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Drug-Resistance in *M. tuberculosis* and the Characterisation of a New Anti-tuberculosis Drug Candidate

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Stockholm 2009

This picture was taken by Dimitris during our course in Infections in the Tropics in Dec, 2005, during which we had the privilege of travelling around Ghana and visiting all units of the Health Care System. This TB-nurse is preparing the daily doses for the TB patients according to DOTS-treatment guidelines. The picture has followed me through my PhD-studies and illustrates multiple aspects of the global fight against TB – the need for new drugs that will simplify & shorten treatment, that are simple to distribute and that are affordable. It further reminds me of the fabulous experience we had in Ghana, where we were warmly welcomed and got to discover this beautiful country. Thank you Sven Britton for a wonderful trip and course!

Published by Karolinska Institutet. Printed by Larserics Digital Print AB.

ISBN 978-91-7409-473-2

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ABSTRACT

The occurrence of multi-drug resistant (MDR) tuberculosis (TB) has been increasing to alarming levels globally, and the spread of extensively drug-resistant (XDR) *M. tuberculosis* is threatening the control of tuberculosis. Understanding the molecular mechanisms behind resistance is an important tool in minimising and preventing its spread, as faster diagnostic methods can de developed and treatment guidelines be optimised. There is also an urgent need to find and develop effective drugs that simplify and shorten the existing treatment regimen, as well as being effective against all forms of TB. The current thesis presents the characterisation of *in vitro*-resistance to rifampicin (RIF), as well as the pre-clinical characterisation of a promising new anti-TB drug candidate.

In **Paper I** we investigated RIF-resistance mutations within the *rpoB* gene of *in vitro*-selected resistant mutants. The array and frequency of mutations, as well as the resistance-levels found in these mutants were similar to those reported for RIF-resistant clinical isolates. Furthermore, we saw that mutants of the Beijing genotype, a family of strains known to be spreading globally and commonly associated with MDR, did not portray a different span of resistance mutations or resistance-level.

Papers II-IV present the pre-clinical characterisation of R207910. R207910 is a new anti-TB drug candidate identified for its high Mycobacterial specificity. This Diarylquinoline, a new class of compounds, was shown to have a strong inhibitory effect on both drug-susceptible and MDR *M. tuberculosis* (Minimum Inhibitory Concentration 0.03μg/ml). In combination with the standard combinatorial TB-treatment regimen, R207910 further had an equal, if not stronger, bactericidal activity in mice than the standard regimen alone. *In vitro* studies showed that resistance occurs through mutations in the bacilli's ATP synthase indicating that the compound targets a unique site; the bacilli's energy-producing ATP synthase. Resistance to R207910 occurred at a relatively slow rate (approx 10^{-8} mutations per cell generation) and this spontaneous acquisition of resistance was prevented at an R207910-concentration of 3 μg/ml, a level deemed attainable within humans without causing adverse effects. R207910 shows the potential of shortening and simplifying the treatment of both DS and MDR-TB. Furthermore, having characterised the dynamics of resistance development to the compound before it reaches clinical use, treatment doses and guidelines can be established that will cure TB-patients as well as prolong, and hopefully prevent, the emergence of clinical R207910-resistance.

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II. Andries, K., Verhasselt, P., Guillemont, J., Gohlmann, H.W., Neefs, J.M., Winkler, H., Van Gestel, J., Timmerman, P., Zhu, M., Lee, E., Williams, P., de Chaffoy, D., **Huitric, E.**, Hoffner, S., Cambau, E., Truffot-Pernot, C., Lounis, N., Jarlier, V.
A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*.
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III. **Huitric, E.**, Verhasselt, P., Andries, K., Hoffner, S.E.
In vitro antimycobacterial spectrum of a diarylquinoline ATP synthase inhibitor.
Antimicrobial Agents and Chemotherapy. 2007. **51**: 4202-4.

IV. **Huitric, E**., Verhasselt, P., Andries, K., Hoffner, S., Andersson, D.
Mycobacterium tuberculosis in vitro resistance-development to a Diarylquinoline ATP synthase inhibitor: Can an optimal dose be identified to prevent clinical resistance?
Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Disease Syndrome
Ala	Alanine
AMK	Amikacin
Asp	Aspartate / Aspartic Acid
BCG	Bacille Calmette-Guérin
cfu	Colony Forming Unit
DARQ	Diarylquinoline
DOTS	Directly Observed Therapy – Short Course
DR	Drug-Resistant
DS	Drug-Susceptible
DST	Drug-Susceptibility Testing
EMB	Ethambutol
ETH	Ethionamide
Glu	Glutamate / Glutamic Acid
His	Histidine
HIV	Humman Immunodeficiency Virus
Ile	Isoleucine
INH	Isoniazid
IS-RFLP	Insertion Sequence-Restriction Fragment Polymorphism
KAN	Kanamycin
Leu	Leucine
MAC	*M. avium* Complex
MDR	Multidrug-Resistant
MED	Minimal Effective Dose
Met	Methionine
MIC	Minimum Inhibitory Concentration
MPC	Mutant Preventive Concentration
mut/Cgen	mutation per cell generation
NTM	Non-Tuberculous Mycobacteria
PAS	Para-Aminosalicylic Acid
Pro	Proline
PZA	Pyrazinamide
RIF	Rifampicin
RRDR	RIF-Resistance Determining Region
Ser	Serine
STR	Streptomycin
TB	Tuberculosis
Tyr	Tyrosine
Val	Valine
WHO	World Health Organisation
XDR	Extensively Drug-Resistant

1 INTRODUCTION

1.1 FROM THE ANCIENT EGYPTIANS TO THE 21ST CENTURY

Phthisis, Consumption, Pott's disease, The Great White Plague and Tuberculosis are all names for a disease that has long afflicted humans. *Tuber* is the Latin name for all forms of degenerative protuberances or tubercles and *tuberculosis* was the term for diseases causing such tubercles (104). Evidence of tuberculosis (TB) in humans has been found as far back as the ancient Egyptian and Aztec times, causing skeletal abnormalities, or "Pott's" deformities. In ancient Greece, Phthisis (meaning wasting away), was described by Hippocrates and was a widely spread disease already at that time. In Western Europe and Northern America, many recall TB as a disease that afflicted ones grandparents but that is now extinct and many are surprised to hear that "TB is coming back". Whilst this view might be the reality for some countries, where the control of TB has been successful and transmission within the population is minimal, this is far from the global reality.

In Western Europe and Northern America, TB reached its peak during the 18th and 19th centuries, during the Age of Industrialisation. During this time there was a mass movement into cities, where people lived and worked in harsh, unhygienic conditions – the perfect setting for the spread of infectious diseases. The Industrial Revolution is known for its over-crowded factories, where people worked in poorly aired workplaces, were poorly paid and lived in cramped houses also with bad aeration. People were undernourished and had little-to-no access to health care. TB had a gained stronghold in the population – it had all it needed to maintain itself in the human population (104, 128).

Many factors have contributed to the drastic decrease in TB disease in these settings. The need for public health systems was recognised, working and living conditions improved as did people's socio-economic situation. Furthermore, the causative agent for TB was finally identified, and, TB was recognised as a contagious disease. This not only led to the ability of preventing disease spread, the continued scientific research at the end of the 19th century and first half of the 20th led to the development of diagnostic methods (acid fast staining and chest X-ray), the development of a vaccine (BCG; Bacille Calmette-Guérin) and lastly, the discovery of anti-tubercular compounds for the treatment of the disease.

Despite the successes in understanding the pathogenicity of TB and finding effective treatment, the epidemic is still at its peak in many parts of our globe, with the most afflicted areas being Sub-Saharan Africa and South East Asia (152). In 1993, the World Health Organisation (WHO) declared TB a global emergency estimating that approximately one third of the world's population was infected with tubercle bacilli. It is known that among these 2 billion infected citizens, active TB disease will develop in 10 % of cases, with 3 % developing the disease within the first three years of infection. In 2007 alone, it was estimated that 1.77 million people died from TB, an equivalent of 4800 deaths per day, amongst which a majority of deaths occurred in low-income, resource-poor countries (152).

With all our knowledge in how to prevent and cure TB, why is it still considered a global emergency? As *Murray* (97) and many others have stated, fighting TB does not only imply providing diagnosis and treatment, but, more importantly, it involves combating poverty and developing a good public health system. In the 1970's Karel Styblo and the International Union Against Tuberculosis and Lung Disease (IUATLD) presented the notion that the control of TB is possible in poor settings provided the following aspects are accounted for: political commitment; effective diagnosis of infectious patients; regular and reliable antibiotics supplies; directly observed treatment and lastly, accurate reporting of treatment results (43, 63). This strategy was the foundation for the development and instigation of the DOTS-program (Directly Observed Therapy – Short Course) by the WHO in the 1990's (63, 97, 154). Although the DOTS-program has proven to be efficient, TB control is far from becoming a reality in many countries, as the instigation of the above-mentioned aspects of the DOTS-program remains a challenge.

The fight against the TB epidemic has, in the past few decades, been further challenged with the emergence of two key obstacles. The first, a man-made phenomenon, is the emergence of resistant forms of TB, against which treatment is more difficult and at times impossible. Resistance was already observed when the first anti-TB drugs came to use (10), and has since steadily increased due to their *misuse*. The second obstacle has been the emergence and pandemic spread of the Human Immunodeficiency Virus (HIV). Like TB, the HIV-epidemic is concentrated in the Sub-Saharan countries of Africa as well as South-East Asia (150). TB is one of the first infections to afflict HIV-positive patients, and HIV-positive individuals are at a 10% risk per year of life of contracting the disease (152, 153). The HIV epidemic has thus proven to be one of the key obstacles in fighting the global TB epidemic, and as *Murray* stated, until one of the two agents in this "cursed duet" are brought to a stop, the prognosis of defeating the TB epidemic looks bleak (97).

1.2 WHAT CAUSES TUBERCULOSIS?

On March the 24th, 1882, Robert Koch stood in front of his peers at the Berlin Physiology Society to present his findings "Concerning TB" ("Über Tuberkulose") (118). Koch had discovered the causative agent of TB, *Mycobacterium tuberculosis*. Applying what was to become the "Koch's postulate", Koch had isolated *M. tuberculosis* from infected individuals and visualised them microscopically using acid-fast staining. Further, he managed to culture *M. tuberculosis* and through the inoculation of animals show that the bacteria were indeed the cause of TB. This scientific breakthrough finally put an end to the myths regarding the cause of TB, and began an era when the disease might finally be combated and eradicated.

