon rifampicin exposure.

Using qRT-PCR we assessed if biological duplicates showed similar up-regulation. This revealed that one cluster of genes (Rv0559c-Rv0560c) was strongly up-regulated in all experiments after exposure to rifampicin in the H37Rv-H526Y. These results were confirmed in a second qRT-PCR experiment in which expression of Rv0559c and Rv0560c was also highly up-regulated in the H37Rv-H526Y strain compared to the H37Rv-WT strain (data not shown). Therefore Rv0559c and Rv0560c are potential candidates for further investigation in rifampicin resistance.

The Rv0559c gene encodes a protein with an unknown function. However, a previous study of Denkin *et al.* showed that Rv0559c is also induced upon salicylate exposure [17]. Rv0560c is also induced by salicylate and probably encodes a benzoquinone methyltransferase [17, 18]. Schuessler *et al.* showed that Rv0559c and Rv0560c have slow induction kinetics after exposure to salicylate and that gene expression is increasing over time [19]. Our results are in agreement with these findings, since our microarray data also revealed that up-regulation of Rv0559c and Rv0560c is time dependent and rifampicin concentration dependent. The data showed an increase in expression of Rv0560c between 30 minutes and 12 hours of rifampicin exposure. An increased expression was also measured for both genes comparing exposure to rifampicin at ¼x the MIC and 1x the MIC. Sun *et al.* suggested that Rv0559c, Rv0560c and Rv0561c are located within the same operon [18], and Schuessler *et al.* showed that only Rv0559c and Rv0560c are regulated by the same promoter. In our microarray analysis we did not find a significant up-regulation of Rv0561c after rifampicin exposure in the H37Rv-H526Y strain, which is in concordance with earlier observations by Schuessler *et al.*

We have found 4 other distinct clusters of genes and 2 different genes differently expressed between both strains after 12 hours of exposure to 1x the MIC of rifampicin. The first cluster of Rv0096 to Rv0101 is strongly down-regulated in the H37Rv-H526Y strain upon 12 hours of rifampicin exposure. The other clusters, Rv0450c to Rv452, Rv0676c to

Rv678 and Rv1216c to Rv1219c are strongly up-regulated in the H37Rv-H526Y. None of these clusters are induced in the H37Rv-WT in response to rifampicin exposure.

Genes of the Rv0096-Rv0101 operon encode a PPE protein family member (Rv0096), a putative oxidoreductase of the *tfdA* dioxygenase family (Rv0097), a type III thioesterase (Rv0098), a fatty acid AMP ligase (Rv0099), an acyl carrier protein (Rv0100) and a nonribosomal peptide synthase (Rv0101). These genes seem to be co-regulated by a two-component regulatory system (*senX3-regX3*) [20] and are involved in biosynthesis of Phthiocerol Dimycocerosate (PDIM) [21]. PDIM is part of the lipid-rich content of the mycobacterial cell envelop and is associated with an increased *Mycobacterium tuberculosis* virulence [22, 23]. Interestingly, no down-regulation was found after microarray analysis for Rv2942 (*MmpL7*), which is also involved in the transport of PDIMs [24]. So, down-regulation of this operon in the H37Rv-H526Y may result in decreased *Mycobacterium tuberculosis* virulence and may be due to a fitness loss as a result of mutations in the RRDR region.

Rv0251c is found up-regulated after 12 hours of exposure to 1x the MIC of rifampicin in the H37Rv-H526Y and is associated with stress response [25]. Furthermore, two up-regulated gene clusters being Rv0450c to Rv452 and Rv0676c to Rv678 were found. Both clusters consist of genes of the MmpL (Mycobacterial membrane protein Large) family, of the MmpS (Mycobacterial membrane protein Small) family and a gene with unknown function. The MmpL proteins belong to the group of resistance, nodulation and cell division proteins (RND) and are associated with drug efflux [26]. The RND family is a group of multidrug resistance pumps that recognize and mediate the transport of a great diversity of compounds and has been well-documented in Gram-negative bacteria. However these MmpL genes does not seem to play a critical role in anti-TB drug resistance in *Mycobacterium tuberculosis*[26]*MmpL4* (Rv0450c) and *MmpL5* (Rv0676c) are transcriptional coupled with *MmpS4* and *MmpS5*, these smaller genes are predicted to encode proteins with one transmembrane domain. Little is known about the MmpS family. In *Mycobacterium smegmatis MmpS4* (Rv0451c) is associated with glycopeptidolipid biosynthesis and export cell surface glycolipids [27]. The up-regulation of Rv0450c to Rv0452 is almost constant in all conditions tested.

Our data showed that the response of Rv0676c to Rv0678 is higher after 30 minutes of exposure to both rifampicin concentrations compared to 12 hours of rifampicin exposure. This suggests that Rv0676c to Rv0678 are early response genes. *MmpL5* and *MmpS5* (Rv0677c) are associated with efflux pump-related azole resistance in *Mycobacterium tuberculosis* and are regulated by Rv0678 [28]. Milano *et al.* showed that Rv0678 has almost 50% homology with the well-characterized *MarR* of *E. coli* [28], this family of regulatory proteins plays an important role in of the development of antibiotic resistance [29]. Based on the already known function of MmpL and MmpS genes and the up-regulation found in the microarray analysis it is likely that in *Mycobacterium tuberculosis MmpL4*, *MmpS4*, *MmpL5* and *MmpS5* are involved in rifampicin efflux out of the cell.

Rv0711 (*atsA*) encodes for a possible arylsulfatase but knowledge about *atsA* is very limited in *Mycobacterium tuberculosis*.

The knowledge about the up-regulated gene cluster Rv1216c-Rv1219c in the H37Rv-H526Y is also very limited. Rv1216c and Rv1217c encode for probable integral membrane proteins. Rv1218c encodes an ABC transporter and previous studies suggest an important role for this gene product in mediating efflux to a wide variety of compounds [30, 31]. However Balganesh *et al.* showed that the MIC of rifampicin is not influenced in Rv1218c knock-out mutants [30]. This suggests that Rv1218c is not primarily involved in regulation of the intracellular concentration of rifampicin. In our rifampicin-resistant H37Rv-H526Y mutant however, Rv1218c is strongly up-regulated, which implies an association between up-regulation of Rv1218c and rifampicin resistance. Rv1219c probably encodes for a transcriptional regulatory protein but further function and regulation is unknown.

To conclude, our results show that several interesting gene clusters associated with efflux, transport and virulence show an altered response in the rifampicin-resistant H37Rv mutant compared to the rifampicin-susceptible H37Rv wild type strain upon exposure to rifampicin. Not all genes could be completely confirmed with qRT-PCR. Although the directions of change were identical, fold changes appeared in general to be lower in qRT-PCR. This lack of concurrence between methods for genes exhibiting low levels of change has been reported by several others [32-35]. Here we show by microarrays and qRT-PCR experiments that Rv0559c and Rv0560c are strongly rifampicin-inducible. This indicates that the induction of both genes is dependent on concentration of rifampicin and exposure time. Therefore this study suggests that Rv0559c and Rv0560c play a pivotal role in rifampicin resistance of *Mycobacterium tuberculosis*. Further investigation on the exact function and regulation of Rv0559c and Rv0560c is needed to understand the mycobacterial mechanisms that are triggered upon rifampicin exposure.

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7

Chapter 8

Assessment of bactericidal drug activity and treatment outcome in a mouse tuberculosis model using a clinical Beijing strain

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ABSTRACT

The increasing emerging *Mycobacterium tuberculosis* Beijing genotype is associated with lower treatment success rates in tuberculosis patients and resistance. Therefore, we explored the impact of using a Beijing strain instead of H37Rv on treatment outcome in our mouse tuberculosis model. Additionally, we assessed the predictive value of bactericidal activity on treatment outcome.

We observed that the treatment success rates of conventional anti-TB regimens ranged between 80-95% in Beijing-infected animals opposed to 100% treatment success observed in previous studies using H37Rv-infected mice. The bactericidal activity was only predictive for treatment outcome after at least 3 months of treatment.

Our data advocate the use of Beijing strains to increase the translational value of mouse TB models. Additionally, our findings support the notion that bactericidal activity in the first 2 months of treatment as measured in clinical phase IIa/b trials, has limited predictive value for TB treatment outcome, thus emphasizing the need for better parameters to guide future phase III trials.

INTRODUCTION

With 1.8 million deaths in 2015, tuberculosis (TB) surpassed HIV as leading cause of death amongst infectious diseases [1]. One factor contributing to this ongoing burden of TB is the rapid emergence of *Mycobacterium tuberculosis* strains of the Beijing genotype [2, 3]. These strains specifically contribute to the spread of drug-resistant TB and are clinically associated with increased rates of treatment failure [3-8].

To overcome this new challenge in TB treatment, novel treatment strategies with increased efficacy are urgently needed. However, clinical trials evaluating TB treatment outcome are expensive, involve large numbers of patients, and may take up to 10 years from drug design to clinical use [9]. Moreover, phase IIa trials, measuring early bactericidal activity (EBA), and phase IIb trials, based on surrogate endpoints such as two month sputum culture status, cannot predict treatment outcome in phase III trials in TB to a satisfying degree [9-11].

Alternatively, phase III trials can be guided by preclinical testing of anti-TB drugs, which is often performed in mouse TB models [12-15]. However, recent disappointing results of phase III clinical trials on moxifloxacin for anti-TB treatment, which were partly based on promising results from mouse experiments, have also raised skepticism regarding the predictive value of preclinical TB models and the need for their improvement [9]. This has led to the formation of multiple international consortia, such as PreDiCT-TB and CPTR, aimed at improving the translational value of preclinical TB models [12].

Approaches that are currently being evaluated include the development of specific *in vitro* models that allow drug activity assessment against *Mycobacterium tuberculosis* in different metabolic states [14], increased appreciation of the pharmacokinetic aspects of treatment [9] and mouse models that develop cavitating lesions, thus better representing human pathology [12].

Currently, most mouse TB models that evaluate treatment outcomes use *Mycobacterium tuberculosis* strains such as H37Rv, which originally derived from a clinical isolate in 1905, but is no longer found in patients [12, 14]. Given the significant clinical impact of Beijing strain infections on treatment outcome, the main outcome parameter of phase clinical III trials, use of Beijing should increase the translational value of preclinical TB models. Therefore, the primary aim of this study was to assess treatment outcomes in mice infected with an *Mycobacterium tuberculosis*-Beijing genotype strain [16-18]. Additionally, since phase IIa/b trials depend on early or late bactericidal activity, we evaluated the predictive value of bactericidal activity on treatment outcome at multiple time points throughout the full 6-months treatment course.

MATERIALS AND METHODS

Bacterial strain

For all experiments, the previously described Beijing VN 2002-1585 (BE-1585) *Mycobacterium tuberculosis* genotype strain was used [18]. This strain was isolated from a patient in Vietnam in 2002 and was verified as a typical Beijing strain based on single nucleotide polymorphism analysis [19]. Susceptibility assays performed according to CLSI guidelines showed minimal inhibitory concentrations for rifampicin of 0.25 mg/L, for isoniazid of 0.125 mg/L, for ethambutol of 5 mg/L and for streptomycin of 2 mg/L [20].

Mice

Specified pathogen-free female BALB/c mice were obtained from Charles River (Les Oncins, France) and acclimatized at least 7 days prior to starting experiments. Mice received food and water ad libitum. At the day of infection, 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 were 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 (117-12-08 and 117-12-13).

Infection

A suspension of *Mycobacterium tuberculosis* stored at -80°C was thawed at room temperature for 30 min and centrifuged for 10 min at 14.000*xg*. The *Mycobacterium tuberculosis* pellet was resuspended and diluted in fresh phosphate buffered saline (PBS). Mice were infected under general anesthesia using a mixture of medetomidine (Sedator®, 0.5 mg/kg; Eurovet Animal Health, Bladel, the Netherlands), midazolam (Midazolam, 5 mg/kg; Actavis, Baarn, the Netherlands) and fentanyl (Fentanyl, 0.05 mg/kg; Hameln Pharmaceuticals, Hameln, Germany), by intratracheal installation of a 40 µl suspension containing 1.4 x 10^5 (0.3 – 2.0 x 10^5) CFU of BE-1585, using a repeating dispenser (Hamilton company; Bonaduz, Switzerland), a 1 mL syringe and a 22-Gauge mouse gavage feeding needle (Fine Science Tools; Heidelberg, Germany), followed by proper inhalation. Mice were antagonized using a mixture of atipamezole (Antisedan®, 2.5 mg/kg; Orion Corporation, Espoo, Finland), flumazenil (Flumazenil, 0.5 mg/kg; Pharmachemie, Haarlem, the Netherlands) and naloxon (Naloxon, 1.2 mg/kg; Orpha-Devel Handels und Vertriebs, Purkersdorf, Germany). Anesthetic and antagonistic agents were administered intraperitoneally, in a total volume of 175 µL and 250 µL, respectively.