Mycobacterium tuberculosis is a slow-growing, acid-fast, non-motile bacillus belonging to the bacterial genus Mycobacterium, a genus that differs substantially from other bacteria due to their exceptionally thick cell wall and high genomic guanine-cytosine content. Similar to gram-positive bacteria, Mycobacteria have a thicker peptidoglycan layer surrounding the cell membrane. In addition, anchored to the cell membrane, Mycobacteria have a thick fatty acid layer surrounding the out layer of the peptidoglycan. This layer, consisting of mycolic acids (long-chain fatty acids) with

protruding glycolipids and proteins, makes the bacteria highly hydrophobic and is the cause for this genus' unusual characteristics. Mycobacteria are highly impermeable, aggregate and/or form biofilms easily and, of clinical importance, are highly resistant to common antimicrobials and disinfectants. The time and energy required for the Mycobacteria to synthesise such a thick outer wall further explains the bacilli's slow growth dynamics, taking up to 28 days to form visible colonies (depending on the species).

M. tuberculosis belongs to the complex of Mycobacteria that cause TB in either humans or animals, the *M. tuberculosis* Complex, and is itself the leading cause of TB in humans. The *M. tuberculosis* Complex consists of *M. tuberculosis*, *M. africanum*, *M. canetti*, *M. bovis*, *M. microti*, *M. caprae*, and *M. pinnipdedii*, the latter two often being considered *M. bovis* subspecies. *M. tuberculosis*, *M. africanum*, and *M. canetti* are typically human pathogens while *M. bovis* and *M. microti* are considered animal pathogens, but are also able to infect humans. *M. microti* was previously considered a strict animal pathogen, but has since been identified in both immune-competent and immune-compromised TB patients (19, 100, 158). *M. caprae* and *M. pinnipedii* were originally identified in goats and sea lions as well as fur seals, respectively, and are commonly considered to cause disease in wild and domesticated animals.

M. bovis is the leading cause of TB in cattle and prior to milk pasteurisation, it was a significant cause of human TB worldwide. In many low-resource countries bovine TB remains endemic and thus remains an important zoonotic source of infection (104). There has been much debate regarding the origin of the Complex's species as it was previously thought that *M. tuberculosis* had evolved from *M. bovis* (27, 130, 134). However, genomic analysis of the *M. tuberculosis* Complex species has shown that *M. bovis* has a smaller genome, suggesting that it is evolutionarily younger (29). Furthermore, genome analysis has suggested that the *M. tuberculosis* Complex species all originated from a common ancestor (29).

Before venturing further into the causes and development of human TB, the main focus of this work, the genus to which the *M. tuberculosis* Complex belongs must be considered, the Mycobacterium genus (7).

1.3 NON-TUBERCULOUS MYCOBACTERIA

Apart from the *M. tuberculosis* Complex, the genus Mycobacterium consists of more than 125 species (7), of which approximately 50 are known to be pathogenic to humans (53, 145). The extensive and ever-growing list of species has earned many names, a few being, *Atypical* Mycobacteria, *Environmental* Mycobacteria, Mycobacterium Other Than Tuberculosis (MOTT), fast-growing Mycobacteria, slow-growing Mycobacteria and the title commonly used today, Non-tuberculous Mycobacteria.

Non-tuberculous Mycobacteria (NTM) share the common trait that they cause neither TB nor leprosy (145). Furthermore, the 50 or so known pathogenic species are considered to be environmental opportunistic pathogens, as no human-to-human or animal-to-human infections have been seen (53). NTMs are commonly found in soil and water (both drinking and natural) and differ in their geographic prevalence.

Depending on the species, as well as the site of infection, NTMs can cause disease in different parts of the human body, causing pulmonary disease, lymphadenitis, skin and soft tissue infections, foreign-object infections (i.e. implantations), central-venous catheter infections, skeletal disease and generalised disseminated disease (53, 145).

Being less virulent than their fellow *M. tuberculosis* Complex species, NTM seldom cause infections in healthy individuals (145), and are typically a threat to immune-compromised individuals (19, 53). The emergence of the HIV-epidemic led to a substantial increase in observed NTM infections with *M. avium* Complex (MAC) infections (see below) being a most common cause of disseminated disease in these individuals (21, 77, 145). Like the *M. tuberculosis* Complex, NTMs are typically very difficult to treat showing high resistance to antimicrobial agents as well as disinfectants.

As mentioned, NTMs can be sub-divided into slow-growers and fast growers, with the former comprising the majority of species. Slow-growing NTM take between 7-28 days to form visible growth and are characterised by their high resistance to common anti-microbial agents (44). The most common and clinically relevant slow-growing NTMs are *M. avium* and *M. intracellulare* which belong to the MAC (21). MAC-infections are a common cause of pulmonary disease in immune-compromised patients, being one of the lead opportunistic infections in AIDS patients, and is also common in patients with chronic pulmonary disease, such as cystic fibrosis (4, 21). MAC infections are difficult to treat and the current treatment guidelines are the use a four-drug regimen consisting of clarithromycin, rifabutin, ethambutol and an aminoglycoside (4).

Other common slow-growing pathogenic NTMs are *M. kansasii, M. malmoense,* and *M. marinum*, with the prevalence of the species varying in different geographical areas (44, 145). *M. kansasii* is the second most common cause of NTM-associated lung disease in the United States whilst *M. malmoense* is the second most common in Scandinavia (145). *M. marinum* is found in water and is a common cause of cutaneous infections in aquarium keepers. An emerging NTM species causing extreme cutaneous infections that can be debilitating, known as Buruli Ulcer, is *M. ulcerans* (140). This NTM is found in West Africa, Australia, South-East Asia and South America in swampy, humid areas and is an extremely slow-growing NTM. *M. ulcerans* is an exceptional NTM as it produces plasmid-encoded toxins, called mycolactones, that play a key role in the severe skin ulcers during infection (47, 98).

Compared to the slow-growers, rapid-growing NTMs form visible colonies in a much shorter time, between 3-7 days, but are still slower than most bacteria (30). The most common rapid-growing pathogenic NTMs belong to two groups; the *M. foruitum* group, with *M. fortuitum* being the main pathogen and the *M. chelonae-abcessus* group. Members of the *M. smegmatis* group are also rapid growing NTM, however these are not considered pathogenic (30, 44). Contrary to slow-growing NTMs, first-line anti-TB drugs are often ineffective against fast growers, and instead, common antimicrobials such as fluoroquinolones and aminoglycosides are more effective (30). Furthermore, susceptibility to specific antimicrobials have been seen to differ depending on the site of infection, as well as to differ between isolates of the same species (30, 145). Whilst *M. fortuitum* infections, commonly causing skin infections, are considered the easiest

fast-growers to treat, *M. absecssus*, typically causing lung disease and *M. chelonae*, causing disseminated disease, are two of the most antibiotic resistant fast-growers. Although these two species belong to the same group, they differ substantially in their susceptibility to drugs and it is thus vital to differ between them to assure adequate treatment regimens (30).

Due to the variation in drug-susceptibility of different NTM species, as well as the variation of susceptibility within the same species, identifying the exact NTM species and running drug-susceptibility testing on patient isolates is vital. Acid-fast staining will identify the bacilli as Mycobacteria and molecular techniques for species identification have been developed (4, 44, 53, 145). However, *in vitro* drug-susceptibility testing of NTM is known to correlate badly with the *in vivo*/treatment success for many anti-microbials, further making NTM-treatment difficult (4, 145).

Mycobacterial infections, whether they cause tuberculosis or NTM-associated infections, share the common trait of being difficult to treat. The NTM genus is vast with new species continuously being identified. Although the majority are rare and non-pathogenic, there are a number of species that cause serious, difficultly treated infections. It is therefore of utter importance to correctly identify the species causing disease so that appropriate treatment can be provided. Of further importance is the lack of effective treatment for several pathogenic NTMs, and therefore the need to find new effective antimicrobials against this ubiquitous group of pathogens.

1.4 CONTRACTING TUBERCULOSIS

As mentioned in the previous chapter, *M. tuberculosis* belongs to the *M. tuberculosis* Complex, a complex whose members can cause TB in humans and animals. For simplicity, as well as for being the focus of this thesis, *M. tuberculosis* will be referred to in the remainder of the text.

M. tuberculosis can cause disease in all organs of the human body. In children, it commonly causes severe miliary and cerebral TB however, as air-borne transmission is the lead source infection today, pulmonary TB is the most common disease-form in adults. A person is infected through the aspiration of contaminated aerosols coughed up by an infectious patient, and the bacilli travel to the pulmonary alveoli where they infect resident macrophages. The bacilli are able to remain within the macrophages' phagosomes without inducing the formation of phago-lysosomes thereby escaping immune attack. In a majority of cases, the host's immune system is able to contain this initial infection and no symptoms are observed. Instead, the bacilli wind down into a low-metabolic state and go into a dormant, non-replicating state, during which infected individuals are neither sick nor infectious (48, 104).

As mentioned, 10% of individuals infected with tubercle bacilli will develop active TB at some point during their life. This typically occurs when the immune system is compromised, be it from HIV-infection, immune-suppressive therapy, malnutrition or a known genetic deficiency in the Interferon-gamma production (70, 104). Regarding pulmonary TB (the focus of this thesis), symptoms of disease are a reflection of the host immune system's inflammatory responses in the lungs which result in tissue

destruction. Patient symptoms are persistent coughing with chest pains, and, when the disease is more advanced, a patients' cough will become mucopurulent (104). Sputum smear microscopy will visualise the acid-fast bacilli, confirming that a patient has active, contagious TB. Ideally diagnosis should be confirmed through culture of sputum samples; however this is not always feasible in resource-poor settings.

The essence of combating TB, as any other infectious disease, is assuring that transmission is prevented between people. Regarding TB, it is vital to: 1. Identify contagious individuals as soon as possible; 2. Assure proper treatment to cure the patient and prevent further transmission and 3. Identify potentially exposed contacts to a patient to provide preventive treatment and reduce their risk of developing disease later in life (28). The ideal for achieving these goals would be to have an effective vaccine, protecting people from infection. In 1908, Albert Calmette and Camille Guérin derived the BCG-vaccine and it became one of the world's most administered vaccines (97). Although the vaccine is known to give protection against disseminated forms of TB in children, the BCG vaccine has to date no known/proven protective effect against adult pulmonary TB (104). A major effort in TB research has been to find a replacement vaccine. Several candidates, based on different mechanisms are currently under development (85), however, as yet, no replacement has been found.

A second vital step in combating TB is the availability of adequate treatment for all; this is the basis of the WHO-derived DOTS-program for TB control (154). The first anti-tubercular drug, streptomycin was discovered in 1944, sixty-two years after Koch's discovery, and within only a few months, the first TB patient ever was cured using chemotherapy (57, 58). Streptomycin (STR) was quickly followed by the introduction of Para-aminosalicylic acid (PAS) (80, 81). There was at long last a cure for TB. However, it was soon noticed that these two drugs alone were inefficient as patients quickly developed resistance; TB had to be treated with a multi-drug regimen attacking the bacilli from several angles in order to cure a patient (10, 13). Despite the long knowledge of clinical resistance-development in TB, preventing this phenomenon has proven to be more difficult than expected. Drug-resistant, Multidrug-resistant and now Extensively drug-resistant tubercle bacilli (all to be discussed below) have emerged globally and are a major threat to the fight against TB.