Antibiotic treatment

All treatment schedules started 14 days after infection. In the experiments assessing bactericidal activity of single anti-TB drugs, mice received 0.5x, 1x or 2x the human phar-

macokinetic equivalent dose (HED) with rifampicin (R) (5, 10 or 20 mg/kg), isoniazid (H) (12.5, 25 or 50 mg/kg), streptomycin (S) (100, 200 or 400 mg/kg), ethambutol (E) (50, 100 or 200 mg/kg) or pyrazinamide (Z) (75, 150 or 300 mg/kg), for 5 days a week, up to 28 days [21]. In the experiments assessing bactericidal activity and treatment outcome of the different anti-TB drug regimens, mice received treatment up to 6 months with different regimens of 1x the HED of each antibiotic 5 days a week. All drugs were administered via oral gavage except streptomycin, which was administered via subcutaneous injections. The different drug regimens are shown in Table 1.

Table 1: schematic overview of the experiments

		D0 [a]	D7	D14	D28	M2[b]	M3	M6	M6+3[c]
R		3 [d]	3	3	3				
H		3	3	3	3				
Z		3	3	3	3				
S		3	3	3	3				
E		3	3	3	3				
RE	(6 RE)	3	3	3	3	3	3	3	20
RZ	(2 RZ / 4 R) [e]	3	3	3	3	3	3	3	15
RH	(6 RH)	3	3	3	3	3	3	3	15
RHZ	(2 RHZ / 4 RH)	3	3	3	3	3	3	3	21
RHZE	(2 RHZE / 4 RH)	3	3	3	3	3	3	3	21
RHZS	(2 RHZE / 4 RH)	3	3	3	3	3	3	3	21
HS	(2 HS / 4 H)	3	3	3	3	3	3	3	9
HZ	(2 HZ / 4 H)	3	3	3	3	3	3	3	20
HE	(6 HE)	3	3	3	3	3	3	3	18
ZES	(2 ZES / 4 E)	3	3	3	3	3	3	3	20

[a] D0= day 0 (start of treatment), [b] M2= 2 months after start of treatment, [c] M6+3= 3 months after stop of a 6-months treatment course, [d] number of mice used for determination of Mtb-loads in the lungs,[e] 2 RZ / 4 R= two months of RZ treatment followed by 4 months of R treatment. Drugs were administered in their human pharmacokinetic equivalent dose, mice were infected at day -14.

Assessment of mycobacterial load in the lungs

In order to assess the mycobacterial load in the lungs, mice were sacrificed by CO_2 exposure. To prevent carry-over of anti-TB drugs on subculture plates, treatment was stopped 72 hours before sacrificing the mice. In addition, activated charcoal (0.4%) was added to the agar to inhibit the antibiotic residue from the tissue samples [22]. The lungs were removed aseptically and homogenized according to protocol using the gentleMACS Octo Dissociator (Miltenyi Biotec BV, Leiden, the Netherlands) in 2 mL PBS. From each tissue homogenate 10-fold serial dilutions were made. Next, 200 µL per dilution was cultured on drug-free 7H10 Middlebrook agar and incubated for 28 days at 37^0C with 5% CO_2

followed by colony enumeration. The time points at which mycobacterial loads were evaluated are shown in the schematic overview of the experiments in Table 1.

Data analysis and statistics

Analyses were performed and graphs were made using PRISM Graphpad 6 (Graphpad software, La Jolla, CA). All data are expressed as median ± range. Student's t-test was used to calculate significance in figure 1. Two-way ANOVA followed by Bonferroni correction was used to calculate significance in table 3. P-values less than 0.05 were considered statistically significant.

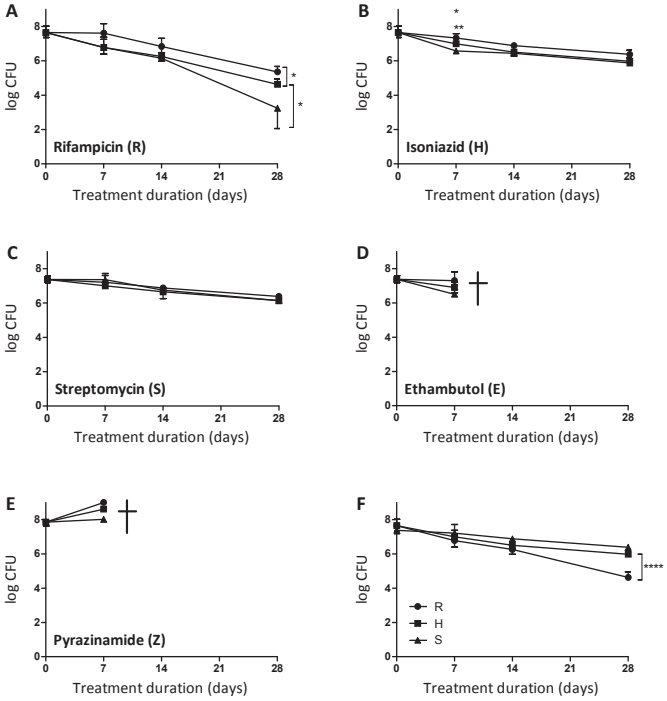

Figure 1. Bactericidal activity after single drug exposure

Mycobacterial loads in the lungs after single drug exposure over a 28-days treatment course, using 0.5x (dots), 1x (squares) and 2x (triangles) the human pharmaco equivalent dose (HED) of the selected drugs. Data are shown as median with ranges with n=3 mice per time point. **A)** Rifampicin showed significant dose responses after 28 days of treatment between 0.5x, 1x HED and 2x HED (*) **B)** Isoniazid showed significant dose responses after 7 days of treatment between 0.5x and 2x HED (**) and between 1x and 2x HED (*). **C)** Streptomycin showed limited bactericidal activity, but prevented mortality **D)** Ethambutol showed no dose responses and could not prevent mortality. **E)** Pyrazinamide showed a significant dose-response between 0.5x and 2x HED after 7 days (**), but none of the administered dosages could prevent mortality. **F)** Comparison of 1x HED treatment with rifampicin, isoniazid and streptomycin shows significantly stronger bactericidal activity of rifampicin compared to the other two drugs after 28 days (****), * $p< 0.05$, ** $p< 0.01$, **** $p<0.0001$.

RESULTS

Mortality and bactericidal activity after single drug exposure

Mice infected with the Beijing strain were treated with isoniazid, rifampicin, ethambutol, pyrazinamide, or streptomycin in 3 different doses. Figure 1 shows mortality and bactericidal activity after 7, 14 and 28 days of single drug exposure. Earlier observations in our model have shown that untreated Beijing-infected mice all become moribund after 3-4 weeks of infection [16]. Treatment with rifampicin, isoniazid and streptomycin was able to prevent mortality, whereas mice treated with pyrazinamide or ethambutol showed similar mortality as untreated mice.

Rifampicin effectively reduced the mycobacterial loads in the lungs and showed a significant dose-dependent bactericidal effect after 28 days (Fig. 1A). Isoniazid also showed bactericidal activity, but significant dose-dependent effects were only observed at day 7 (Fig. 1B). Streptomycin reduced mycobacterial loads, but did not show dose-dependent effects (Fig. 1C). Ethambutol showed bactericidal activity after 7 days that was comparable to rifampicin or isoniazid, but failed to prevent mortality (Fig. 1D). Pyrazinamide did not display bactericidal activity in any of the dosages tested and did not prevent mortality (Fig. 1E).

Comparison of the bactericidal activity of rifampicin, isoniazid and streptomycin over the 28-days exposure window showed no significant differences between the different drugs after 7 and 14 days of treatment (Fig. 1F). After 28 days rifampicin showed markedly stronger bactericidal activity than the other two drugs.

Treatment outcome and bactericidal activity after treatment with different anti-TB drug regimens

Treatment outcome after a 6-months treatment course for 10 different anti-TB drug regimens is shown in Table 2. Unexpectedly, none of the regimens achieved 100% treatment success.

We also found that treatment success depended critically on rifampicin. Rifampicin-containing-regimens showed treatment success rates between 80-95%, compared to 0% treatment success of all non-rifampicin containing regimens (Table 2). Among the different rifampicin-containing regimens themselves, no significant differences in treatment success could be detected. This indicates limited contribution of anti-TB drugs other than rifampicin on treatment outcome. Notably, the rifampicin-pyrazinamide regimens even appeared to perform better than the rifampicin-isoniazid-pyrazinamide regimen.

Next, we determined whether the degree of bactericidal activity after any given treatment duration could predict the impact of rifampicin on treatment outcome as observed in Table 2. To this aim, we ranked the bactericidal activity of the different rifampicin-

containing regimens and non-rifampicin-containing regimens after 7, 14 and 28 days and after 2, 3 and 6 months are shown in Table 3.

Table 2: Treatment outcome against a Beijing strain

Drug regimen		Treatment success[a]	
RZ	(2 RZ / 4R)[b]	95%	(20/21)[c]
RHZS	(2 RHZS / 4 RH)	95%	(20/21)
RHZE	(2 RHZE / 4 RH)	90%	(19/21)
RH	(6 RH)	87%	(13/15)
RE	(6 RE)	85%	(17/20)
RHZ	(2 RHZ / 4 RH)	80%	(17/21)
HS	(2 HS / 4 H)	0%	(0/9)
HZ	(2 HZ / 4 H)	0%	(0/20)
HE	(6 HE)	0%	(0/18)
ZES	(2 ZES / 4 E)	0%	(0/20)

[a] percentage of mice with culture-negative lungs 3 months after stop of a 6-months treatment course, [b] (2 RZ / 4 R) = 2 months RZ treatment followed by 4 months treatment with R only, [c] (20/21) = 20 mice with culture-negative lungs out of 21 mice assessed. All rifampicin-containing regimens are marked grey. R = rifampicin, H = isoniazid, Z = pyrazinamide, S = streptomycin and E = ethambutol.

After 7 and 14 days, no significant differences in the mycobacterial load could be found between rifampicin-containing regimens and non-rifampicin-containing regimens. After 28 days, the rifampicin-containing regimens started to show a trend towards stronger bactericidal activity compared to non-rifampicin-containing regimens. At this time point, two out of six rifampicin-containing regimens showed significantly lower mycobacterial loads in the lungs compared to all non-rifampicin-containing regimens tested. After 2 months of treatment, four out of six rifampicin-containing regimens showed significant lower mycobacterial loads compared to all non-rifampicin-containing regimens. However, a clear distinction in bactericidal activity between all rifampicin-containing regimens and all non-rifampicin-regimens to similar extent as observed for treatment outcome in Table 2, could only be made after 3 months of treatment (Table 3).

Lastly, at the end of the 6-months treatment course no mycobacteria could be cultured from the lungs of nearly all mice treated with rifampicin-containing regimens. Of the non-rifampicin-containing regimens, all mice treated with H in the continuous phase (HE, HZ and HS) showed reductions in mycobacterial load, but mycobacteria could still be cultured from the lungs. Two exceptions were the RZ and the HS group, which both contained 1 out of 3 mice with culture-positive lungs. Mice of the ZES group, which were treated with E in the continuous phase actually showed an increase in mycobacterial loads compared to

Table 3: Bacterial loads over a 6-months treatment course

Intensive phase (all drugs administered)					Continues phase (no Z/S/E)[b]	
D0	**D7** [a]	**D14**	**D28**	**M2**	**M3**	**M6**
RHZE 7,3	RH 6,7	RHZ 6,1	RHZS 4,3*	RHZS[c] 0,9****	RE 1,4****	RE 0
RZ 7,7	HS 6,8	HS 6,1	RE 4,3*	RHZE 2,3****	RH 1,4****	RH 0
HS 7,7	RHZE 6,9	RHZS 6,1	RZ 4,5	RZ 3,0**	RHZS 1,6****	RHZS 0
RH 7,7	RHZ 6,9	ZES 6,2	RH 4,7	RE 3,1*	RZ 1,8****	RHZ 0
HZ 7,7	RHZS 7,0	RH 6,2	RHZ 4,9	RHZ 3,8	RHZE 2,1****	RHZE 0
HE 7,9	HE 7,2	RHZE 6,3	RHZE 5,3	RH 3,9	RHZ 2,3****	HS[d] 0,4
RHZ 8,0	HZ 7,2	RZ 6,4	ZES 5,3	ZES 4,3	HS 3,9	RZ[d] 1,1
RE 8,0	ZES 7,3	HZ 6,6	HS 5,5	HS 4,3	ZES 4,0	HE 3,0
RHZS 8,0	RE 7,3	HE 6,7	HE 5,8	HZ 4,9	HZ 4,0	HZ 3,4
ZES 8,0	RZ 7,6	RE 6,7	HZ 6,2	HE 5,2	HE 4,9	ZES 4,9

The different anti-TB drug regimens were ranked based on the mean log value of colony forming units (CFU) of mycobacteria in the lungs of n=3 mice per time point. Rifampicin-containing regimens are marked grey. Mice were infected at day -14 and treatment was started at day 0. [a] D7=7 days after start of treatment, M2= 2 months after start of treatment, etc. [b] after 2 months Z, S and E were stopped, with the exception of the ZES, RE and HE regimen. [c] 2/3 mice of the RHZS group were culture negative at M2, [d] 2/3 mice of the HS and RZ group were culture negative at M6. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ after Bonferroni correction for multiple comparisons. Significance for rifampicin-containing regimens was calculated against all non-rifampicin-containing regimens at that point. R = rifampicin, H = isoniazid, Z = pyrazinamide, S = streptomycin and E = ethambutol

3 months of treatment. Thus, bactericidal activity only correlated with treatment outcome after 3 months of treatment.