1.5 TREATING TUBERCULOSIS

The decades following the introduction of STR and PAS were marked by the discovery of several new anti-TB drugs, and since, the WHO has developed and recommended a standardised treatment regimen based on these compounds (154). The current anti-TB drugs are divided into first- and second-line drugs, the first-line drugs being the most effective and enabling the shortest treatment time (6-8 months). According to *Mitchison*, tubercle bacilli can reside within the human host in four different populations: actively growing bacilli, bacteria in spurts of metabolism, bacteria with low metabolic activity that reside in acidic environments and lastly "dormant", non-replicating bacteria (91, 92). The different drugs are active on bacilli that are in the first three mentioned phases of growth. However, to date there is no anti-TB drug that is fully effective against dormant bacilli (91). **Figure 1** depicts the first-line and the main second-line drugs and their targets within the bacilli.

1.5.1 Treatment of Drug-Susceptible Tuberculosis

The five first-line anti-TB drugs are rifampicin (RIF), isoniazid (INH), ethambutol (EMB), pyrazimamide (PZA) and streptomycin (STR), of which RIF and INH are the most effective (154). Thioacetazone is also within this group, however due to its toxicity, its use is discouraged (154). By recommendation of the WHO, all newly identified TB cases where resistance is not suspected should be treated for a period of 6-8 months using the described first-line drugs. During the first two months, the initial phase, treatment should consist of RIF, INH, PZA and EMB. The following four to six months (six months if the patient remains smear/culture positive after the initial phase), the continuation phase, treatment should continue with a regimen of RIF and INH (154). As the drugs have different targets within the bacilli, this combinatorial treatment prevents the development of drug-resistance, provided treatment is correctly followed and completed.

RIF is a broad-spectrum, bactericidal RNA polymerase inhibitor. It targets the ß subunit of the bacilli's DNA-dependent RNA polymerase, thereby blocking transcription (90). **INH**, a cell wall synthesis inhibitor, is a prodrug that, upon activation by catalase-peroxidase produces harmful reactive oxygen species, as well as reactive organic radicals. Furthermore, the active form of INH inhibits mycolic acid synthesis by forming a ternary complex with the enoyl ACP reductase enzyme (encoded by *inhA*) and its NAD dehydrogenase II (ndh)-cofactor (156, 159).

EMB, also a cell wall synthesis inhibitor, targets arabinosyl transferase and interferes with the arabinogalactan synthesis, a key cell wall polysaccharide (136, 159). The protein synthesis inhibiting aminoglycoside, **STR**, targets the 16S rRNA and S12 ribosomal protein of the small 30S ribosomal subunit, affecting mRNA translation thereby inhibiting protein synthesis (46, 132, 159). Due to the compound's toxicity, need to be administered intravenously and commonly seen resistance, replacing STR with the membrane energy-depleting compound, **PZA**, is recommended. The prodrug, nicotinamide-analogue, PZA, is activated by the pyrazinamidase (PZase) enzyme to produce the active substance, pyrazinoic acid (POA) (123, 159, 161). POA is active in an acidic environment where its protonated form crosses the bacilli's cell membrane, thus acidifying the bacterial cytoplasm. The resulting disturbance of the cell's membrane potential collapses the proton motive force, and thereby the membrane energy metabolism (160).

All five first-line drugs are bactericidal. However, as PZA and EMB are only active under specific conditions (e.g. in acidic environments, at higher drug concentrations or lower bacterial loads), these are considered to be bacteriostatic (159). At different stages of treatment, a patient's response is assessed through sputum smear microscopy and/or culture. In cases of treatment failure, drug-resistance should be suspected and if resources permit, drug-susceptibility testing (DST) should be run (if this has not already been conducted at the time of diagnosis). Treatment can then be adapted to an effective regimen.

1.5.2 Treatment of Drug-Resistant Tuberculosis

Clinical forms of drug-resistant (DR)-TB are a man-made phenomenon resulting from the ill use of the current drugs. This has been due to the erroneous drug-prescribing by health care providers and the lack of patient compliance to treatment. Resistant TB is an increasing global threat to the fight against the disease and the increase in Multidrug-Resistant (MDR)-TB, termed as isolates resistant to at least RIF and INH, the two most effective anti-TB drugs, is a cause for increasing concern. Due to the lack of efforts in TB-drug discovery and development, the last TB drug *specifically* active against *M. tuberculosis* to have been developed was INH in 1952 (RIF, a *broad-spectrum* antimicrobial, was later discovered in the 1960's and its strong activity on *M. tuberculosis* quickly led to its introduction in TB-treatment). The treatment of MDR-TB resorts to much longer regimens (up to two years) combining second-line compounds that are often less active, more toxic, and more expensive. There are numerous second-line drugs of which some commonly used ones are; fluoroquinolones, aminoglycosides, capreomycin and ethionamide (ETH). **Figure 1** depicts these different drugs mode of action.

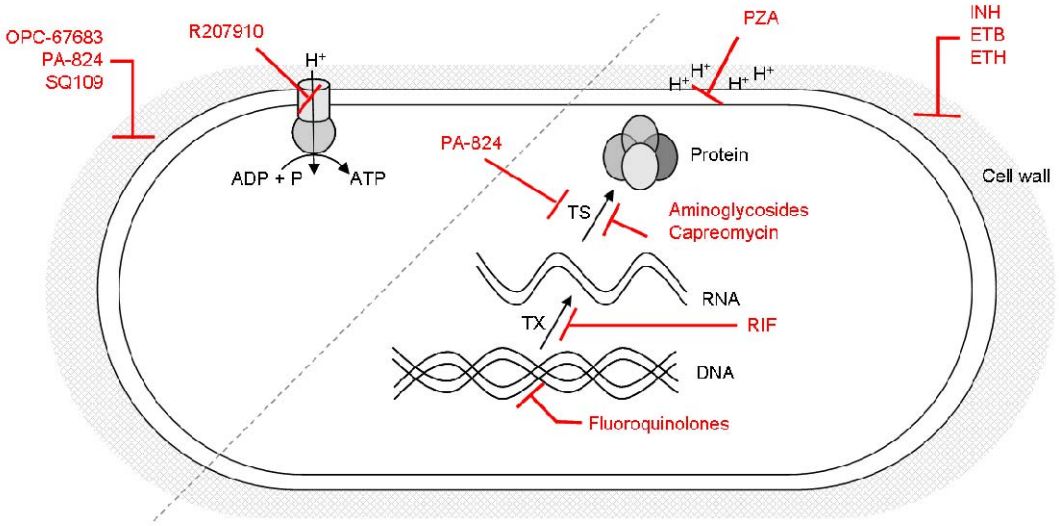


Figure 1: Mechanisms of action of first- and second-line anti-TB drugs on *M. tubersulosis* (to the right) as well as four drug-candidates under clinical development (to the left). (Adopted from *Sassetti, C & Rubin, EJ*. 2007 (122)) . TX- transcription; TS – translation.

Of the second-line drugs, the broad-spectrum, DNA synthesis inhibitors, **fluoroquinolones**, have shown high activity against the bacilli (64), and the most commonly used are ofloxacin and ciprofloxacin. This class of drugs blocks DNA synthesis by binding and interfering with the topoisomerases DNA gyrase and topoisomerase IV, of which *M. tuberculosis* only has the former (50). Similarly to streptomycin, the two **aminoglycosides**, amikacin (AMK) and kanamycin (KAN), interfere with protein synthesis at the ribosomal level (159). This group of drugs can cause nerve toxicity, and due to their bad gastro-intestinal uptake, must be administered parenterally (1, 8, 41). Like the aminoglycosides, the polypeptide injectable antibiotic, **capreomycin**, interferes with the bacilli's protein synthesis, although it is not

completely understood how (3). **Ethionamide**, although activated through a different pathway, is identical to INH in regards to its mode of action. This compound's S-oxide metabolite is thought to inhibit the enoyl-ACP reductase involved in the synthesis of the cell wall mycolic acid (69).

The WHO recommends a 6-month initial treatment phase consisting of a minimum of four drugs and should include at least one fluoroquinolone and one injectable drug (i.e. AMK, KAN, or capreomycin). This should be followed by a 12-18 month continuation phase consisting of a minimum of three of the most effective and tolerated drugs (154). As this regimen implies, the treatment of MDR-TB is much longer and more difficult than that of drug-susceptible (DS) TB. To ensure that further resistance does not develop it is vital to ensure patients follow treatment, a demanding task on both the patient and the health care system.

MDR-TB is an increasing global threat to TB-control and elimination. In the latest WHO Global Report on Anti-TB Drug-Resistance, it was estimated that 4.8% of the global TB cases are MDR (151, 157). The proportion of cases does however vary widely in different countries. Among new TB-cases, defined as patients who have not previously received anti-TB treatment, several countries have reported no MDR-cases whilst in Baku, Azerbaijan as much as 22.3% of new TB cases are MDR, pointing to a high level of transmission within the area. Of the countries reporting the highest proportion of MDR cases, India and China are estimated to carry about 50% of the MDR-burden and the Russian Federation approximately 7% (151, 157).

In response to the urgency of preventing further spread and development of drug-resistance, the DOTSplus-program, a supplement to the DOTS-program, has been developed with guidelines on how to manage MDR-TB (154). In order to support poor-resource settings with a high MDR-burden, the WHO has initiated the Green Light Committee, dedicated at assisting TB control programs to access to high-quality second-line drugs at feasible prices (51, 154). However, these responses have not been quick and effective enough and patient isolates that are resistant to almost all available anti-TB drugs have been observed. Extensively Drug-Resistant TB (XDR-TB; termed MDR-TB isolates with further resistance to a fluoroquinolone and a second-line injectable drug) has been reported in 45 countries, with former USSR states being highly affected (151). With no alternative treatment regimens for such resistant bacilli there has never been a greater need to find new effective anti-TB drugs since the time prior to the discovery of the first anti-TB drugs.

1.6 DRUG-RESISTANT TUBERCULOSIS

1.6.1 Mechanisms of Drug-Resistance

Having discussed the forms of TB treatment, the different compounds' modes of action as well as a description of clinical drug-resistance, the next to be considered is *how M. tuberculosis* acquires drug-resistance. As already mentioned, *M. tuberculosis*' natural resistance to numerous antimicrobial agents is due to the bacilli's impermeable, lipid-rich cell wall. This is further strengthened by the expression of efflux pumps able to excrete antimicrobial substances as well as the expression of degrading or inactivating enzymes, as for example ß-lactamases (33, 36, 76, 119). However, how

does *M. tuberculosis* develop resistance to the drugs used for treatment? Understanding the mechanisms behind resistance is not only important in supporting the optimisation of the drugs used to prevent resistance development, but can further assist in identifying and developing new anti-TB drugs.