DISCUSSION

Two important findings in this study were that infection with an *Mycobacterium tuberculosis*-Beijing genotype strain in mice is associated with lower treatment success rates compared to other strains in literature [13, 17, 23, 24] and that bactericidal activity is an unreliable predictor for treatment outcome in TB when assessed in the first 2 months of treatment.

Infections with Beijing strains are associated with treatment failure in TB patients [3, 5-8]. The data obtained in our mouse TB model reflect these clinical findings. None of the regimens tested, including the standard of care regimen 2RZH/4RH, achieved 100% treatment success. In contrast, at least four different studies using *Mycobacterium tuberculosis*-H37Rv genotype strains, including one previous study in our own model, showed 100% treatment success of the 2RHZ/4RH regimen in BALB/c mice [13, 17, 23, 24]. In TB patients, treatment success rates with 2RHZE/4RH in controlled trial settings are 92% or less [25]. This indicates that the repeatedly found 100% treatment success rates in preclinical mouse TB models using H37Rv might overestimate clinical treatment success rates. The 90% treatment success rate for 2RHZE/4RH observed in our model using a Beijing strain might better represent the clinical situation.

The difference in treatment outcome between Beijing and H37Rv strains might be explained by the observation that only Beijing strains constitutively express proteins belonging to the DosR dormancy regulon [26, 27]. These proteins regulate the mycobacterial metabolic state in response to stressors induced by the host-response. This could result in a more rapid or more profound conversion by Beijing genotype strains to a metabolic state in which the mycobacteria are less susceptible to anti-TB drugs. Other possibilities include the ability of Beijing strains to circumvent and manipulate host-responses more effectively than H37Rv [28, 29], resulting in better localization in (intracellular) niches, shielded from anti-TB drugs [30].

In TB, clinical phase IIa trials were found to be a poor predictor for treatment outcome [9, 31]. These studies measure early bactericidal activity (EBA) in patient sputum samples between 2-7 days or between 2-14 days in case of the extended EBA [11]. Our mouse TB model clearly supports this clinical finding, as it is impossible to distinguish the rifampicin-containing regimens from the non-rifampicin-containing regimens after 7 or 14 days of treatment, despite their markedly different treatment outcome after 6 months of treatment.

Our single-drug exposure experiments showed that rifampicin only starts to show significantly stronger bactericidal activity compared to other anti-TB drugs after a minimum of 28 days of treatment. Two clinical studies that continued EBA measurements up to 28 days

indeed found a markedly stronger association between bactericidal activity and treatment outcome for anti-TB drug regimens containing pyrazinamide and rifampicin [32, 33]. These studies indicate that extending EBA to 28 days might be a better predictor for treatment outcome. In our regimen-experiments we found that after 28 days the rifampicin-containing regimens showed a trend towards lower mycobacterial loads in the lungs compared to the non-rifampicin-containing regimens. However, a significant distinction in bactericidal activity between all rifampicin-containing regimens and all non-rifampicin-containing regimens could still not be made. Moreover, after 28 days the standard of care RHZE-regimen showed similar mycobacterial loads in the lungs as the non-rifampicin-containing ZES-regimen, while having markedly different treatment outcomes. Thus, based on our data we conclude that extending EBA for up to 28 days is more informative compared to 7 or 14 days, but remains an unreliable parameter for predicting treatment outcome.

Clinical phase IIb trials measure bactericidal activity over a 2-months period with sputum culture status as surrogate endpoint for treatment outcome [31]. These studies were initially thought indicative for phase III trial outcomes in TB [31], but the disappointing results of the recent phase III REMox trials show otherwise [9, 25]. Our study shows that after 2 months of treatment, the rifampicin-containing regimens RH and RHZ still do not show significant differences in lung mycobacterial loads compared to the non-rifampicin-containing regimens. The inability at this time point to significantly distinguish between regimens with a markedly different treatment outcome after 6 months supports the limited predictive value of measuring bactericidal activity during longer treatment durations in TB.

Our mouse TB model did show a clear distinction in lung mycobacterial loads between rifampicin-containing regimens and non-rifampicin-containing regimens after 3 months of treatment. However, the value of such a late time point in clinical studies is highly questionable, especially when phase III trials strive to shorten treatment duration to 4 months [25].

In conclusion, multiple approaches are currently evaluated for their potential to further increase the translational value of preclinical TB models. Implementation of mouse strains that better mimic human disease are likely to improve future anti-TB drug research. Furthermore, integration of advanced biostatistics to generate more informative models and increased appreciation for pharmacokinetics,- and dynamic aspects of treatment are also necessary to optimize anti-TB drug research. Our data complement these developments by advocating the use of *Mycobacterium tuberculosis*-Beijing genotype strains to increase the translational value of preclinical models assessing treatment outcomes. Also, our data in this mouse TB model support the notion that bactericidal activity in the first 2 months of treatment as measured in clinical phase IIa/b trials has limited predictive value for treatment outcome, which emphasizes the need for better biomarker to guide future phase III trials.

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Chapter 9

Assessment of Treatment Response by Colony Forming Units, Time to Culture Positivity and the Molecular Bacterial Load Assay Compared in a Mouse Tuberculosis Model

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ABSTRACT

The aim of the study is to compare counting of colony forming units (CFU), the time to positivity (TTP) assay and the molecular bacterial load (MBL) assay, and explore whether the last assays can detect a subpopulation which is unable to grown on solid media. CFU counting, TTP and the MBL assay were used to determine the mycobacterial load in matched lung samples of a murine tuberculosis model. Mice were treated for 24 weeks with 4 treatment arms: isoniazid (H) - rifampicin (R) - pyrazinamide (Z), HRZ-Streptomycin (S), HRZ – ethambutol (E) or ZES.

Inverse relationships were observed when comparing TPP with CFU or MBL. Positive associations were observed when comparing CFU with MBL. Description of the net elimination of bacteria was performed for CFU vs. time, MBL vs. time and 1/TTP vs. time and fitted by nonlinear regression. CFU vs. time and 1/TTP vs. time showed bi-phasic declines with the exception of HRZE. A similar rank order, based on the alpha slope, was found comparing CFU vs. time and TTP vs. time, respectively HRZE, HRZ, HRZS and ZES. In contrast, MBL vs. time showed a mono-phasic decline with a flat gradient of elimination and a different rank order respectively, ZES, HRZ, HRZE and HRZS. The correlations found between methods reflects the ability of each to discern the general mycobacterial load. Based on the description of net elimination, we conclude that the MBL assay can detect a subpopulation of *Mycobacterium tuberculosis* which is not detected by the CFU or TTP assays.

INTRODUCTION

Tuberculosis (TB) is a bacterial infection that affects more than ten million people globally every year, with 1.8 million deaths recorded in 2015 and is ranked as a leading cause of death from infectious diseases worldwide [1].

Mycobacterial populations in TB patients are heterogeneous; several subpopulations of bacteria have been identified in the lungs, in different microenvironments [2]. Environmental stress and antibiotic pressure can drive *Mycobacterium tuberculosis* into a non-replicating state [3, 4]. As a consequence, extensive and long-term treatment is required to eradicate all these subpopulations of *Mycobacterium tuberculosis*[5]. In order to shorten the treatment duration, new drugs and novel combinations are essential and provide the best opportunity to eliminate all mycobacterial subpopulations.

For the development of new drugs and regimens, it is important at an early stage to determine a drug's and regimen's potency to prevent relapse, which will determine the required treatment duration with this regimen [6].

Monitoring treatment response provides information that can be used to make inferences regarding treatment success. Current methods to monitor treatment response such as sputum smear or sputum serial colony counting have their drawbacks including loss of sensitivity, being laboratory intensive [7], or being sensitive to contamination [8]. As a result, these methods do not predict individual success with enough certainty, but also seem to lack precision in predicting the important long-term clinical outcome of a regimen [9]. Furthermore, it is rather unlikely that all different subpopulations of *Mycobacterium tuberculosis* are detected using these methods [10].

In vitro experiments and animal models often use counting of colony forming units (CFU) as a parameter to determine the total bacterial load. It has been shown by Hu *et al*. that CFU counts are not always representative for the total population present in an *in vitro* system, as well as in the lungs of TB infected mice [11]. Besides the CFU counts found, there was also a population of *Mycobacterium tuberculosis* present that could only be detected when they were resuscitated using resuscitation promoting factors (RPFs), indicating that basic CFU counting is maybe not the most optimal parameter to determine the total bacterial load. Therefore new techniques, which are less dependent on culture viability, are necessary to provide this information.

In the present study, we used matched lung samples of our murine TB model to compare CFU counting, liquid culture/time to positivity (TTP) and the molecular bacterial load (MBL) assay [12]. This MBL assay detects *Mycobacterium tuberculosis* 16S ribosomal RNA with a robust internal control (IC) which normalizes for RNA loss during extraction and the presence of sample inhibitors. By measuring 16S ribosomal RNA the MBL assay is therefore not depending on actively growing mycobacteria.

The aim of the study is to compare CFU counting, TTP and the MBL assay, and explore whether the last assays can detect a mycobacterial subpopulation which is unable to grown on solid media.

MATERIALS AND METHODS

Mouse TB model

Specified pathogen-free female BALB/c mice were obtained from Charles River (Les Oncins, France). At the day of infection, animals were 13-15 weeks old and weighed 20-25 g.

Four groups of mice were infected with *Mycobacterium tuberculosis* as described previously [13]. In short, mice under anesthesia were infected by intra-tracheal instillation of a suspension (40 µL) containing 1.3 x 10^5 CFU (0.7 – 1.7 x 10^5) of the Beijing VN 2002-1585 genotype strain [14], followed by proper inhalation to ensure the formation of a bilateral TB infection.

Antibiotic therapy

Four treatment arms, each with a total length of 6 months (24 weeks) were designed, as part of the research conducted within the PreDiCT-TB consortium: HRZ (2HRZ / 4HR), ZES (2ZES / 4E), HRZE (2HRZE / 4HR) and HRZS (2HRZS / 4HR). All treatment arms started 2 weeks after infection.

Human equivalent doses of antibiotics were used: isoniazid (H) 25 mg/kg, rifampicin (R) 10 mg/kg, pyrazinamide (Z) 150 mg/kg, streptomycin (S) 200 mg/kg and ethambutol (E) 100 mg/kg [15]. All drugs were dissolved in water and given orally (0.2 mL) as a bolus, 5x/week, except for S, which was administered subcutaneously.

Determination of the mycobacterial load in infected lungs

To determine the mycobacterial load in infected lungs, mice were sacrificed by CO_2 exposure at the start of therapy and after 1, 2, 4, 8, 12 and 24 weeks of therapy (n=3). To prevent carry-over of TB-drugs, therapy was stopped 72 hours before sacrificing the mice.

The lungs were removed aseptically and homogenized within 5 minutes in M-tubes with the gentleMACS Octo Dissociator using the RNA program (Miltenyi Biotec BV, Leiden, the Netherlands) in 2 mL Phosphate Buffered Saline (PBS).

Colony forming units counts

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 (0.4%) , and incubated for 28 days at 37^0C with 5% CO_2 to perform colony counting.

Activated charcoal was added to the agar to inhibit the antibiotic residue from the tissue samples and prevent carry-over [16]. Lower limit of quantification is 10 CFU per lung.

Time to Positivity

From each tissue homogenate 200 µL was added to a mycobacterial growth indicator tube (BBL MGIT; Becton, Dickinson and Company, MD, USA) in combination with 800 µL OADC enrichment. Tubes were incubated in the Bactec MGIT 960 (Becton, Dickinson and Company, MD, USA) and TTP was automatically recorded.

MBL assay

From each tissue homogenate 300 µl was directly preserved in guanidine thiocyanate (GTC) containing 1% ß-mercaptoethanol and stored at -80°C. MBL assay was performed as previously described by Honeyborne *et al.*[12]. In short, following thawing, 50 ng of the internal control (IC), a 1,957-bp RNA molecule that contain parts of the *sol* gene from potato tuber (*Solanum tuberosum*) [12], was spiked into each tissue sample. The IC normalizes for RNA loss during extraction and the presence of sample inhibitors. To assign cycle threshold values to bacterial loads, a standard curve was prepared. Naïve lung homogenate in GTC was spiked with serial diluted concentrations of a *Mycobacterium tuberculosis* stock ranging from 1.3 x 10^7 down to 1 CFU/mL, including the IC (table 1).

Table 1. Standard curve of CFU and CT values obtained from a serially diluted *Mycobacterium tuberculosis* stock spiked in naïve lung homogenate.

	CFU *Mycobacterium tuberculosis* / 1mL lunghomogenate							
CFU	1,3E+07	1,3E+06	1,3E+05	1,3E+04	1,3E+03	1,3E+02	1,3E+01	0,0E+00
Mean CT	11,99	13,26	19,05	24,17	31,02	35,23	>40	-
SD	0,77	0,71	0,78	0,78	1,82	1,71	-	-

Lung homogenate in GTC plus IC was centrifuged (14,000 x *g*;20 minutes) at room temperature, supernatant was discarded and 1 mL of RNApro (FastRNA pro Blue kit; MP Biomedicals) was added to the pellet. RNA extraction was performed according to the manufacturer's instructions. DNase I (Turbo DNA-fee kit; Ambion) was used to remove contaminating DNA.

Expression of 16S-ROX and IC-JOE was measured using multiplex reverse transcriptase-quantitative PCR (RT-qPCR). RT-qPCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Bleiswijk, the Netherlands) using a one-step qRT-PCR MasterMix for probe Assay Low ROX (Eurogentec, Maastricht, the Netherlands). Primers and dual-labelled probes were also purchased from Eurogentec.

Data Analysis

Data 0-24 weeks of treatment (2-26 weeks following infection) were used to investigate elimination of bacteria under the four therapeutic conditions (HRZ, ZES, HRZE, HRZS). Contaminated CFU (n=2) and TTP (n=11) samples were excluded. Correlations between measurements obtained by CFU, TTP and MBL assay over time collectively were evaluated by Spearman's Rank ($p < 0.017$ was considered statistically significant at the 5% level incorporating a Bonferroni correction for 3 pairwise comparisons for each treatment). Given that data from 3 distinct mice were available at each time point, naïve pooling was undertaken for analysis [17]. Mathematical models to describe the profiles of elimination of bacteria under treatment were fitted by nonlinear regression to the observed profiles of bacterial load vs. time as measured by CFU, 1/TTP, and MBL (TTP in broad terms being inversely proportional to the number of bacteria in the system). Parameter estimates for this modelling analysis were determined using the nonlinear least squares optimization function "lsqnonlin" as part of the "pracma" package in R (www.r-project.org). Standard errors were calculated using previously described methods by Landaw *et al.* with the Jacobian of model parameter sensitivities estimated using a numerical central difference method [18]. Exponential growth and death of bacteria cannot be differentiated with this data (the growth and death processes may occur simultaneously across the bacterial population as a whole) therefore exponential rate constants describing the "net" rate of bacterial growth or death were estimated from the quantitative timecourse profiles of bacterial load as determined by the 3 assays. These net rate constants take a negative value corresponding to net elimination, and a positive one to describe net growth.

RESULTS

The data of lung CFU, MBL and TTP (TTP data is converted to 1/TTP) over 24 weeks of treatment are illustrated (Figure 1) and statistical relationships between assays are summarized (Table 2). Overall, inverse correlations were observed between TTP with CFU and MBL and positive associations between CFU and MBL with statistical significance for all regimens with the exception of TTP *vs.* MBL for ZES and HRZE and CFU *vs.* MBL for HRZS (Table 2). For HRZ, HRZE and HRZS, MBL consistently reported lower than CFU between 2-8 weeks post-infection and higher than CFU to 14 weeks and 26 weeks post-infection (Figure 1).

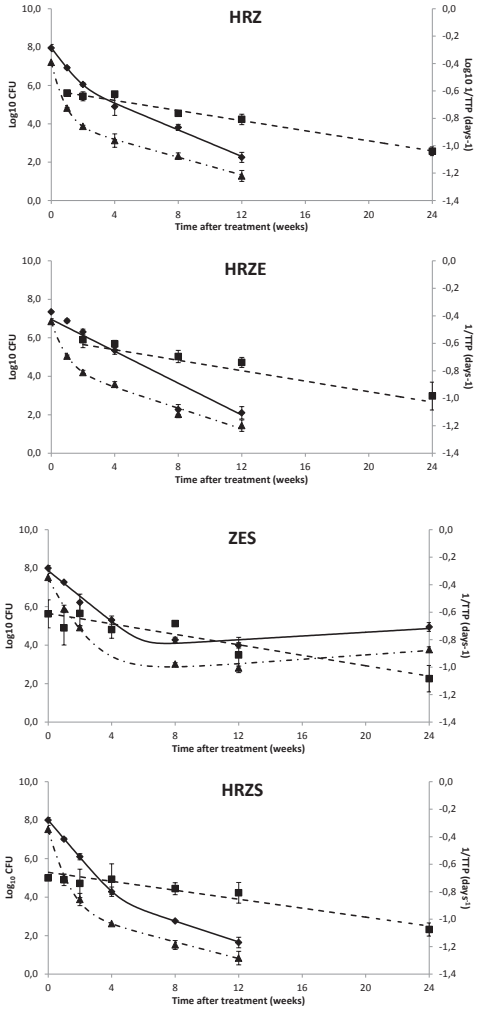

Figure 1. Mouse lung CFU, MBL and TTP over 24 weeks post-treatment (26 weeks post-*Mycobacterium tuberculosis* infection) with HRZ, ZES, HRZE and HRZS. Data are presented as mean value (n=3 mice per time point) ± SD with CFU and MBL on the left y-axis and 1/TTP on the right. The solid and dashed lines represent the modelled fit to the data.

Figure legend

◆ Observed CFU ------- Predicted CFU

■ Observed MBL ------- Predicted MBL

▲ Observed 1/TTP ------- Predicted 1/TTP

CFU: Colony Forming Units; MBL: Molecular Bacterial Load; TTP: Time To Positivity

H: isoniazid; R: rifampicin; Z: pyrazinamide; S: streptomycin; E: ethambutol

Table 2. Summary of spearman rank correlation coefficients evaluating the relationships between assays for measurement of *Mycobacterium tuberculosis* in mouse lung following 24 weeks treatment with HRZ, HRZE, HRZS, ZES (analyses from week 2-26 post- *Mycobacterium tuberculosis* infection). P values highlighted in **bold** type represent statistically significant correlations with Bonferroni correction of 3 per drug combination for multiple comparisons (p< 0.017)

Treatment	Comparison	Rho* (95% CI)	P value
HRZ	TTP vs. CFU	-0.993 (-0.998 to -0.978)	**<0.0001**
	CFU vs. MBL	0.846 (0.529 to 0.956)	**0.0008**
	TTP vs. MBL	-0.874 (-0.964 to -0.602)	**0.0003**
HRZE	TTP vs. CFU	-0.766 (-0.911 to -0.452)	**0.0005**
	CFU vs. MBL	0.797 (0.412 to 0.941)	**0.0027**
	TTP vs. MBL	-0.382 (-0.799 to -0.283)	0.248
HRZS	TTP vs. CFU	-0.965 (-0.989 to -0.890)	**<0.0001**
	CFU vs. MBL	0.507 (-0.0069 to 0.809)	0.054
	TTP vs. MBL	-0.753 (-0.922 to -0.345)	**0.0044**
ZES	TTP vs. CFU	-0.982 (-0.994 to -0.952)	**<0.0001**
	CFU vs. MBL	0.492 (0.0888 to 0.757)	0.010
	TTP vs. MBL	-0.457 (-0.762 to 0.0120)	0.028

* Spearman's rank correlation co-efficient

CFU: Colony Forming Units; MBL: Molecular Bacterial Load; TTP: Time To Positivity; H: isoniazid; R: rifampicin; Z: pyrazinamide; S: streptomycin; E: ethambutol; CI: confidence interval

Modelling of CFU *vs.* time

For CFU profiles over time (t) from the lung while under therapy the best description was given by a bi-exponential decline of the form CFU(t) = A*10^(-α*t)+B*10^(-β*t) with the exception of HRZE for which a mono-phasic decline was supported [CFU(t) = A*10^(-α*t)] (Figure 1). The first order exponential rate constants alpha (α) and beta (β) (in broad terms the "slopes" of the phases of the profile vs. time when plotted on a semi-logarithmic scale) are summarized (Table 3). Based on the β exponential rate constant, governing the 2nd phase of a multiexponential profile (if 2 exponential phases are present, and governing the only phase if the profile is mono-phasic) a rank order HRZE > HRZ > HRZS > ZES was shown, i.e. HRZE produced the steepest gradient on a logarithmic scale corresponding to a more rapid rate of elimination of bacteria, in contrast to ZES which actually showed signs of regrowth of bacteria during the second phase of its profile, though at a slow rate close to stasis over the 12-24 week period of the study (β of -0.414 vs. +0.049; Table 3). The β exponential rate constant is potentially a more interesting parameter of comparison as it is likely to reflect more the activity of treatment against a slower dying, less drug susceptible mycobacterial subpopulation similarly though, based on α slope, rate of eliminations were in the order of HRZ > HRZS > ZES (Table 2) with HRZE for unknown reasons showing only a mono-phasic decline in this experiment.

Table 3. Parameter estimates and associated percentage relative standard errors (%RSE) for α and β exponential rate constants describing net bacterial growth/elimination for each treatment regimen between weeks 2-24 of infection. Data analysed by fitting a bi or mono-exponential model as appropriate.

CFU *vs* time				
Treatment	**α (wk^{-1})**	**%RSE**	**β (wk^{-1})**	**%RSE**
HRZE*	-	-	**-0.414**	**9.4**
HRZ	-1.127	30.4	**-0.346**	**7.6**
HRZS	-0.971	11.2	**-0.270**	**19.0**
ZES	-0.664	9.8	**+0.049**	**25.6**
MBL *vs* time				
Treatment	**α (wk^{-1})**	**%RSE**	**β (wk^{-1})**	**%RSE**
ZES*	-	-	**-0.136**	**11.4**
HRZ*	-	-	**-0.132**	**4.5**
HRZE*	-	-	**-0.130**	**10.0**
HRZS*	-	-	**-0.116**	**10.9**
(1/TTP) *vs* time				
Treatment	**α (wk^{-1})**	**%RSE**	**β (wk^{-1})**	**%RSE**
HRZE	-0.605	19.7	**-0.038**	**10.4**
HRZ	-0.718	14.2	**-0.032**	**9.2**
HRZS	-0.526	12.6	**-0.030**	**17.8**
ZES	-0.276	7.6	**+0.008**	**18.6**

* Mono-exponential fitting

CFU: Colony Forming Units; MBL: Molecular Bacterial Load; TTP: Time To Positivity; H: isoniazid; R: rifampicin; Z: pyrazinamide; S: streptomycin; E: ethambutol; %RSE: percentage Relative Standard Error of parameter estimate.

Modelling of MBL *vs*. time

Profiles of MBL over time during therapy were described by mono-phasic declines (Figure 1). The gradients of the elimination slopes on the logarithmic scale for each combination therapy were relatively flat and similar numerically suggesting slow bacterial elimination for all therapies (Table 3).

Modelling of TTP *vs*. time

Given the inverse relationships observed between TTP with CFU and MBL a bi-exponential elimination model was fitted to 1/TTP vs. time data in order to determine a comparable elimination rate for TTP (Figure 1) (1/TTP being a more directly comparable measure of bacterial load). Based on the β rate constant, rate of bacterial decline was of an identical order to that observed for CFU (HRZE > HRZ > HRZS > ZES) with ZES also showing potential regrowth (i.e. a positive β rate constant) in the 12-24 week period as it did in its CFU profile.

DISCUSSION

Identification of subpopulations of *Mycobacterium tuberculosis* is important for drug development, refinement of existing or novel drug combinations to ultimately achieve therapeutic success in a clinical setting. Animal models can be particularly useful to evaluate potential methods of quantifying different populations of *Mycobacterium tuberculosis*[11, 19]. One of the benefits of an animal model is the ability to study the total population of *Mycobacterium tuberculosis* in the lung, including the more hidden bacteria in granuloma like formations, whereas in sputum from TB patient only the secreted bacteria can be studied [20]. However, animal studies from the past using CFU counts as a parameter are maybe not predictive enough because it has been shown by Hu *et al.* that CFU counts are not representative for the total population present in an *in vitro* systems as well as in the lungs of TB infected mice, using RPFs to resuscitate a persistent and non-growing population [11].

We have presented for the first time the comparison of CFU, MBL and TTP methods using matched lung samples from a mouse TB model and explored the use of mathematical modelling to further summarize and scrutinize the data, describing the elimination of *Mycobacterium tuberculosis* over time for each method under different therapeutic conditions.

The ability to detect different *Mycobacterium tuberculosis* populations in our mouse TB model using the three methods can be explained by the principles of the techniques. In contrast to DNA based assays like the GeneXpert® MTB/RIF assay, the MBL assay detects the presence and amount of 16S rRNA and is therefore less hampered by the presence of genomic material of already dead cells [21]. The 16S rRNA is much more unstable compared to DNA and degrades much faster [22]. It has been shown that RNA, in particular mRNA, correlated with solid culture but more closely with growth in liquid culture [23]. However, for culture on solid media or in liquid media actual growth is still required, thereby presenting only the fittest mycobacteria which were capable of growing. In contrast, for the MBL assay a particular population of *Mycobacterium tuberculosis* should be alive, and have metabolic activity, but actual growth is not required.