Bacteria typically acquire resistance to antibiotics through the alteration or acquisition of genes directly or indirectly involved in a compound's target. This can be through spontaneous chromosomal mutations of the genes, or through the horizontal transfer of genes between bacteria, by means of conjugation (plasmids), transduction or transfection (bacteriophages). Unlike other bacteria however, *M. tuberculosis* only acquires resistance through spontaneous chromosomal mutations in genes involved in a drug's target (25). Point mutations, insertions or deletions can occur in: genes specifically expressing the drug's target, inhibiting or decreasing the drug's ability to bind to its target; in genes expressing enzymes needed to activate prodrugs, inhibiting or decreasing the drug's activation; or in genes expressing efflux pumps, increasing their expression will increase the expulsion of the drug from the bacteria (25, 59). Mutations affecting the *expression* level of the genes (e.g. in the promotor region) may also convey resistance, increasing for example the presence of the drug-target, as is seen for INH-resistant *M. tuberculosis* (69, 111).

Although bacteria's overall chromosomal mutation rate is constant, (0.0033 mutations per DNA replication), a reflection of the natural errors occurring during DNA replication, the rate of mutation between different genes is known to vary; a phenomenon not fully understood (49). Thus, with regards to drug-resistance, the rate of resistance-mutations varies for different drugs. This is not only a reflection that different genes are involved in resistance to the different drugs, but also a reflection of the *number* of genes in which mutations may confer resistance. For example, resistance to RIF is in more than 95% of times observed in one single gene, the *rpoB* gene, whilst INH-resistance mutations are known to occur in several genes, two of which are involved in 70-80% of INH-resistant clinical isolates (*katG* and *inhA*) (111). Subsequently, RIF has a slower mutation/resistance rate than INH.

The success of a resistance mutation will ultimately depend on the extent to which it affects the bacteria's biological functions (16, 17). Antimicrobial compounds typically target essential bacterial functions, so although resistance-conferring mutations interfere with a drug's ability to bind its target, they will also affect the gene product's *function* ((**R**) **Figure 2)**. In other words, a resistance mutation will give the bacteria a selective advantage when exposed to the drug, but in the absence of the drug, they will have a disadvantage relative to their isogenic, mutation-free, susceptible strain. The level of fitness cost, and thus the ability for a resistant mutant to stabilise itself in the bacterial population, will depend on how important the gene product is for the *cell*'s function and to which extent the mutation affects the gene product's *function*.

If resistance-conferring mutations impose a biological cost on the bacteria, and thus a selective disadvantage when the drug is not in the environment, then drug-resistance should be easily defeated – simply stop using antibiotics for a while and the susceptible strains will take over. Unfortunately, this has proven to be too good to be true. Drug-resistant bacteria, not the least *M. tuberculosis*, have been able to stabilise

themselves in the human host and community, a phenomenon explained by two means. Firstly, if endowed with only a low-level or negligible fitness cost, as has been seen for some STR resistance mutations, resistant mutants will be able to stabilise themselves in a bacterial population, even in the absence of the drug ((**R^**) **Figure 2**) (26). It is enough that a single resistance-mutation has a negligible fitness cost for clinical resistance to establish itself (25, 26).

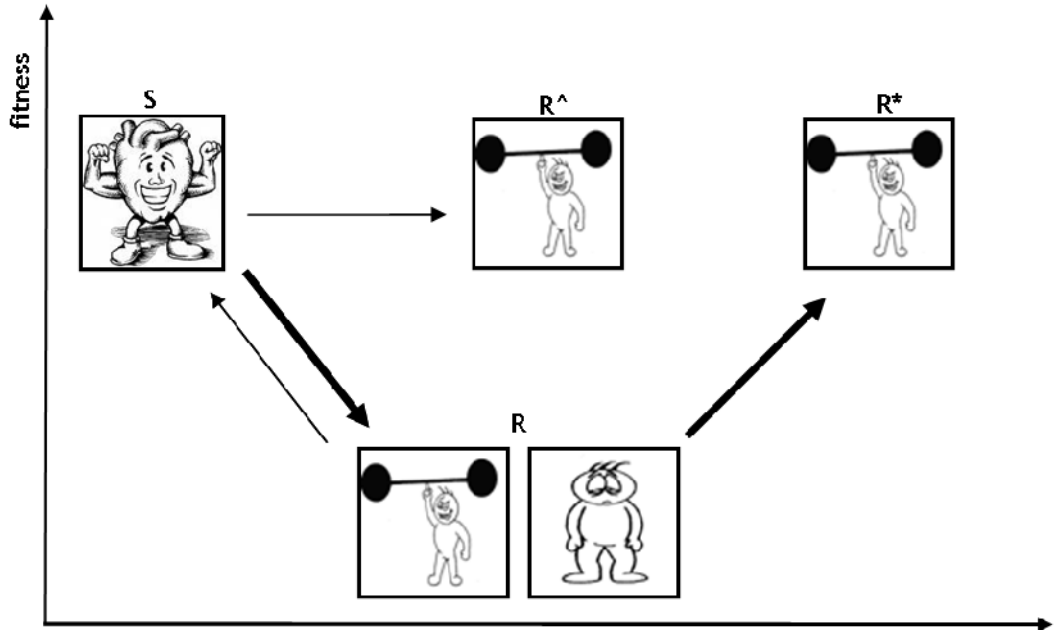


Figure 2: Dynamics of resistance-development in bacteria. Resistance-mutations often confer a fitness cost giving it a selective disadvantage in environments without the drug pressure (R). Additional spontaneous mutations can occur that restore the bacteria's fitness whilst maintaining resistance (R*). Depending on the importance of the gene's function, or the mutational site within the gene, a mutation may have no effect on the organisms function, i.e. no fitness cost is conferred (R^). (Adopted from *Paulander, W* 2007 (106))

Secondly, fitness costs can be compensated for through the acquisition of additional spontaneous chromosomal mutations in genes that will ameliorate the loss of function, without affecting the bacteria's acquired resistance ((**R***) **Figure 2**) (16, 17, 25). Although reversion of the resistance-mutation to its *wild-type* sequence is also possible, which will restore the bacteria's fitness at the cost of its resistance, the statistical chances are significantly lower as such a mutation has to occur in one specific site. The chances of a compensatory mutation occurring are higher as there are several sites at which fitness might be restored. The dynamics of resistance development, fitness cost and compensatory evolution can be visualised in **Figure 2**.

1.6.2 Studying Resistance Development

Calculating the frequency and rate at which resistance occurs can be an important aid in understanding drug-resistance and guide to developing optimal treatment regimens. The fluctuation assay devised by *Luria and Delbruck* in the 1940's to show that mutational events were indeed random events as apposed to being a response to stimuli (e.g. antibiotic exposure) (84), can be used to calculate the mutation frequency,

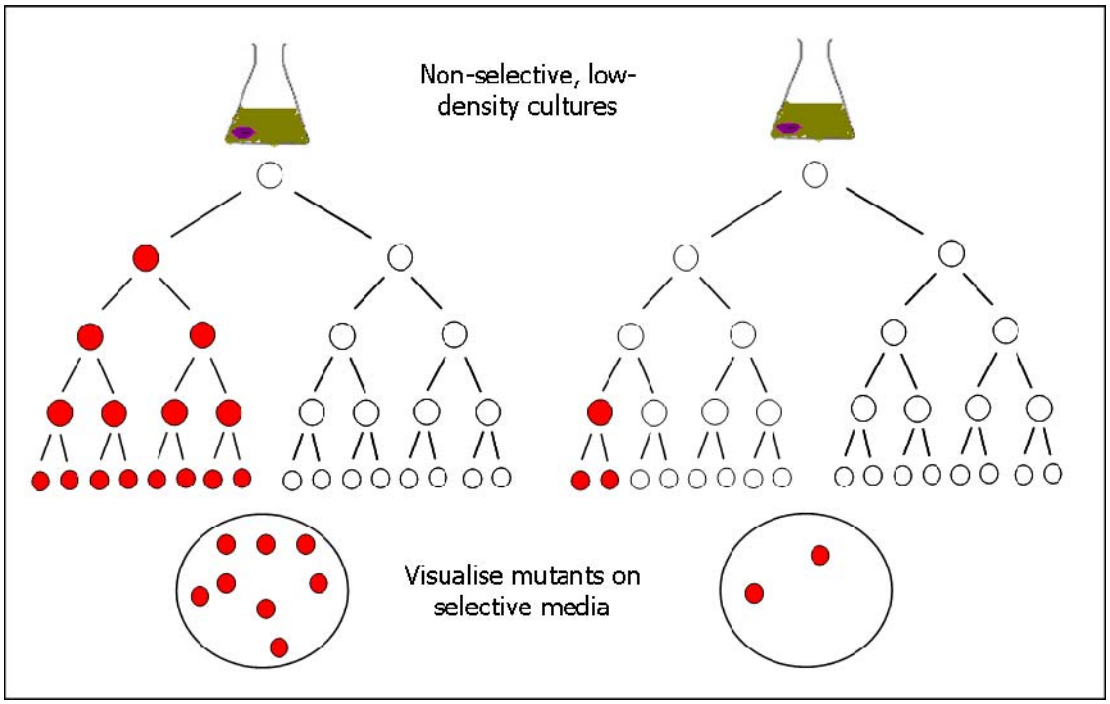


Figure 3: Fluctuation assay, shown as the number of mutant cfu in two independent cultures. Low-density non-selective cultures are prepared and grown to allow one resistance-mutation to occur. Mutant growth is measured by plating on selective agar. The mutation frequency is the proportion of mutant cfu over the total bacterial population (measured by plating on non-selective media). To the left is a jackpot culture; the high cfu count reflects that a mutation occurred early during growth. Red dots represent mutant colonies. White dots represent susceptible wild-type colonies.

expressing the fraction of mutant bacteria in a determined population, and the mutation rate, the probability that a cell will sustain a mutation during its lifetime.

In a fluctuation assay, a low-density, non-selective bacterial culture (e.g. *M. tuberculosis*) is prepared and evenly distributed into 12-25 culture flasks. The low-density cultures allow the assumption that no resistant bacteria are present in the cultures (116). The parallel cultures are then left to grow to a population size that will allow a single resistance-conferring mutation to occur and the resistant bacteria are visualised and enumerated by plating the cultures on selective and non-selective agar. As the mutational events occur randomly, the resistance-conferring mutation can occur at any stage during the cultures' growth and will be reflected by the number of mutant Colony Forming Units (cfu) appearing on the selective agar. As **Figure 3** shows, resistance-conferring mutations occurring early during culture will result in a high number of mutant colonies upon selection, known as a jackpot culture, compared to if the mutational event occurs later during the culture growth. Following a Poisson Distribution, there will also be a number of cultures in which no mutational events will occur, resulting in zero mutant colonies (116).