Statistically significant correlations between assays were evident for the majority of regimens tested. Lack of association between TTP *vs.* MBL for ZES and HRZE and CFU *vs.* MBL for HRZS was possibly due to small sample size in these groups. Unsurprisingly, TTP was inversely related to CFU and MBL as a shorter time to positivity would be expected with high bacterial load measured with CFU as well as with the MBL assay. Conversely, if the viable mycobacterial count CFU was zero, the MGIT will not measure fluorescence as a result of oxygen consumption and time to positivity would be infinite, because there are no growing mycobacteria present.

Mathematical modelling allowed estimation of the elimination rate of bacteria under each of the four drug combinations. The rates of elimination of bacteria for HRZ, ZES,

HRZE and HRZS as measured by CFU and TTP were of a similar rank order and were both typically bi-exponential in this study. This suggests that CFU and TTP are measuring similar bacterial populations that show net elimination under chemotherapy. A study conducted by Diacon *et al.* showed that CFU can be substituted with TTP in early bactericidal activity (EBA) studies [7], but on the other hand, a sample taken at the end of treatment can take longer to signal positive in MGIT compared to a sample taken early with a similar CFU count as shown by Bowness and colleagues [24]. They showed that the relationship between CFU and TTP changes over time in early response to treatment (14 days). These observations imply that MGIT detects an extra sub-population of *Mycobacterium tuberculosis* and that this sub-population decreases in number during drug exposure and treatment. A similar analysis to Bowness and colleagues was not feasible however with the data in our study due to the limited number of samples per time point (or groups of time-points). We therefore cannot conclude that CFU and TTP are detecting different sub-populations of *Mycobacterium tuberculosis* in our mouse model.

Compared to the bi-phasic decline of CFU and TTP over time (with the exception of HRZE), description of MBL was mono-phasic for all four treatments with gradients on the logarithmic scale close to zero, but MBL counts remained positive during treatment in all four treatment arms, suggesting the presence of a mycobacterial subpopulation which decreases slowly under treatment, but cannot be detected by TTP and CFU counting. However, elimination rate constants derived from MBL data cannot arms tested. All four treatment arms have MBL elimination rate constants of similar magnitude, whose differences cannot be considered statistically significant. In contrast to the mono-phasic elimination in the present study, a bi-phasic decline with the MBL was observed by Honeyborne *et al.*[12]. However, the study reported an EBA measured over 14 days with the first 3 days showing a steeper gradient compared to EBA 3-14 days. The difference in observations between the Honeyborne study and the present study may be a result of the richer initial time course studied by Honeyborne *et al*. compared to sampling 1 week after start treatment in our study. Thereby potentially failing to capture a bi-phasic decline of the first 3 days, which is a limitation of the current study.

At week 12, MBL numbers were higher compared to CFU and TTP (with the exception of ZES) and at week 24 CFU counts were negative for HRZ, HRZE and HRZS but the MBL numbers were still positive. Because rRNA is downregulated in bacteria entering dormancy [25], we could speculate this could infer that the MBL assay maybe even underestimates the mycobacterial load during the continuation phase of treatment. This could for instance explain the failure of the MBL assay to differentiate between the efficacy of ZES and the HRZ-based regimens at the end of treatment.

In the present study, MBL also measured lower than CFU in the early phase of treatment, indicating a less sensitive assay to detect high numbers of actively replicating *Myco-*

bacterium tuberculosis. This was also shown by van der Vliet *et al*. [26], demonstrating that conversion of 16S rRNA to CFU must be optimized.

Also notable, , is the disparity between CFU and MBL counts after 12 weeks with rifampicin and isoniazid containing regimens (HRZ, HRZE and HRZS), compared with the observation of CFU and MBL after 12 weeks of the ZES treatment where minimal difference is found between the two. This observation is in keeping with a pattern where during the early phase of treatment the metabolically active *Mycobacterium tuberculosis* are eliminated by isoniazid, while rifampicin is one of the drugs responsible for the elimination during the sterilizing phase [27]. The positive MBL counts at later time points which are not counted by the CFU and TTP assay possibly represent a subpopulation of *Mycobacterium tuberculosis,* which are in a dormant state. To add on to this, Malherbe *et al.* recently showed that mRNA can still be found in a bronchoalveolar lavage samples at the end of treatment in cured patients, also indicating that there is a subpopulation of mycobacteria which cannot be detected on solid media but is still present in the lungs [28]. This suggest an important complementary role for the hosts immune system to maintain a disease-free state and shows that sterilizing or host-directed therapies are needed for the development of shortened and improved treatment regimens [28].

A study by de Steenwinkel *et al.,* aiming for a shortened treatment regimen found that increasing the rifampicin dose up to 80 mg/kg/day reduced the treatment duration by four months, without relapse of infection [29]. To add on to this, Hu *et al.* recently found that increasing the rifampicin dose eventually also eliminate the non-culturable population in their model [11]. However, Kayigire *et al.* showed that rifampicin significantly reduced the CFU counts, but also significantly changed the proportions of non-growing, lipid body-containing mycobacteria an viable mycobacteria, indicating that treatment can drive mycobacteria in a non-culturable state [30].

Therefore, additional investigation of the use of the MBL assay with high doses of rifampicin in combination with RPFs is warranted to further characterize the non-culturable subpopulation.

In conclusion, we show strong correlations between CFU, TTP, and the MBL assay reflecting the ability of each to determine the mycobacterial load in our mouse TB model. The findings also show that the MBL assay tells "a different story" to that of CFU counts and TTP, by detecting a subpopulation of *Mycobacterium tuberculosis* which is not detected by CFU or TTP assays.

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Chapter 10

Summarizing discussion

SUMMARIZING DISCUSSION

The overarching aim of this thesis is to contribute to better tuberculosis (TB) therapy. TB therapy can be improved in many ways, this work focussed on the additional value of efflux pump inhibition. Moreover, we set ourselves the goal to further optimize the predictive value of our preclinical mouse TB model.

The primary objectives were:

- To assess the therapeutic potential of co-administration of efflux pump inhibitors, i.e. thioridazine or SILA-421 with the standard treatment regimen
- To study a moxifloxacin and linezolid combination as a novel treatment regimen for MDR-TB and potentiate this combination with efflux pump inhibitors and cell wall destabilizing drugs
- To characterize the effect of rifampicin on the transcription profile of a rifampicin-susceptible and a rifampicin-resistant *Mycobacterium tuberculosis* strain
- To study the added value of using a *Mycobacterium tuberculosis* strain of the clinical highly relevant Beijing genotype in our mouse TB model
- To compare colony forming units counting, the time to positivity assay and the molecular bacterial load assay, and explore whether the last assays can detect a mycobacterial subpopulation which is unable to grow on solid media

To attain these objectives we studied the *in vitro* and *in vivo* activity of 'classic' and novel TB drugs in combination with efflux pump inhibitors against *Mycobacterium tuberculosis*

Therapeutic potential of coadministration of efflux pump inhibitors with the standard TB regimen

Thioridazine is described in literature as a potent efflux pump inhibitor [1-9]. Therefore, we set out to potentiate the standard isoniazid-rifampicin-pyrazinamide (HRZ) combination *in vitro* as well as *in vivo* **(chapter 2)**. The addition of thioridazine as a companion drug, was prompted by promising results from other groups [5, 6, 8]. Using thioridazine as a companion drug in our *in vitro* model we were able to potentiate the activity of both mono-exposure of isoniazid and rifampicin against *Mycobacterium tuberculosis,* and concluded that thioridazine could work synergistically with both drugs. The synergy also was found on the prevention of selection of isoniazid resistance. In spite of the promising *in vitro* results, translation to our mouse TB model was less successful as the addition of thioridazine to the HRZ-regimen was not associated with shorter treatment duration. This was in contrast to a previous study where a significant reduction of the mycobacterial load was observed when the HRZ-regimen was combined with thioridazine at a dose of 32 mg/kg compared to HRZ only treatment [8]. Also a study by Dutta *et al.* reported a trend towards enhanced clearance when thioridazine at a dose of 25 mg/kg was combined with the HRZ treatment [5]. Due to severe toxic events the maximum tolerated dose in our mice was much lower than

previously published thioridazine doses in mice [5, 8]. However, our final dose of 18 mg/kg/day is close to the 25 mg/kg/day dose used by others that yields exposures of thioridazine in mice similar to those in humans after a 25 mg/kg/day dose [6]. In addition, over time thioridazine accumulates in lung tissue up to 30-fold relative to serum [6]. But nevertheless, a 3-months HRZ-regimen in combination with thioridazine was not successful and relapse of infection occurred. Unfortunately, we did not assessed relapse of infection after four or five months treatment to study the possibility of shortening treatment duration by only one or two months.

In a guinea pig TB model exposure to thioridazine also causes severe toxic events and limited bactericidal activity was found against extracellular mycobacteria within granulomas [10]. No support could be found in our studies for the use of thioridazine in combination with the standard TB regimen. Our results and the literature on thioridazine use as a TB drug taken together, the question remains whether it is still worth to study thioridazine as a potential TB drug. Alternatively, we may focus on more promising and safer efflux pump inhibitors e.g. verapamil in combination with bedaquiline.

To potentiate the activity of TB drugs *in vitro* and the HRZ-regimen *in vivo,* we also used the organosilicon compound SILA-421 **(chapter 3)**. Little is known about the mode of action of SILA-421, but it is associated with efflux pump inhibition [11-14]. We found a clear antimycobacterial effect of SILA-421 as single drug and synergistic activity in combination with isoniazid against drug-sensitive *Mycobacterium tuberculosis*. SILA-421 as single drug is also active against rifampicin-resistant strains. In combination with high concentrations of rifampicin it works synergistically and can completely eliminate the rifampicin-resistant *Mycobacterium tuberculosis*. However, despite promising *in vitro* results, we found no added value of SILA-421 in our mouse TB model when it was used for three months in combination with the standard HRZ-regime. SILA-421 is a novel compound and there was no assay available to measure SILA-421 levels in murine plasma. A PK/PD study that might explain why SILA-421 failed to improve the HRZ-regimen was therefore not possible. A dose finding study in combination with a pharmacokinetic study is needed to define the optimal SILA-421 dose in mice. Similar to chapter 2, we did not study the relapse of infection after four or five months treatment with the HRZ-regimen together with SILA-421. To better assess the sterilizing capacity of this combination, assessment of relapse of infection after different treatment lengths should be included.

A novel treatment regimen for MDR-TB based on moxifloxacin and linezolid

Treatment options for multidrug-resistant TB (MDR-TB) are limited [15]. Second-line treatment usually consist of various TB drug combinations administered for many months and with success rates of only 50% [15, 16]. We therefore explored the effectiveness of a novel regimen consisting of moxifloxacin and linezolid in different combinations with two efflux

pump inhibitors (verapamil and timcodar) and/or the cell wall destabilizers colistin and SQ109 **(chapter 4)**.

These efflux pump inhibitors and cell wall destabilizers can potentially increase the intra mycobacterial concentrations of moxifloxacin and linezolid and possibly enhance the activity of the drugs. *In vitro*, both moxifloxacin and linezolid were effective as single drugs against *Mycobacterium tuberculosis*. While moxifloxacin was associated with 99% killing with as little as 1/16x the fC_{max}, exposure to 1x fC_{max} of linezolid resulted in 90% killing. There is every reason, therefore, to propose that moxifloxacin may serve as a key drug to treat MDR-TB [17, 18].

The WHO recently upgraded linezolid to group C, which includes 'other core second-line agents' for the treatment of drug resistant TB, but warned that more (pre-) clinical data of linezolid is urgently needed [18]. Our data shows indeed that linezolid acts against *Mycobacterium tuberculosis* at relative low concentrations. We also showed that the combination of moxifloxacin plus linezolid is active against *Mycobacterium tuberculosis* at concentrations that can be achieved in TB patients.

Nevertheless, administration of especially linezolid carries the risk of toxicity [19]. Adding potentiator drugs, we thought, would optimize the activity of the moxifloxacin - linezolid regimen and avoid high systemic drug concentrations, which are associated with toxicity [20].

To this aim we used moxifloxacin and linezolid concentrations which in combination would only inhibit growth, and not result in killing. The addition of colistin to the moxifloxacin plus linezolid resulted in an increased elimination of *Mycobacterium tuberculosis.* This, however, did not meet our criterion of synergy of a 2 log difference between the combination and the most active single drug of the combination [21]. Verapamil, timcodar and SQ109 did not increase the *in vitro* activity. We concluded that *Mycobacterium tuberculosis* could be effectively eliminated with the moxifloxacin – linezolid combination, and that colistin could act as potentiator compound. Our data support the usefulness of moxifloxacin and linezolid in the treatment of MDR-TB. To evaluate the bactericidal activity and sterilizing capacity of this combination, the results from our extracellular *in vitro* model must be translated to our *in vivo* mouse TB model.

To study the moxifloxacin and linezolid regimen in mice we needed to know what doses would achieve a human pharmacokinetic equivalent exposure **(chapters 5 & 6)**. Using increasing dosages and a pharmacokinetic analysis we obtained different parameters upon which we could perform the comparisons of mouse and humane data. In this studies, we also assessed the usability of the Dried Blood Spot (DBS) analysis method for measuring moxifloxacin and linezolid concentrations in murine blood spots. DBS analysis can reduce the number of animals needed in preclinical drug development because it allows for the assessment of drug concentrations in small blood volumes over time within a single mouse. In addition it may serve as a method for therapeutic drug monitoring (TDM) during activity and efficacy experiments in mice. For instance, TDM can be used to study drug-drug

interactions for their impact on the drug exposure of drugs studied [22]. We found that DBS concentrations of moxifloxacin and linezolid can predict plasma concentrations with good precision, so DBS analysis can be of use in preclinical murine models for its capacity of reducing the number of animals needed.

For moxifloxacin, the AUC relative to the minimal inhibitory concentration has been suggested as the best parameter to predict *in vivo* efficacy against *Mycobacterium tuberculosis*[23]. Based on the protein binding, which seems slightly lower in mice compared to humans and the $AUC_{0-\infty}$ calculated in the current study, we concluded that a single dose of 200 mg/kg moxifloxacin in mice would approximate a human equivalent free drug exposure (AUC_{0-24} 24.8 mg*h/L / $fAUC_{0-24}$ 17.3 mg*h/L [24]). In line with this, Poissy *et al.* reported AUC values close to those found in humans with the use of a similar dose as well as the highest decline in CFU with this dose [25].

For linezolid we found that a dose of 50 mg/kg resulted in an $AUC_{0-\infty}$ of 78 mg/L*h in BALB/c mice, which approximates free linezolid exposures of a dose between 300 and 600 mg in humans. The literature is divided on the optimal linezolid dose associated with treatment efficacy in humans. Initial treatment of MDR-TB with 600 mg of linezolid twice daily has been limited by adverse effects [26, 27]. Lower doses mostly seemed to retain effectiveness and to limit toxicity [20, 26, 27]. Therefore, the linezolid doses ≥100 mg/kg tested in mice by other groups probably by far exceed clinically useable doses and exposures [28, 29].

Characterization of the transcription profile after rifampicin exposure

A small side-step in this thesis is presented in **chapter 7**. We studied the transcriptome response following rifampicin exposure of an isogenic pair of strains, one rifampicin-sensitive and one rifampicin-resistant *Mycobacterium tuberculosis* strain. Rifampicin resistance in *Mycobacterium tuberculosis* is caused for almost 95% by non-synonymous single nucleotide polymorphisms in the 81 base pair region in the *rpoB* gene [30]. Still, rifampicin can actively induce the activity of efflux pump genes and transporters genes, at the level of gene expression [31].

With this study, we aimed to provide more insight in the influence of rifampicin resistance on the overall gene expression upon rifampicin exposure. We were particularly interested in whether efflux pumps and transporter-associated genes were affected. Microarray analysis revealed resistance-dependent differences in response between the rifampicin-sensitive and the rifampicin-resistant strain. Gene clusters associated with efflux, transport and virulence were altered in the rifampicin-resistant mutant compared to the rifampicin-susceptible wildtype strain after exposure to rifampicin. Two up-regulated gene clusters, that is Rv0450c to Rv452 and Rv0676c to Rv678, respectively, were found which both consist of genes of the MmpL (Mycobacterial membrane protein Large) family, and the MmpS (Mycobacterial membrane protein Small) family. Recently, mutations in the Rv0678 gene were shown to lead to subsequent transcriptional upregulation of all three

genes (rv0678, *mmpS5*, and *mmpL5*) of the locus. As a consequence, partial resistance to both clofazimine and bedaquiline was found [32]. These data, together with the results of Milano *et al.*[33] and the results presented in chapter 7, confirms the role of the gene cluster (rv0678, *mmpS5*, and *mmpL5*) that is involved in drug efflux. Functions of the other upregulated gene clusters are still unknown. RNA-sequencing, the new version of microarray analysis, would be an interesting option for further research.

Evaluation and optimisation of our mouse TB model with a Beijing isolate as a preclinical model

Evidence continues to accumulate indicating that TB caused by strains of the Beijing genotype are clinically associated with treatment failure [34-37]. However, the H37Rv laboratory strain is used in many preclinical TB models, which might not optimally represent the virulence of strains that cause TB in patients. Therefore, we aimed to assess the bactericidal activity and sterilizing capacity of TB drugs in our preclinical mouse TB model with a Beijing genotype strain **(chapter 8)**.

We found that bactericidal activity after 2 and 4 weeks treatment was primarily driven by rifampicin, were in TB-patients isoniazid showed the best bactericidal activity after 2 weeks treatment [38]. In the sterilizing capacity experiments only the rifampicin-containing combinations reached culture-negativity of lungs after a 6-months treatment course. Based on this we concluded that rifampicin is essential in the treatment of drug-susceptible TB. Nevertheless, even these rifampicin-containing combinations failed to reach complete sterilizing capacity. The remarkable difference between the rifampicin-pyrazinamide (RZ) combination (5% relapse) and the rifampicin-isoniazid-pyrazinamide (RHZ) combination (20% relapse) is interesting. This difference was not significant, but our results are complementary to those of an earlier study assessing sterilizing activity after a 6-months treatment course with RZ or RHZ after intravenous infection of Swiss mice [39].

In line with clinical studies [40], bactericidal activity did not correlate with sterilizing capacity in our model. This is particularly true for RHZ-treated mice, which showed the highest bactericidal activity between 0-14 days but also the highest relapse rate of 20%.

In our previous studies and in studies by other groups, the RHZ regimen against the H37Rv strain in BALB/c mice resulted in 100% sterilizing capacity, without relapse of infection [41-44]. Moreover, other mouse TB models such as C3HeB/FeJ mice also show 100% sterilizing capacity using the RHZ regimen against infection with H37Rv [45, 46]. In this study we clearly demonstrated that each of the studied combinations of conventional TB drugs, including the RHZ, regimen did not achieve 100% sterilizing capacity against a clinical Beijing isolate.

We concluded that testing sterilizing capacity of TB drugs against different mycobacterial strains is important to improve the predictive value of our mouse TB model. Furthermore, we concluded that bactericidal activity is not predictive for the sterilizing capacity.

The method of colony forming units (CFU) counting is often used to monitor the mycobacterial load in preclinical models. Bowness *et al.* have suggested that liquid media is more suitable to detect a population of cells that are viable but non-culturable on solid agar [47]. Also the Molecular Bacterial Load (MBL) assay is thought to be a good replacement of solid culture [48]. We therefore optimized the MBL assay for use in our studies, together with the time to positivity (TTP) assay, and the standard CFU counting to monitor changes in the mycobacterial load and thereby the treatment response **(chapter 9)**. Mice infected with *Mycobacterium tuberculosis* were treated with four different treatment arms i.e. HRZ, HRZS, HRZE and ZES. The obtained CFU counts at multiple time points during treatment were compared to the results obtained with the TTP assay and MBL assay.

We found significant correlations between the three methods, and concluded that all methods were able to discern a general mycobacterial load of *Mycobacterium tuberculosis* in our mouse TB model. However, when fitting the net elimination of mycobacteria with nonlinear regression, we found that both CFU counting and the TTP assay describe the data in a bi-phasic manner with a small but steep α-slope during the first phase of treatment, followed by a β-slope during the rest of treatment. In contrast the MBL showed a mono-exponential decline with a flat gradient of elimination. This would explain why CFU counting and the TTP assay indicated culture negativity after three months treatment whereas the MBL assay was still positive. Maybe the MBL assay is detecting a subpopulation of mycobacteria which is gone unnoticed by CFU counts or the TTP assay. This subpopulation is possibly in a dormant state and therefore not able to grow. Obviously, only drug combinations that can successfully eradicate this dormant population can reach true culture negativity and can prevent relapse of disease. Therefore it is pivotal to detect, visualize and closely monitor the dormant population of mycobacteria in preclinical models as well as in TB-patients.

FUTURE PERSPECTIVES

The use of efflux pump inhibition

Regardless of the limited success of efflux pump inhibition described in this thesis, there is clearly a role of efflux pump inhibition in the treatment of TB, as shown by Gupta *et al.*[49-51]. They performed experiments in mice using verapamil in combination with the standard regimen as well as in combination with bedaquiline. Verapamil added as potentiator drug to the HRZ-regimen accelerated the mycobacterial killing, and addition of verapamil to bedaquiline increased the bactericidal activity of bedaquiline [49-51]. Recent WHO guidelines recommend bedaquiline and delamanid separately as an 'add on agent' in the treatment of MDR-TB and XDR-TB. So far, no clinical trials have studied the combination of bedaquiline and delamanid. However, two recent case reports describe good short-term

outcomes for this combination in XDR-TB patients, although side effects were seen [52, 53]. Our mouse TB model is suitable to evaluate the bactericidal activity and sterilizing capacity of a bedaquiline and delamanid combination, and to provide preclinical data which can be used to design a clinical trial. This combination can be administered with the efflux pump inhibitor verapamil to increase the activity of bedaquiline. By optimizing the bedaquiline and verapamil combination, it might be possible to lower the bedaquiline dose without loss of activity and efficacy and avoid toxicity.

Further optimize our mouse TB model

In an experimental setting the most important factor describing treatment success is the relapse rate. Therefore, relapse of infection was also a parameter in some of the studies included in this thesis. There is no standard method of performing relapse assessment studies, however, and many variations are possible, e.g. sample size, duration of the treatment free period and the use of Cornell model.

The current method of relapse assessment is not informative enough; it lacks dynamics. Describing the relapse in a more dynamic way could help improve the predictive value of our mouse TB model. For future studies it is essential that we optimize and standardize the way we do relapse studies. Continuous assessment of the relapse mycobacterial load after different durations might enable to characterize the dynamics of relapse and the underlying microbiological response.

Evaluate the bactericidal activity and sterilizing capacity against non-replicating *Mycobacterium tuberculosis*

Our findings that CFU counting might lead to underestimation of the total population of mycobacteria shows that better tools are needed to monitor treatment response. By using the MBL assay a population of bacteria was detected that was not able to grow on agar or in liquid media. It is thought that this persisting population is responsible for the relapse of TB and that prolonged treatment is needed to eliminate these persisting mycobacteria. Several recent clinical trials of shortened regimens containing fluoroquinolones failed to show non-inferiority mainly due to increased relapse rates, despite a more rapid clearance of mycobacteria in both mice and patients [17, 54-56]. This highlights the important role of the hidden, non-culturable mycobacterial population. Great effort must be made to study this hidden population and its response to treatment. One way to characterize this population is by using resuscitation-promoting-factors (RPFs). RPFs are a family of secreted peptidoglycan-remodelling enzymes, which are required for the regrowth of dormant mycobacteria [57]. RPF-dependent mycobacteria are highly abundant in sputum from TB-patients and can also be recovered from infected lungs [58, 59]. These RPFs can drive *Mycobacterium tuberculosis* into an active state in which they can be cultured. The use of RPFs must be evaluated in the time-kill kinetic experiments in order to identify TB drugs

that are real sterilizing drugs instead of drugs that inhibit growth and drive *Mycobacterium tuberculosis* in a non-replicating state.

To prove that the subpopulation found using the MBL assay is an actual non-replicating subpopulation, we have to resuscitate the bacteria present in the lung homogenate of our mouse TB model using RPFs. If we can increase the CFU numbers identical to the MBL counts, we can combine CFU counts with RPFs as an optimized tool to monitor the mycobacterial load.

At the start of this thesis we referred to the 'END TB STRATEGY' of the WHO and the targets set in that document. We hope that this thesis forms a piece of the enormous TB eradication puzzle and contribute to a slightly better view and understanding of the challenges and putative solutions that will lead to the end of TB.

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Chapter 11

Nederlandse samenvatting

SAMENVATTING

Tuberculose (TBC) is een infectieziekten en door de geschiedenis heen heeft TBC wereldwijd de meeste doden op zijn naam staan ten opzichte van andere infectieziekten. Geschat wordt dat het er meer dan 1 miljard zijn in de afgelopen 200 jaar. Ook vandaag de dag is TBC de infectieziekte die de meeste doden tot gevolg heeft. De Wereldgezondheidsorganisatie (WHO) schat dat in 2015 1,8 miljoen mensen zijn overleden aan de gevolgen van TBC en dat er 10,4 mensen TBC hebben opgelopen, waaronder 1 miljoen kinderen. Om deze kille cijfers meer gevoel te geven; dit betekent dat er wereldwijd elke minuut drie mensen overlijden aan TBC. Om deze ziekte een halt toe te roepen, heeft de WHO in mei 2014 de 'END TB STRATEGY' gelanceerd. Het doel is een wereld zonder TBC. Om dit te realiseren zijn ambitieuze doelen gesteld, zoals een 95% reductie van het aantal doden ten gevolge van TBC en een 90% reductie van de TBC-incidentie in 2035 ten opzichte van 2015. Het is bijzonder ambitieus, omdat de WHO ook inschat dat 2 tot 3 miljard mensen wereldwijd zijn besmet met de bacterie die TBC veroorzaakt. Deze mensen zijn latent geïnfecteerd, maar zijn niet ziek. Van deze groep ontwikkelt 5 tot 15% alsnog TBC gedurende zijn of haar leven. Uiteindelijk kan iedereen TBC krijgen, maar er zijn een aantal factoren bekend die het risico vergroten op het oplopen van TBC. Dit zijn diabetes, roken en het gebruik van verdovende middelen, maar ook problemen met de gezondheid waarbij het immuunsysteem is aangetast, zoals HIV. Daarnaast is TBC een aan armoede gerelateerde ziekte.