The mutation frequency for resistance to a given drug is calculated by taking the median fraction of mutant colony growth of the independent cultures over the total bacterial growth, the median being used to cancel out the effect of a jackpot culture. If a large enough number of parallel cultures is set, decreasing the effect of jackpot cultures

and/or zero mutant cultures on the median number of mutant colonies, the mutation rate can be calculated. Several methods have been devised to calculate this probability and are based on the fluctuation in mutant culture sizes of the independent cultures (116). Being a measure of a *cell's* chance of sustaining a resistance *mutation*, as opposed to a measure of the number of *mutants* in a *determined population*, measuring the mutation rate is a more accurate and representative measure of resistance-development (116).

Using the fluctuation assay to select drug-resistant mutants not only enables the calculation of the mutation frequency and rate, but also further characterisation of the selected mutants. As each mutant colony obtained from the parallel cultures represents independent mutational events, a library of mutants is obtained and can be further scrutinised. Resistance-mechanisms can be studied by sequencing known resistance genes and/or searching for unknown mechanisms, the fitness cost of the resistance can be determined and compensatory evolution can be characterised.

1.6.3 Resistance-Development in *M. tuberculosis*

Much research has been dedicated to understanding the mechanisms by which *M. tuberculosis* develops resistance to anti-TB drugs. Below is a general description of the resistance-mechanisms to the first-line anti-TB drugs and the key second-line drugs. As one of the projects in this thesis focuses on RIF-resistance, special attention will be given to the resistance to this drug.

As mentioned above, **INH** is a cell wall inhibitor prodrug that is activated by catalase-peroxidase. Resistance occurs at an approximate frequency of 3.5×10^{-6} and the mutation rate has been measured to be 2.6×10^{-8} mutations per cell generation (mut/Cgen) (34, 69). Mutations in the *katG*, *inhA* and *ahpC* genes are the most commonly seen, with *katG* and *inhA* mutations accounting for 70-80% of resistance mutations in INH-resistant clinical isolates (111). Deletions or point mutations in the *katG* gene, which encodes the INH-activating enzyme, catalase-peroxidase, inhibit or partially decrease the level of INH-activation. The loss of catalase-peroxidase activity is thought to be compensated for by the increased expression of the *ahpC* gene, encoding the alkyl hydroperoxidase reductase, also involved in inactivating organic peroxides (111, 127). Mutations in the promoter region of *ahpC* occur in 10% of INH-resistant clinical isolates and are typically associated with *katG* mutations (114). Mutations in the *inhA* gene, commonly seen in the gene's promotor region, lead to the over-expression of enoyl-ACP reductase. Resistance is conferred by outnumbering the amount of this enzyme relative to the exposure of INH. This resistance-mechanism only confers low-level resistance (69).

The *in vitro* mutation rate for **EMB** resistance has been measured to be 1.0×10^{-7} mut/Cgen (34). Approximately 70% of EMB-resistant clinical isolates have been seen to contain point mutations in the *embCAB* genes, encoding the drug's target, arabinyl transferase, with amino acid substitutions within the *embB* gene being the most common (111, 112, 133). Phenotypic DST to EMB is known to be inefficient, correlating badly with the clinical susceptibility (79). The molecular determination of resistance *embCAB* gene mutations can therefore be a complementing diagnostic tool in determining resistance (69). Mutations in the *rrs* and *rpsL* genes, encoding the 16S

rRNA and S12 ribosomal protein, respectively, account for 65-67% of the identified resistance-mutations in **STR**-resistant clinical isolates (111). Resistance to **PZA** is conferred by means of interfering with the drug's activation. Mutations in the *pncA* gene, encoding the activating enzyme PZase, inhibit the formation of POA from PZA, thereby conferring resistance (123). A polymorphism in the *pncA* of *M. bovis*, which is naturally resistant to PZA, also shows the role of the gene in resistance (123). Needing an acidic environment for PZA to be active, phenotypic DST of PZA both in broth and solid media, can be cumbersome and makes molecular DST a potential diagnostic tool. Additional resistance-mechanisms do remain to be identified, as 30% of PZA-resistant clinical isolates have an intact, unaltered *pncA* gene (111).

Fluoroquinolones interfere with the negative supercoiling of DNA by targeting the organism's DNA gyrase. The DNA gyrase's two subunits are encoded by the *gyrA* and *gyrB* genes and mutations in the Quinolone Resistance Determining Region of *gyrA* are seen between 42 to 85% of clinical isolates (50, 111). Although *gyrB* mutations have been identified in *in vitro*-selected mutants, they are seldomly seen in clinical isolates (50). Lastly, the **aminoglycosides** AMK and KAN. Similar to STR, these two drugs interfere with protein synthesis on the ribosomal level. However, the presence of different resistance-genes, as well as the lack of cross-resistance with STR, suggests that AMK and KAN target protein synthesis differently. Resistance mutations within the *rrs* gene, encoding the 16S rRNA, account for high-level resistance to these two drugs (69). Contrary to STR, resistance to either AMK or KAN give cross-resistance to the other (15). Additional studies have however shown that KAN-resistance does not always confer resistance to AMK (75).

Rifampicin is one of the two key anti-TB drugs endowed with a strong bactericidal effect and inhibits *M. tuberculosis* by targeting the RNA polymerase's ß subunit (90). The RNA polymerase consists of two α, one ß, one ω, one ß' subunits interacting with a σ factor, and it is thought that RIF sterically blocks the elongation phase of transcription (31). Following transcription initiation, where the RNA polymerase binds to the DNA template to form the transcription bubble, elongation begins by the incorporation of the first two nucleotides in the bubble's active site. Under normal conditions, the polymerase continues reading the DNA template pushing the new mRNA template through the complex (see **Figure 4**). However, in the presence of RIF, which is thought to be bound in close proximity of the bubble's active site, the passage of the mRNA strand is sterically blocked, abrogating the bacilli's transcription (31).

RIF-resistance in *M. tuberculosis* occurs at a rate of $1x10^{-8}$ mut/Cgen (61), and similar to resistance in *E. coli* (67), is caused by point mutations, in frame insertions or in frame deletions within the *rpoB* gene, the gene encoding the RNA polymerase's ß subunit (139). Mutations in the 81-bp RIF-Resistance Determining Region (RRDR) of the *rpoB* gene are seen in more than 95% of RIF-resistant clinical isolates (111). Mutations upstream and downstream of the RRDR have also been identified in clinical isolates, however at a much lower frequency (56, 137). Point mutations leading to amino acid substitutions are the most abundant RRDR mutations, with the substitutions of Serine (Ser) for Leucine (Leu) at codon 531 and Histidine (His) to Tyrosine (Tyr) at codon 526, being the most common (111). *In vitro* studies, in which RIF-resistant

mutants were selected and RRDR mutations characterised, have seen a similar predominance of 531 and 526 substitutions (23, 56, 88, 96).

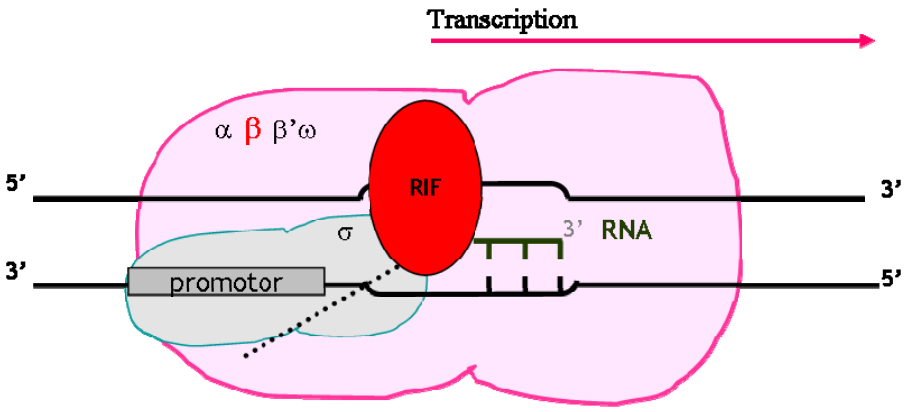


Figure 4: Steric blocking of transcription elongation by RIF. RIF sterically blocks the elongation process of transcription by preventing the passage of the new RNA strand through the polymerase. This leads to the dissociation of the RNA polymerase, abrogating transcription and gene expression.

Fitness assays on *in vitro*-selected RIF-resistant mutants have shown that resistance to the drug is coupled with a biological cost (23, 45, 88). *Billington et al*. measured that Ser531Leu mutations conferred the smallest fitness cost when mutants were tested in competition assays with their isogenic susceptible strains (23). This was also observed by *Mariam et al*, however, they also observed that the measured fitness levels can vary depending on the experimental model used (88). The differences in fitness levels of the different substitutions offer an explanation for their varied frequency in clinical isolates; for example, having a smaller fitness cost, Ser531Leu mutants can survive in the human host and establish an infection. Fitness assays between patients' initial, DS-isolate and the later RIF-resistant isolate, have also shown that Ser531Leu mutations have a negligible fitness cost (45). It cannot however be excluded that clinical isolates, after developing resistance in the human host, have had the time to acquire compensatory mutations, thereby restoring the bacilli's fitness.

1.6.4 The Beijing Genotype

The Beijing genotype is a family of *M. tuberculosis* strains is highly prevalent globally and commonly associated with MDR-TB, especially in countries of the former USSR, Cuba, Vietnam and South Africa (2, 52, 94, 99, 105, 109, 142, 143). The genotype is identified by its characteristic genetic pattern as determined by spoligotyping, and has further been sub-divided into two subgroups, the ancestral "atypical" subgroup and the modern "typical" subgroup, based on differences in insertion sequence (IS) 6110 Restriction Fragment Length Polymorphisms (RFLP) patterns (71, 93, 95, 146). Further analysis of the genotype has shown that the Beijing *M. tuberculosis* strains associated with MDR and that have a high rate of spread worldwide, are of the modern, typical subgroup (2, 55, 147).

Much research has been designated to understanding the association of this genotype with MDR-TB as well as its global dissemination. *In vitro* studies have shown that clinical isolates of the Beijing genotype do not show a higher mutation rate with regards to RIF-resistance development, and, as discussed in **Paper I**, RIF-resistant Beijing mutants do not exhibit a different span of RRDR-resistance mutations and resistance-level (61). It has been proposed that Beijing isolates are more apt to circumvent BCG-induced immunity. *Kremer et al*. recently reported a higher association between BCG-vaccination and patients infected with *typical* Beijing strains, than BCG-vaccination among patients infected with atypical Beijing strains (74). The "success" of the modern, typical Beijing subtype might thus be explained by the strains' ability to circumvent BCG-induced immunity (74, 82).