De bacterie die al deze ellende veroorzaakt, is *Mycobacterium tuberculosis*. Het is een bacterie die al duizenden jaren deze ziekte veroorzaakt en zelfs al ver voor Christus is beschreven in bijvoorbeeld de Bijbel (Lev 26:16). De bacterie is voor het eerst ontdekt door Robert Koch in 1882, en is geclassificeerd als een gram positieve bacterie. De overdracht van mens naar mens gebeurt meestal via kleine waterdruppeltjes die worden uitgehoest door de patiënt. De klassieke symptomen van TBC zijn hoesten, significant gewichtsverlies, nachtzweten en koorts. Als de infectie niet wordt behandeld, overlijdt ongeveer 70% van de mensen binnen tien jaar na infectie.

Voor de Tweede Wereldoorlog waren er geen antibiotica beschikbaar om TBC te behandelen. In 1944 werd streptomycine ontdekt en in 1952 isoniazide. Door deze antibiotica te combineren was TBC te behandelen, maar de behandelduur was minstens achttien maanden. In 1967 werd rifampicine voor het eerst gebruikt ter bestrijding van TBC en dat is vandaag de dag nog steeds het middel om TBC mee te behandelen in combinatie met isoniazide, pyrazinamide en ethambutol. De totale behandelduur is nu zes maanden in het geval van een niet resistente *Mycobacterium tuberculosis*. De mycobacteriële bacteriepopulatie in een patiënt is gevarieerd en bestaat uit diverse subpopulaties, en al deze subpopulaties kunnen verschillend reageren op antibiotica. Voor een succesvolle behandeling van TBC is het extreem belangrijk dat de combinatie van antibiotica actief is tegen alle subpopulaties, om zo de kans op 'relapse' van TBC en resistentie te voorkomen.

Resistentie maakt het moeilijk om de wereldwijde epidemie van TBC te stoppen. Op dit moment zijn er volgens de WHO per jaar bijna een half miljoen gevallen van multidrug resistente TBC. De behandeling van deze vorm van TBC is moeilijk, omdat de bacteriën resistent zijn voor de meest gebruikte antibiotica. Een alternatieve behandeling kan tot wel twintig maanden duren, is complex en bestaat uit veel verschillende pillen en injecties, samen dit kan oplopen tot wel 14.000 stuks over de gehele periode. En uiteindelijk is er slechts in 50% van de gevallen genezing mogelijk. Een groot probleem van deze tweede keus antibiotica zijn de vele bijwerkingen, zelfs zo erg dat patiënten de bijwerkingen van de antibiotica ervaren als erger dan de ziekte zelf. De extreem lange behandelduur en de vele serieuze bijwerkingen leiden ertoe dat patiënten tijdens de behandeling afhaken, met als resultaat dat de bacteriën alleen nog maar resistenter worden. Het is dan ook duidelijk dat de behandeling van TBC moet worden geoptimaliseerd.

Het onderzoek

Voor de optimalisatie zijn er meerdere wegen die naar Rome kunnen leiden, meerdere strategieën zijn mogelijk. De eerste optie de we hebben bestudeerd om TBC efficiënter te bestrijden is de hoeveelheid van de antibiotica op de plaats van infectie te vergroten. De hoofdstukken 2, 3 en 4 beschrijven onderzoek naar hoe we door middel van effluxpompremmers de activiteit en effectiviteit van verschillende antibiotica hebben geprobeerd te optimaliseren. Dit kan bijvoorbeeld bereikt worden door effluxpompen te blokkeren. Deze effluxpompen bestaan uit eiwitcomplexen in de celwand van bacteriën en kunnen allerlei stoffen, waaronder antibiotica, de bacterie uit pompen. Door deze effluxpompen te blokkeren kunnen antibiotica niet meer, of in mindere mate, de bacterie of cel uit gepompt worden. Ook kan met antibiotica worden gezorgd dat de celwand van de bacterie wordt verzwakt, waardoor deze beter doorlaatbaar wordt en andere antibiotica makkelijker de bacterie in kunnen.

Een andere manier om de behandeling van TBC te optimaliseren is het gebruik van nieuwe antibiotica. Na decennia van relatieve stilte op het gebied van nieuwe antibiotica voor TBC, zijn er nu verschillende nieuwe antibiotica beschikbaar en zijn er aantal in ontwikkeling. Voordat deze nieuwe stoffen kunnen worden ingezet als antibiotica worden zeer uitgebreide testen gedaan, maar deze preklinische fase duurt jaren. Om dit proces te optimaliseren en te versnellen zijn wereldwijd verschillende onderzoek consortia, waaronder PreDiCT-TB, bezig om methoden te ontwikkelen om zo in een vroege fase van antibiotica-ontwikkeling de meest veelbelovende stoffen te identificeren en verder te ontwikkelen. Een deel van het werk beschreven in dit proefschrift (hoofdstuk 8 en 9) maakt deel uit van PreDiCT-TB. In hoofdstuk 8 hebben we in ons muis TBC-model met een klinische *Mycobacterium tuberculosis* stam van het Beijing genotype de bacteriële activiteit en steriliserende capaciteit van verschillende antibiotica tegen TB bepaald. De data als resultaat van deze experimenten wordt in wiskundige modellen gebruikt om de voorspel-

lende waarde van de verschillende preklinische modellen te berekenen. In hoofdstuk 9 hebben we drie verschillende methoden onderling vergeleken die kunnen worden gebruikt voor de monitoring van de hoeveelheid mycobacteriën in de long. Dit met als doel het onderzoeken of één van deze methoden in staat is een subpopulatie van mycobacteriën aan te tonen die niet groeit op vaste voedingsbodems. De drie gebruikte methoden zijn 'Colony Forming Units (CFU) counting' in een longhomogenaat op vaste voedingsbodems, de 'Time to Positivity' (TTP) assay vanuit een longhomogenaat in vloeibaar medium en de 'Molecular Bacterial Load' (MBL) assay voor het bepalen van de hoeveelheid mycobacterieel ribosomaal RNA in een longhomogenaat.

Resultaten

Het therapeutisch potentieel van het combineren van effluxpomp-remmers met de standaard TB-behandeling

In **hoofdstuk 2** hebben we de activiteit van thioridazine tegen *Mycobacterium tuberculosis* onderzocht, allereerst alleen en vervolgens samen met de isoniazide-rifampicine-pyrazinamide (HRZ) combinatie *in vitro*. Vervolgens hebben we thioridazine ook toegevoegd aan de HRZ-combinatie in ons muis TB-model. De rationale achter het gebruik van thioridazine is het feit dat thioridazine in verschillende literatuur is beschreven als potente effluxpomp-remmer, zowel *in vitro* als *in vivo* als daadwerkelijk in TB-patiënten. Onze resultaten van de *in vitro* experimenten zijn veelbelovend. Thioridazine heeft van zichzelf activiteit tegen *Mycobacterium tuberculosis* en de combinatie van isoniazide met thioridazine werkt synergistisch ten opzichte van de activiteit van beide middelen los. Dit effect van thioridazine zagen we ook als het werd toegevoegd aan rifampicine. De combinatie van isoniazide met thioridazine zorgde ook voor de eliminatie van isoniazide-resistente mycobacteriën die worden gevonden na blootstelling aan isoniazide alleen. De toevoeging van thioridazine aan de HRZ-combinatie in het muis TB-model was minder succesvol, de beoogde therapieduur verkorting werd niet behaald, dit in contrast met sommige andere studies. Vanwege toxische bijwerking was de maximum toelaatbare dosis in onze muizen lager dan in eerdere publicaties waarbij thioridazine is gebruikt in muizen. Ook in een cavia TB-model was toxiciteit een probleem en is een zeer beperkte bactericide werking gerapporteerd. Concluderend zijn er dus tegenstrijdige resultaten gepubliceerd en moeten we ons afvragen of het nog steeds zinvol is om onderzoek te doen naar thioridazine als middel dat de activiteit en effectiviteit kan versterken van de HRZ-combinatie.

In **hoofdstuk 3** hebben we SILA-421, een organosilicon, gebruikt om de activiteit en effectiviteit te versterken van isoniazide of rifampicine *in vitro*, of de HRZ-combinatie *in vivo* in een vergelijkbare opzet als beschreven in hoofdstuk 2. Ook SILA-421 heeft een anti-mycobacteriële werking en werkt synergistisch in combinatie met isoniazide. Daarnaast is SILA-421 ook actief tegen rifampicine-resistente *Mycobacterium tuberculosis*, in

combinatie met hoge concentraties rifampicine is volledige eliminatie van deze rifampicine-resistente mycobacteriën mogelijk. Vergelijkbaar met de resultaten met thioridazine, leidt ook toevoeging van SILA-421 aan de HRZ-combinatie in ons muis TB-model niet tot een succesvolle verkorting van de behandelduur tot drie maanden.

Een nieuwe behandeling van multidrug-resistente tuberculose gebaseerd op moxifloxacin en linezolid

In **hoofdstuk 4** hebben we, met als doel een nieuwe combinatie te maken van antibiotica voor de behandeling van MDR-TBC, eerst de activiteit van moxifloxacin en linezolid tegen *Mycobacterium tuberculosis* onderzocht. Vervolgens hebben we onderzocht of we met beide middelen ook een combinatie konden maken die effectief is tegen *Mycobacterium tuberculosis*. Daarnaast is van twee effluxpomp-remmers (verapamil en timcodar) en twee middelen die de mycobacteriële celwand destabiliseren (colistine en SQ109) de activiteit bepaald, om vervolgens deze middelen toe te voegen aan de moxifloxacin-linezolid combinatie met als doel deze combinatie te versterken door middel van remming van effluxpompen of celwand destabilisatie. Zowel moxifloxacin als linezolid is in staat tot het afdoden van *Mycobacterium tuberculosis* in concentraties die ook haalbaar zijn in patiënten, en ook een combinatie van beide antibiotica is mogelijk. Zowel verapamil als timcodar als colistine heeft als middel op zichzelf weinig activiteit tegen *Mycobacterium tuberculosis,* dit in contrast met SQ109 wat een heel duidelijke dosis-respons laat zien en zeer effectief is in het afdoden van *Mycobacterium tuberculosis.* Als we vervolgens deze vier middelen één voor één toevoegen aan de moxifloxacin-linezolid combinatie, kunnen we concluderen dat zowel verapamil als timcodar geen toegevoegde waarde hebben in deze combinatie. De toevoeging van SQ109 leidt tot antagonisme en dus een verminderde werking van de combinatie. Toevoeging van colistine zorgt wel voor een verbetering van de activiteit, maar deze verbetering voldeed nog niet aan onze eisen om het daadwerkelijk synergistisch te noemen. Vervolgonderzoek zal moeten uitwijzen of de *in vitro* gevonden resultaten zijn te vertalen naar *in vivo* experimenten en mogelijk uiteindelijk ook naar patiënten.

Om in ons muis TB-model een humaan equivalente blootstelling van moxifloxacin en linezolid te gebruiken, hebben we in **hoofdstuk 5 en 6** de farmacokinetiek bepaald van beide antibiotica om de dosis te bepalen die resulteert in de humaan equivalente blootstelling. Ook is het gebruikt van 'Dried Bloot Spot' (DBS)-analyse gevalideerd voor het bepalen van moxifloxacin en linezolid concentraties in muizenbloed. De DBS methode wordt in de praktijk in patiënten gebruikt om in bijvoorbeeld een druppel bloed verkregen na een vingerprik de concentraties van antibiotica te bepalen. In ons muismodel kan het gebruik van DBS-analyse het gebruik van muizen voor farmacokinetiek onderzoek reduceren, omdat we door middel van DBS-analyse de antibiotica concentraties kunnen bepalen in een klein sample volume. Hierdoor kunnen we meerdere monsters uit één muis nemen in plaats van één monster per muis per tijdsmoment. Op basis van de resultaten beschreven in hoofdstuk

5 en 6 kunnen we concluderen dat DBS-analyse geschikt is om moxifloxacin en linezolid concentraties te bepalen in bloed en te vertalen naar plasma concentraties, en dat we deze techniek kunnen gebruiken in ons muis TB-model. Voor gebruik van moxifloxacin in ons muis TB-model resulteert een dosis van 200 mg/kg in een blootstelling die de blootstelling in patiënten benadert. De daarbij behorende C_{max} is wel hoger dan gevonden in patiënten, een aanvullende studie kan uitwijzen of een dosis van 200 mg/kg gesplitst in 2x 100 mg/kg resulteert in een gelijke blootstelling met een lagere en humaan vergelijkbare C_{max}. Een linezolid dosis van 50 mg/kg resulteert in een blootstelling die tussen de blootstelling ligt bij het gebruik van 300 mg/kg en 600 mg/kg dosis in patiënten en is geschikt om te gebruiken in ons muis TB-model.

Karakterisatie van het transcriptieprofiel van *Mycobacterium tuberculosis* na rifampicine blootstelling

In **hoofdstuk 7** hebben we geprobeerd meer inzicht te verkrijgen in de verandering in genexpressie nadat *Mycobacterium tuberculosis* is blootgesteld aan rifampicine. We hebben onderzocht of er in het bijzonder verschillen in genexpressie zijn in aan efflux gerelateerde genen, na blootstelling aan rifampicine tussen een rifampicine-gevoelige stam en rifampicine-resistente stam. Dit onderzoek hebben we uitgevoerd met behulp van microarray analyse. Op basis van de resultaten beschreven in hoofdstuk 7 kunnen we concluderen dat er verschil is in respons tussen de rifampicine-gevoelige stam en rifampicine-resistente stam. In de rifampicine-resistente stam is er bij een aantal genenclusters een significant verhoogde expressie, waarbij twee genenclusters opvallen omdat ze coderen voor membraam eiwitten. Van één van deze clusters (rv0678, *mmpS5* en *mmpL5*) is recent aangetoond dat een mutatie in rv0678 leidt tot een verhoogde expressie van *mmpS5* en *mmpL5* met als gevolg dat de stam deels ongevoelig werd voor clofazimine en bedaquiline. We kunnen op basis van de resultaten en de literatuur concluderen dat dit cluster van genen betrokken is bij efflux. De functie van de andere genen en clusters van genen met een verhoogde expressie is nog onduidelijk, hiervoor is meer onderzoek vereist.

Evaluatie en optimalisatie van ons muis TBC model als preklinisch model

De resultaten beschreven in **hoofdstuk 8 en 9** zijn onderdeel van het PreDiCT-TB project dat, zoals eerder beschreven, tot doel heeft om een de preklinische fase van antibiotica tegen TBC te optimaliseren. In dat kader hebben we in **hoofdstuk 8** in ons muis TB-model de activiteit bepaald van de conventionele TBC antibiotica tegen een *Mycobacterium tuberculosis* stam behorend tot het Beijing genotype. Ook hebben we de activiteit en effectiviteit van tien combinaties van antibiotica onderzocht. De gegenereerde data wordt gebruikt in mathematische modellen die door middel van wiskundige formules de voorspellende waarde van de verschillende experimenten proberen te vergroten. Los van het bovenstaande vonden we dat van alle antibiotica rifampicine de sterkste anti-

mycobacteriële activiteit heeft na vier weken blootstelling. In de effectiviteitsstudie vonden we dat alleen de antibioticacombinaties die rifampicine bevatten na 24 weken behandeling resulteerde in kweek-negatieve longen. In aanvulling daarop, deze rifampicine combinaties laten na een twaalf weken durende periode zonder behandeling veel minder gevallen van 'relapse' van TBC zien vergeleken met de niet rifampicine combinaties. Op basis hiervan kunnen we concluderen dat rifampicine inderdaad, zoals al eerder was beschreven, het belangrijkste antibioticum is om TBC te behandelen. Verder kunnen we op basis van deze data concluderen dat de 'early bactericidal activity' (EBA 0-14) de uitkomst van de effectiviteitsexperimenten niet goed kan voorspellen en dat kweek-negatieve organen na behandeling niet genoeg voorspellende waarde hebben voor de incidentie van 'relapse' van TBC. Een mogelijke oorzaak hiervan kan zijn dat de methode waarmee we de daling van het aantal mycobacteriën monitoren in *in vitro* experimenten en *in vivo* experimenten niet de meest optimale methode is. Het is eerder aangetoond in de literatuur dat vloeibaar groeimedium in staat is een populatie van mycobacteriën te detecteren die niet of nauwelijks groeien op de vaste groeimedia. PreDiCT-TB heeft als subdoel het ontwikkelen en testen van nieuwe technieken om de preklinische fase van TBC-antibiotica te optimaliseren. Als resultaat hiervan is de MBL assay ontwikkeld. Deze MBL assay detecteert de aanwezigheid van 16S ribosomaal RNA van *Mycobacterium tuberculosis*, deze assay is daarom niet afhankelijk van groeicapaciteit van de bacteriën. Dit in tegenstelling tot CFU counting -met andere woorden het tellen van kolonies op een vaste voedingsbodem- en de TTP assay waarbij de tijd tot groei in vloeibaar groeimedium een maat is voor de hoeveel aanwezige mycobacteriën. In **hoofdstuk 9** hebben we deze drie methoden met elkaar vergeleken in ons muis TB-model. We hebben gevonden dat we met alle drie de methoden vrij vergelijkbaar de hoeveelheid mycobacteriën in de longen van muizen konden bepalen tijdens vier verschillende soorten therapieën. Maar aan het einde van de therapieduur waren CFU counting en de TTP assay negatief bij drie van de vier TBC-behandelingen terwijl de MBL assay nog steeds positief was. Op basis hiervan concluderen wij dat CFU counting en de TTP assay de hoeveelheid bacteriën onderschatten, en dat de MBL assay een populatie van bacteriën kan aantonen die niet meer in staat zijn om te groeien maar wel aanwezig zijn. En juist deze persisterende populatie van mycobacteriën is van groot belang, omdat deze voor een groot deel het succes bepalen van een behandeling van TBC.

We hebben eerder gerefereerd aan de 'END TB STRATEGY' van de WHO en de ambitieuze doelen die daarbij zijn gesteld. We hopen dan ook dat dit onderzoek een stukje is van de enorme TBC-puzzel en bijdraagt aan meer kennis van de uitdagingen en oplossingen die kunnen leiden tot het einde van TBC.

Dankwoord

Na pakweg 170.000 kilometer, 10.000 liter brandstof, zeven sets banden, een handjevol flitsfoto's en twee motoren ligt er dan eindelijk een boekje op tafel. Gedurende de hele rit om tot dit boekje te komen, zijn er aantal mensen betrokken geweest. Hen wil ik graag bedanken voor hun bijdrage aan dit resultaat.

Irma: Allereerst dank voor het vertrouwen in mij om dit stuk van het TB-onderzoek te mogen uitvoeren. Het is al door veel mensen benoemd, maar je grondige en nauwkeurige manier van werken is van onschatbare waarde. Dit leidt er wel toe dat 'even met Irma overleggen' eigenlijk niet mogelijk is, je bent voor je het weet vijftien minuten verder. Door de jaren heen heb ik enorm veel van je geleerd, al is het maar zoiets kleins als de grafiek lay-out, als je zorgt dat deze Irma-proof is dan ben je al een flink eind op weg met je onderzoek. Dank voor alle revisies van manuscripten, presentaties en andere stukken. Het deed soms een beetje pijn als ik door alle handgeschreven correcties en geel gemarkeerde aanpassingen mijn eigen tekst niet meer terug vond, maar het is er uiteindelijk zoveel beter van geworden.

Jurriaan: Jij en Irma hebben me door de jaren heen opgeleid van jonge analist naar de onderzoeker die ik nu ben, met als resultaat dit boekje. Dank voor je begeleiding. We werken al tien jaar samen en dat is niet voor niets. Ik heb het altijd als bijzonder prettig ervaren om op jouw relaxte en informele manier te overleggen over hoe we de dierstudies eens gingen aanpakken, of even op de achterkant van een oud A4'tje lekker een paar goede experimenten te bedenken en uit te schrijven (die dan uiteindelijk een paar maanden werk waren). Dank daarvoor. Dank ook voor alle lol en gezelligheid op reis naar een meeting/congres. Lekker in het zonnetje op Schiphol een uurtje voor de vlucht alvast een Juupje drinken. Of zodra we aankwamen, even naar een supermarkt om wat lokaal bier, pure chocolade, chips en misschien iets gezonds te kopen. En bij dat lokale bier zit af en toe een hele goede, maar ik kan me de smaak van een ranzig 'Murphy's stout beer' met een pingpongbal erin helaas nog goed herinneren. Het was een bijzonder mooie en waardevolle tijd.

Hubert en Johan: Het is een ietwat ongebruikelijke situatie dat de promotor (of zelfs twee) pas in het laatste jaar van het promotieonderzoek worden benoemd. Desondanks ook jullie bedankt voor jullie rol in deze promotie, het corrigeren van de laatste stukken van het boekje en het in goede banen leiden van de papierwinkel rondom de promotie.

Aart, Bas, Corné, Heleen, Irma, Jurriaan, Marian en Sanne: Dank voor al het werk en alle hulp en gezelligheid in het vitrolab en in het muizenlab. We hebben daar de afgelopen jaren vele uren doorgebracht en vele honderden muizen geïnfecteerd en behandeld. Tijdens het muizen infecteren werd er straf doorgewerkt volgens een vier of vijf minuten-schema, maar het was altijd erg gezellig. Ook het geven van de therapie duurde soms eindeloos, maar ook dan was het allemaal ontspannen. Een beetje ouwehoeren over vrouwen, auto's, kinderen, werk en wat al niet meer en tussendoor werd er dan ook nog even met twee loodzware isolatoren 'gedanst'. Het was een bijzondere en leuke tijd, dank voor alles. Dank

ook voor alle hulp met het uitvoeren van de vele killingcurves, het waren er echt heel veel. En dan moesten er na die curves ook nog eens eindeloze stapels platen met bacteriën geteld worden. De vraag blijft nog steeds: wat is de juiste manier van het stickeren en stapelen van de platen ;-)? Marian in het bijzonder bedankt, ik heb veel van je geleerd en het heeft me een betere analist en onderzoeker gemaakt.

Carla: Al die muisexperimenten en killingcurves konden niet worden uitgevoerd zonder die duizenden agarplaten en hectoliters broth, die altijd op een vrij flexibele manier door de keuken werden geleverd. 'Kun jij voor overmorgen wat agarplaten maken?' 'Ja hoor'. 'Kun je misschien acht liter (vierhonderd platen) maken?' Het was altijd mogelijk, dank voor je inzet en flexibiliteit.

Bewoners Na-902: Een kantoortuin zonder deur of functionele muren voor vijftien enthousiaste onderzoekers lijkt op papier misschien een geniaal plan, maar het blijkt dat er in de praktijk toch wel een paar haken en ogen aan zitten. Ik denk dat we kunnen concluderen dat het vooral tot gevolg heeft dat het er vrij druk maar gezellig is en dat dat weinig bevorderend werkt voor een goede concentratie. Een goede koptelefoon behoort hierdoor eigenlijk tot de standaarduitrusting van een bewoner van Na-902. Maar vanwege de informele sfeer zijn korte overleg-lijntjes goed mogelijk, en ook hobbybeest-overstijgend overleg is op deze manier erg praktisch. Maar uiteindelijk is het er ook altijd super gezellig, wordt er soms werkelijk van alles gedeeld en zijn er echt goede gesprekken. Dank voor alles.

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PhD portfolio

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PHD TRAINING

Conferences

2011

– Scientific spring meeting of the Dutch society for Medical Microbiology (NVMM), Papendal, the Netherlands

2012

– Scientific spring meeting of the Dutch society for Medical Microbiology (NVMM), Papendal, the Netherlands (poster presentation)
– 5th International workshop on Clinical Pharmacology of TB Drugs, San Francisco, USA (oral presentation)
– Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), San Francisco, USA (poster presentation)

2013

– Scientific spring meeting of the Dutch society for Medical Microbiology (NVMM), Papendal, the Netherlands (poster presentation)

2014

– Nederlandstalige Tuberculose Diagnostiek Dagen (NTTD), Rotterdam, the Netherlands (oral presentation)

2016

– Scientific spring meeting of the Dutch society for Medical Microbiology (NVMM), Papendal, the Netherlands (poster presentation)
– European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Amsterdam, the Netherlands (poster presentation)
– 9th International workshop on Clinical Pharmacology of TB Drugs, Liverpool, UK (poster presentation)

– The 47th Union World Conference on Lung Health, Liverpool, UK (oral presentation)

2017

– Scientific spring meeting of the Dutch society for Medical Microbiology (NVMM), Papendal, the Netherlands (poster presentation)

Scientific Meetings

2012

– Departmental research day, Rotterdam, the Netherlands (oral presentation)

2012-2017

– PreDiCT-TB consortium meeting (oral presentations)
– ZonMw project meetings (oral presentations)

2012-2017

– Departmental Journal clubs
– Departmental Research meetings

Scientific award

– 3th Poster price NVMM 2012

Specific Courses

– Determinants of drug response and variability in early clinical development (2013), Tres Cantos, Spain
– Scientific integrity and ethics (ErasmusMC)
– Workshop Presenting skills for junior researchers (ErasmusMC)

Teaching

2007-2017

– Supervision of 2nd year medical students, "Vaardigheidsonderwijs Infectieziekten"

2014-2016

– Lecturer in the summercourse of the Research Master "Infection and Immunity" of the Erasmus University Rotterdam

CV – about the author

Gerjo de Knegt was born 1984 in Gouda, the Netherlands. He graduated from the Hogeschool Utrecht in 2006 with a Bachelor of Applied Sciences, specializing in Life Sciences. The same year Gerjo started as a technician in the Tuberculosis Research Group under the direct supervision of doctor Irma Bakker-Woudenberg and doctor Jurriaan de Steenwinkel. His PhD-work started in 2012 within the same group.