Another explored suggestion has been that Beijing strains have a defective DNA repair system, thereby making them more prone to develop resistance. Drug-resistant isolates of the Beijing genotype have been seen to contain polymorphisms within putative mutator genes, suggesting that such isolates are more prone to adapting to new environments (110). However, Beijing genotype have not been seen to have a higher mutation rate (148).

1.7 IN THE SEARCH FOR NEW ANTI-TUBERCULOSIS DRUGS

There are several reasons behind the urgent call for new anti-TB drugs and the development of new treatment regimens, of which some have already been highlighted. The current TB treatment regimen, although effective against DS-TB, is long, demands several drugs that are to be taken daily, several of which cause side effects, the treatment regimen cross-reacts negatively with anti-retroviral drugs and it is ineffective against latent TB (6, 138). International partnerships, such as the StopTB Partnerhip and the TB Alliance for TB Drug Development, have been founded with the aim of bringing together academia, pharmaceutical industries, public research institutes and Non-Governmental Organisations (NGOs) to respond to this call and catalyse the discovery and development of new TB drugs (6, 12).

The ultimate characteristic of a new anti-TB drug and/or regimen is that it will:

1. Substantially decrease time to cure
2. Show no cross-resistance with other drugs (i.e.effective on MDR-TB)
3. Be compatible with anti-retroviral drugs
4. Improve the treatment of latent TB

To fulfil these criteria, compounds showing high activity against tubercle bacilli and that target the bacilli from new angles are needed, i.e. new targets or Achilles heels in the bacilli need to be identified and attacked. The pharmacokinetics of compounds need to be optimal, allowing a rapid uptake of the drug, preferentially after oral intake, and with minimal toxic effects. Compounds with long half-lives are promising as they will enable longer dosing-intervals during treatment, facilitating a patient's treatment and increasing chances of compliance. As TB-treatment will always consist of combination therapy to prevent resistance-development, it is essential that new drugs do not interact negatively with other drugs, be it existing anti-TB drugs or new compounds. Finding optimal treatment doses that are both highly effective against the bacilli and that will

prevent the development of clinical drug-resistance are key to decreasing the time of TB-treatment, as well as key to ensuring the longevity of the drug. Lastly, it is vital that new drugs can be produced, distributed and handled at an affordable price, to ensure their access to the populations in greatest need of effective, rapid anti-TB treatment (6, 138).

Following decades of stagnation, TB-drug discovery and development began re-blooming in the 1990's and the anti-TB drug pipeline has at long last begun to thicken. The pool of new compounds is growing, with numerous under preclinical phases of development (115). Also, known compounds (both anti-TB agents and antimicrobials used against other bacteria) are being modified and re-tested, as for example moxifloxacin and gatifloxacin (5, 9, 68). This increase in research and development has led to successes and, for the first time in decades, a number of new compounds are in late stages of clinical development, holding promise of becoming new anti-TB drugs (6, 12, 115). Below a brief description of the new compounds already in clinical trials is given. **Figure 1** depicts the compounds' target within the *M. tuberculosis*.

1.7.1 SQ109

SQ109 is a lead compound of the diamine EMB-analogues and will enter phase Ib clinical trials in 2009 (108, 124, 126). The compound has shown specificity for Mycobacteria, has a strong *in vitro* effect against *M. tuberculosis*, showing inhibitory concentrations at 0.25-5 μg/ml, and is equally active against RIF-, INH, and EMB-resistant isolates (32, 108). The compound is thought to target the bacilli's cell wall synthesis although the exact target is not known, and the lack of cross-resistance indicates the mechanism is different to that of EMB (108). SQ109 has shown *in vitro* synergistic effects when combined with either RIF or INH, shows no antagonism with other first-line drugs, and has synergistic effects in combination with RIF, INH and PZA *in vivo* (32, 101). Interestingly, combining SQ109 with RIF *in vitro* was highly active against RIF-resistant strains and is thought to be due to the increased activation of SQ109 by RIF-induced *M. tuberculosis* cytochrome P450 (32). Phase Ia trials in healthy individuals have shown that the compound does not have serious side effects following a single oral dose, that it is well distributed to tissues, including the lungs, and its long half-life indicates its potential to be given in once weekly doses (125).

1.7.2 PA-824

The bicyclic 4-nitroimidazole, **PA-824**, belongs to the class of nitroimidazoles known to be active against anaerobic microbial infections (42). The compound has shown high *in vitro* activity against *M. tuberculosis* (Minimum Inhibitory Concentration (MIC) 0.015-0.25 μg/ml), with no cross-resistance to first-line drugs (135). Furthermore, it is active *in vitro* against non-replicating bacilli, showing potential for the treatment of latent TB (135). PA-824 is a prodrug that is activated by the *M. tuberculosis* glucose-6-phosphate dehydrogenase (FGD1) and F420 cofactor and in its reduced, active form, PA-824 inhibits protein and cell wall synthesis (87). *In vitro* resistance occurs at a frequency similar to that of INH (6.6×10^{-7}) and results in high-level resistance. The loss of FGD1 or F420 cofactor function in mutants suggests that resistance is mediated by blocking the compound's activation (87, 135). *In vivo*, PA-824 has shown activity comparable to INH and to be effective in late phases of

infection, indicating the compound's effect on persistent bacilli (103, 144). PA-824 is currently under Phase II clinical trials.

1.7.3 OPC-67683

OPC-67683 is a nitro-dihydro-imidazooxazole prodrug that targets the synthesis of *M. tuberculosis* cell wall mycolic acid. Like the other compounds discussed, OPC-67683 has a high *in vitro* activity against both DS and DR *M. tuberculosis* (MIC 0.012 μg/ml) (89, 121). The compound's long half-life, lack of cytochrome P450-metabolism and its efficacy in immune-compromised *M. tuberculosis* infected mice, suggest it will be effective in TB/HIV-coinfected patients (89). OPC-67683 shows no antagonistic effects *in vitro* when given with RIF, INH, EMB or STR, and when combined with the first line drugs in the mouse model, OPC-67683 is highly effective at three months of treatment, eliminating the bacilli at four months (89). This compound shows a good sterilising effect, and with its long half-life, holds potential to substantially shorten treatment time as well as dosing frequency. A phase II trial assessing OPC-67683's safety, efficacy and pharmacokinetics in patients following 14 days of monotherapy, has been completed (11).

1.7.4 R207910

R207910, also named **TMC207**, belongs to the class of diarylquinolines (DARQ) which has shown high activity and specificity for Mycobacteria (18, 60, 107). R207910 has a strong *in vitro* activity against DS, DR, MDR and XDR *M. tuberculosis* isolates (MIC 0.03 μg/ml) and is a reflection of the compound's unique mechanism of action (18, 60). DARQs are the first anti-TB compounds shown to target the bacilli's membrane-bound ATP synthase, inhibiting ATP production of both actively growing and non-replicating M. *tuberculosis* strains (18, 73). This makes the ATP synthase a vulnerable spot even during latent *M. tuberculosis* infection.

R207910 is highly active *in vivo* (mouse model) when given alone, but more importantly, when combined with the first-line drugs RIF, INH and PZA, it is more active than the first-line regimen alone, resulting in a significant bactericidal effect after one month of treatment (18, 62, 83, 149). In combination with second-line drugs, R207910 also shows a strong effect in the mouse model. After two months of treatment the combination of R207910 with AMK, ethionamide and moxifloxacin was more active than the second-line regimen alone (83).

The lack of cross-resistance as well as R207910's strong activity in combination with existing anti-TB drugs have shown the compound's potential to decrease treatment time as well as to be used on all forms (DS, DR, MDR or XDR) of TB. Furthermore, R207910 is well absorbed when taken orally and its long half-life holds potential for the drug to be taken with longer intervals (18). R207910 has now entered phase IIb clinical trials for the treatment of MDR-TB and has already shown substantial bactericidal activity when combined with a standard-of-care second-line treatment regimen (141).

1.8 THE ATP SYNTHASE – A NEW ANTI-TB DRUG TARGET

The ATP synthase is a membrane-bound enzyme-complex that catalyzes the formation of ATP during oxidative phosphorylation. During oxidative respiration, the electron transport chain generates an electrochemical proton gradient by pumping H^+ ions out of the bacterial cell through membrane electron carriers. The ATP synthase then shuttles these H^+ ions through its F0-unit to its intracellular F1-unit to catalyse the formation of ATP from ADP and inorganic phosphate. The enzyme can also work in reverse, as an ATPase, pumping H^+ out of the cell when the extracellular proton gradient is low or ATP levels are high in the cell (37).

As mentioned, the ATP synthase consists of two units, the membrane-bound, H^+ translocating F0 complex and the intracellular, catalytic F1-complex. The F0 complex consists of three subunits (a, b, c), with one a, two b and approximately ten c subunits which are encoded by the *atpB*, *atpE* and *atpF* gene, respectively. The catalytic F1 complex is built up of 5 subunits (α,β,γ,δ,ε) with three α-, three β-, and one each of γ-, δ-, and ε-subunits each encoded by the *atpH*, *atpA*, *atpG*, *atpD* and *atpC*, respectively (37).

Contrary to other bacteria, the ATP synthase is essential for *M. tuberculosis* growth and survival, both during active growth and when the bacilli are in a non-replicating latent phase. *Koul et al.* observed that, although substantially down-regulated, dormant *M. tuberculosis* do maintain a sub-level of ATP synthase expression. Upon exposing these bacilli to the ATP synthase inhibitor, R207910, they observed that the bacilli were indeed efficiently killed by the compound (73). Exposing non-replicating hypoxic *M. tuberculosis* to various inhibitors of the respiratory chain, *Rao et al*. also showed that *de novo* ATP production, although at a lower level, is still essential for the bacilli's survival (113). ATP production via the ATP synthase is essential for *M. tuberculosis* survival in any environment, thus making it an optimal and interesting target for new anti-TB drugs.

2 AIMS

Increasing our knowledge of the mechanisms by which *M. tuberculosis* acquires drug-resistance is an important resource in preventing resistance-development, as well as in identifying new targets against which drugs can be developed. Discovering and developing new effective drugs are urgently needed to shorten and simplify TB-treatment and more importantly to counteract the spread of MDR-TB.

Considering the above statement, the general and specific aims of this thesis were:

1. To characterise RIF-resistance mutations of *in vitro*-selected *M. tuberculosis* mutants and compare them to RIF-resistant clinical isolates (**Paper I**).

2. To characterise the activity of the new anti-TB drug candidate, R207910.

   In **Paper II** we aimed to thoroughly characterise the compound. This included determining R207910's: chemical structure; *in vitro* anti-mycobacterial activity; bacterial target and bacterial resistance-mechanisms; pharmacokinetic and pharmacodynamic activity in animal models; and lastly its safety, tolerability and pharmacokinetics in healthy human individuals.

   Based on this first study, in **Paper III** we aimed to continue investigating R20710's antimycobacterial spectrum, focusing on drug-susceptible and MDR-TB isolates, as well as non-tubercular Mycobacteria.

   Lastly, in **Paper IV** we aimed to determine the *in vitro* rate, level, mechanisms and fitness cost of *M. tuberculosis* resistance to R207910.

3 RESULTS AND DISCUSSION

3.1 PAPER I

Rif-Resistance in *in vitro*-Selected *M. tuberculosis* Mutants

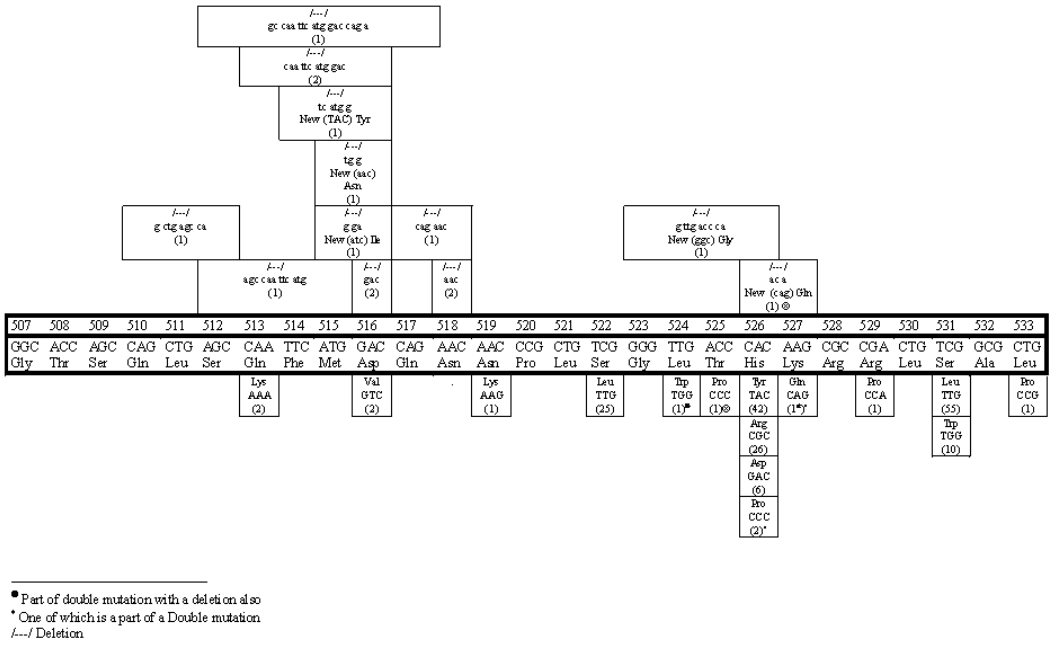
In **Paper I** we determined the frequency, distribution and resistance-level of RRDR mutations among *in vitro*-selected RIF-resistant *M. tuberculosis* mutants and compare these to RIF-resistant clinical isolates. Having previously shown that *M. tuberculosis* clinical isolates of the Beijing family do not exhibit a higher mutation rate for RIF-resistance (148), we aimed to determine whether there is a difference of the mentioned three parameters between mutants of the Beijing genotype and mutants of other genotypes.

In the previous referred study, the *Luria et Delbruck* fluctuation assay was used to select independent, spontaneous RIF-resistant mutants from four Beijing and four non-Beijing *M. tuberculosis* clinical isolates, as well as from the H37Rv reference strain (148). These mutant clones formed the basis for our study. A total of 189 independent RIF-resistant mutants were studied, of which 89/189 had a Beijing genotype, as determined by spoligotyping (71). We sequenced the mutants' 81-bp RRDR region of the *rpoB* gene and determined the clones' resistance-level to RIF through MIC-determinations on solid media. A more thorough description of the methods can be found in **Paper I**.

A mutation in the RRDR was observed in all but one of the 189 RIF-resistant clones. Twelve different single nucleotide mutations, affecting 7 codons, were observed in 172 mutants. Substitutions in amino acids 531, 526 and 522 were the most frequent. Eleven in frame deletions were detected in 14 mutants and one double mutation and one triple mutation were detected. These latter two mutants were both of the Beijing genotype. A similar distribution and frequency of RRDR mutations were seen between Beijing and non-Beijing mutants. The mutants showed a high level of resistance (MIC $\geq$ 36 μg/ml). Interestingly, 10 mutants with the Ser522Leu substitution exhibited lower resistance-levels (MIC between 8-16 μg/ml). **Figure 5,** as well as **Table 1** in **Paper I** depict the mutation distribution and resistance-level of the studied mutants.

Similar to clinical isolate studies and previous *in vitro* studies on RIF-resistant mutants, substitutions in amino acids 531 and 526 were the most frequent among our mutants, with Ser531Leu (55/189, 29%) and His526Tyr (42/189, 22%) being the predominant (14, 23, 24, 56, 86, 88, 96, 111, 120, 131). The high frequency and resistance-level suggest that these two mutation sites have a smaller biological cost relative to other substitutions. Among *in vitro*-selected mutants, the Ser531Leu substitution is coupled with a high relative fitness (between 0.5 and 1.05, depending on the model used) and His526Tyr mutants have shown a relative fitness of around 0.8 (23, 45, 88). Comparing the relative fitness of *in vitro*-selected mutants and RIF-resistant clinical isolates, *Gagneux et al.* further confirmed that within the human host, isolates with 531 and 526 substitutions also have a high relative fitness (45). Clinical isolates with the Ser531Leu substitution even exhibited a higher fitness (relative fitness > 1) than the RIF-susceptible progenitor. The elevated fitness of these clinical isolates could be a reflection of compensatory evolution following long prevalence within the human host.

Contrary to RIF-resistant clinical isolates, where it is rarely seen, the Ser522Leu substitution was the third most common mutation among our mutants. It has been seen that mutants harbouring this mutation are endowed with a significant fitness cost in competition assays between the mutant and its isogenic susceptible strain (relative fitness between 0.54-0.88) (45, 88). Although able to survive *in vitro*, the low fitness must impose Ser522Leu mutants with a selective disadvantage when confronted by the pressures of the human host.



* Part of double mutation with a deletion also
* One of which is a part of a Double mutation
/---/ Deletion

Figure 5: Distribution and frequency of RRDR (*rpoB*) mutations in 188/189 *in vitro*-selected *M. tuberculosis* mutants. Amino Acid numerated according to *E. coli* sequence. (Adopted from *Ramaswamy et al.* 1998 (111).)

As mentioned, a similar span, frequency and resistance-level of mutants with a Beijing genotype and those of another genotype. The only difference seen was that the two observed multiple mutations (one double and one triple) were within mutants of the Beijing genotype. Although not significant, this observation is similar to that seen by *Tracevska et al*. in which multiple RRDR mutations were observed in 8.2% of Beijing clinical isolates compared to 2% in isolates of other genotypes (143). It has been suggested that the Beijing family genotype has an acquired mutator phenotype as a result of polymorphisms in putative mutator genes involved in DNA repair, which would explain the genotypes higher tendency to acquire resistance (78, 110). However, a higher mutation rate would be expected if these polymorphisms caused a mutator phenotype, which has not been seen (22, 61). Several other studies have been dedicated to analyzing mutator phenotypes within drug-resistant *M. tuberculosis* in general (20, 78, 102, 110). However, no general trend is seen, and results vary between studies. If mutator phenotypes do lie behind different genotypes' ability to develop drug-resistance, one would expect to see a higher mutation rate among such isolates. It would be interesting to conduct fluctuation assays on isolates exhibiting polymorphisms in the mentioned mutator genes, to determine whether these have a higher mutation rate.

In conclusion, our study showed that *in vitro*-selected RIF-resistant mutants, originating from distinct *M. tuberculosis* clinical isolates, exhibit a similar span of RRDR *rpoB* gene mutations and resistance-levels as are seen in clinical isolates. Although frequent within our *in vitro* mutants, the Ser522Leu substitution's low frequency in clinical isolates is most likely the reflection of a fitness cost and lower resistance-level. Lastly, a similar span, frequency or resistance-level was seen between mutants of the Beijing genotype and mutants of other genotypes. The cause for the Beijing genotype's success in spreading globally and its correlation with MDR-TB remains to be explained. Studies on the genotypes virulence, pathogenicity or transmissibility are other possible explanations to this question.

3.2 PAPER II

Characterisation of a Novel Diarylquinoline Anti-TB Drug Candidate

Paper II presents the in depth characterisation of R207910, a new anti-TB drug candidate with strong anti-mycobacterial activity. The paper is the result of a large collaborative effort, involving several laboratories, led by researchers at Johnson & Johnson Pharmaceutical R&D. Having been involved in the *in vitro*-characterisation, this section will present a more detailed description and discussion of these results.

The DARQs were identified and developed as a new class of potential anti-TB compounds. Upon testing against the H37Rv reference strain, 20 DARQ molecules showed strong inhibitory effects with MIC's below 0.5 μg/ml, and the DARQ R207910 was identified as the most active. For the chemical structure of the compound please refer to **Figure 1.** in **Paper II** (18).

Minimum Inhibitory Concentrations of R207910 against DS and DR *M. tuberculosis* clinical isolates showed that R207910 has a potent effect (MIC range 0.03-0.120 μg/ml), regardless of the isolates' susceptibility to anti-TB drugs. The lack of cross-resistance with first- and second-line anti-TB drugs led us to test R207910's potential for the treatment of MDR-TB. Using the Bactec 460 Radiometric system, a liquid media culture system commonly used for standard DST (129), we showed that all 30 MDR *M. tuberculosis* clinical isolates tested were susceptible to 0.1 μg/ml of R207910, with 17 (57%) being susceptible to 0.01 μg/ml. These results formed the basis for our continued *in vitro* characterisation of the compound. As discussed in **Paper III**, MIC-determinations on these isolates confirmed the equal MIC distribution of R207910 on DS and MDR *M. tuberculosis* clinical isolates (60). R207910's strong inhibitory effect on DS, DR and MDR *M. tuberculosis* supports its future potential as a first-line anti-TB drug, but, more importantly, its potential to be used for the treatment of MDR-TB.

MIC determinations on other Mycobacterial species (NTMs), as well as on other bacterial species further confirmed the compound's high specificity against the Mycobacterial genus. As can be seen in **Table 1** in **Paper II**, clinically relevant Mycobacteria were high susceptibility to R207910 (MIC range 0.003-0.5 μg/ml) (18). Contrarily, R207910 had little effect on other bacterial species. In **Paper III** we confirmed the compound's efficacy on a larger selection of Mycobacterial species, and thus R207910's additional potential to be used for the treatment of NTM infections

(60). In a footpad infection mouse model of, *Ji, B et al.* have further shown that R207910 has good activity against *M. leprae* and *M. ulcerans*, two clinically important NTMs (65, 66).

The lack of cross-resistance with other drugs suggested that R207910 targets a novel site within the bacilli. At 8xMIC ($MIC_{M.\ smegmatis}$ = 0.003μg/ml; $MIC_{M.\ tuberculosis}$ = 0.03μg/ml), the proportion of resistant mutants for both *M. smegmatis* ($1x10^{-8}$) and *M. tuberculosis* ($5x10^{-8}$) was similar to the proportion of RIF-resistant mutants ($10^{-7}/10^{-8}$). Upon exposure to first or second line anti-TB drugs, the R207910-resistant mutants did not show cross-resistance, suggesting further that the compound has a unique bacterial target. Whole-genome sequencing of two *M. smegmatis* mutants, one *M. tuberculosis* mutant and the *wild-type M. smegmatis* progenitor identified nucleotide changes within the *atpE* gene of the mutants. The mutants had one of two point mutations within the *atpE* gene (*M. smegmatis* mutant: Asp32Val; *M. tuberculosis* mutant: Ala63Pro), the gene expressing the membrane-bound c subunit of the ATP synthase. Complementation assays of the Asp32Val mutation further confirmed that mutations in the *atpE* gene conferred the observed R207910-resistance.

These results suggested that R207910 targets *M. tuberculosis* ATP production by interfering with the membrane-bound F0 section of the ATP synthase, which was at that stage not yet identified as a potential anti-TB drug target. Through ATP measurement assays, *Koul et al*. validated the target by showing that *M. tuberculosis* ATP levels decrease in a dose-dependent manner upon drug-exposure, whilst levels in R207910-resistant mutants were not affected. Furthermore, the authors demonstrated an interaction of the compound with the ATP synthase target protein through binding assays between the two entities (72). These results confirm that R207910 does target the *M. tuberculosis* ATP synthesis, exhibiting its inhibition by abrogating the bacilli's ATP production.

Additional *atpE* mutations have been identified in subsequent *in vitro*-resistance studies, further suggesting the role of *atpE* genes in resistance-development. *Petrella et al*. observed the Ala63Pro substitution described above, as well as an additional *atpE* point mutation (Ile66Met) in *in vitro*-selected R207910-resistant mutants (107). Furthermore, in **Paper IV**, we identified two additional amino acid substitutions (Asp28Pro and Glu61Asp) among *in vitro*-selected resistant mutants of *M. tuberculosis*. Computational structure analysis of the *M. tuberculosis* ATP synthase have suggested that the compound binds to glutamate at amino acid 61 in the c subunit thereby blocking H^+ passage through the channel and subsequently preventing ATP synthesis (35). The observed mutations in the vicinity of this binding site might thus confer resistance by preventing R207910's interaction with the subunit. This would enable the H^+ flow and thus bacterial ATP production.

Pharmacokinetic and pharmacodynamic studies were conducted in the mouse model to establish R207910's efficacy. R207910 exhibited good absorption (plasma C_{max} of 1.1-1.3 μg/ml within 2-4 hours), good tissue distribution as well as a long half-life (43-64 hours in plasma and 28-92 hours in tissue). The long half-life reflects the compound's slow redistribution in the body. A single dose of R207910 in the

non-established infection model in mice showed a bacteriostatic effect with a 50 mg/kg single dose (0.5 log decrease in the lungs). However, the compound was bactericidal when this dose was increased to 100 mg/kg, resulting in a 2.5 log decrease that lasted 8 days. This observation has further been confirmed by *Veziris et al.* in a recent paper assessing several once-weekly R207910-containing regimens in the mouse model (149). Of the different combinations tested, the once weekly regimen consisting of R207910 (125 mg/kg) + rifapentine + PZA showed the strongest effect, yielding 9/10 mice culture-negativity after two months of treatment. The compound's long effect together with its long half-life supported R207910's potential to be administered with longer dosing intervals.

A Minimal Effective Dose (MED, defined as preventing gross lung lesions) of 6.5 mg/ml was measured when infected mice were treated with multiple doses (5 times weekly) over a four-week period. Furthermore, dosing at 12.5 mg/kg led to a 5-2 log decrease in the lung, showing that the compound's Minimal Bactericidal Dose (i.e. dose needed to eradicate the bacilli) is very close to that of the MED. *In vitro*, this strong bactericidal effect, was not increased if concentrations were increased. Together, these results indicated that R207910 has a time-dependent rather than concentration-dependent bactericidal activity.

Administered R207910 monotherapy in infected mice was at least as effective as the standard first-line treatment regimen for TB (RIF + INH + PZA). More importantly, when R207910 was *combined* with this first line regimen, a greater effect was seen than the standard regimen alone. After one month of treatment, 25 mg/kg R207910 combined with RIF, INH and PZA led to a substantially greater decrease (2 log greater decrease) in the lung bacterial load compared to the standard regimen alone. By two months this decrease was another 1 log greater. Furthermore, when one of the first-line drugs (RIF, INH or PZA) was replaced with R207910, a stronger activity was observed, especially following 1 month of treatment. These results can be seen in **Figure 4** in **Paper II**.

Further studies on the combination of R207910 with first and second-line drugs have since been conducted and continued to show the strong bactericidal effect of the compound (62, 83, 149). Of most interest has been the synergistic activity seen when R207910 is combined with PZA (62, 149). Upon five daily doses of R207910 (25 mg/kg) and PZA, 100 % of the treated mice became culture negative after two months of treatment (62). A similar effect was seen with only once weekly doses of R207910 combined with PZA and Rifapentine; 9/10 mice were culture negative at two months (149). This synergistic effect is not entirely unexpected as these compounds, although through different mechanisms, target the bacilli's energy production; PZA affecting the membrane potential needed to drive the ATP synthase proton pump (160), and R207910 directly inhibiting ATP production through its interaction with the ATP synthase. A multi-drug regimen against TB will always be needed in order to prevent the development of resistance, and it is essential that new anti-TB compounds combine well with other agents. R207910 has shown optimal activity when combined with both first- and second line anti-TB drugs, and has even shown to *increase* the efficacy of the standard regimen.

A double-blind, randomized, placebo-controlled trial in healthy male individuals determined R207910's safety, tolerability and pharmacokinetics in humans. Both single and multiple oral dosing showed the compound was well absorbed, reaching peak concentrations within 5 hours with an effective half-life of 24 hours. Both single and multiple dosing showed good safety and tolerability. ATP measurements in mammalian cells exposed to R207910 have indeed shown that human mitochondrial ATP synthase were 20,000-fold less sensitive to R207910 than to *M. tuberculosis* (54). Interestingly, upon sequence comparison of the human *atpE* gene with that of *M. tuberculosis* as well as the R207910-resistant mutants, mammalian mitochondria displayed a methionine at amino acid 63, which is the same point mutation that has been identified in the resistant mutants (54). The mammalian ATP synthase's low sensitivity to R207910, possibly explained by the polymorphism seen in the *atpE* gene, offers a rationale for the low toxicity seen for R207910 in humans.

Paper II's extensive characterisation of R207910 established this compound's potential to becoming a new anti-TB drug for the treatment of both DS and MDR-TB. Since then, numerous studies have followed further characterizing and most importantly, phase II clinical trials for the treatment of both DS and MDR-TB have been ongoing (117, 141). In the first Phase IIa trial for the treatment of DS-TB patients (giving 7 daily R207910-doses), R207910 (termed TMC207) had a delayed Bactericidal Activity compared to that of RIF and INH, taking four days to exhibit a significant effect (compared to 3 days for RIF and INH) (117). This observation confirms the time-dependent activity seen for the compound in the *in vitro* and animal studies. This same trial further confirmed the compound's good safety and tolerability in patients. Recently, the first results from a Phase IIb clinical trial for the treatment of MDR-TB have shown that TMC207, when combined with a standard-of-care second-line treatment regimen, had a significant effect in reducing patients' bacterial loads. After eight weeks of treatment, 47% of patients were culture negative (as assessed by MGIT cultures), compared to 8.7% of patients treated with the second-line regimen alone (38). These results further underline the compound's strong antimycobacterial activity and potential to decrease treatment duration in patients. At the same time, these data clinically validate the ATP synthase as an exciting new target for TB drug discovery. The second stage of this clinical trial will further assess TMC207's activity in 24-week treatment regimen using the same second line treatment regimen (141).

3.3 PAPER III

R207910's Anti-Mycobacterial Spectrum

In **Paper III** we aimed to further characterise R207910's activity against the Mycobacterium genus. The MIC was determined against DS- and MDR *M. tuberculosis* isolates as well as against MAC isolates and 18 other NTM species.

Although susceptibility tests showed R207910's high activity on both DS and MDR isolates (18), to support the process of clinical trials for the treatment of MDR-TB patients, R207910's specific activity against MDR *M. tuberculosis* was determined. As we saw in **Paper II**, no difference in R207910-susceptibility was seen between DS and MDR *M. tuberculosis* clinical isolates (18). All but one isolate had MIC levels below or

equal to 0.06 μg/ml (MIC range 0.002-0.120 μg/ml; MIC_{50} = 0.03 μg/ml). The one identified XDR isolate, which was MDR with additional resistance to amikacin and ofloxacin, exhibited an MIC of 0.03 μg/ml. This finding further showed R207910's lack of cross-resistance with second line anti-TB drugs. The first results of the phase Phase IIb clinical trial for the treatment of MDR-TB patients have indeed shown R207910's (TMC207) high activity against MDR *M. tuberculosis*, substantially decreasing the time to culture conversion in patients following 8 weeks of combinatorial therapy (38).

Seventeen of the 20 tested NTM species showed high susceptibility to R207910, with 8/20 showing MIC-levels below or equal to 0.03 μg/ml (MIC range: 0.03-0.5 μg/ml) with no difference being seen between fast and slow growing NTM. **Table 1** in **Paper III** shows a detailed list of these results (60). As it was seen in **Paper II**, R207910 showed high activity against the clinically relevant MAC (18). All the 22 tested MAC strains, representing 13 different serotypes, were inhibited at 0.25 μg/ml (MIC range: 0.03-0.25 μg/ml). MAC-infections, a leading cause of opportunistic infections in immune suppressed patients, are known to be naturally resistant to several antibiotics (4). R207910's strong activity against MAC-isolates shows its potential to treat such infections.

R207910 strong *in vitro* activity against the NTM support the compound's future potential for the treatment of such infections. However, knowing that the correlation between *in vitro*-drug susceptibility and drugs' effect during treatment can vary for certain species, further *in vivo* studies and clinical studies would be needed to confirm the effect for the specific species (4, 53).

Three NTM species, *M. xenopi*, *M shimoidei* and *M. novocastrense*, were found to be naturally resistant to R207190, showing MIC-levels between 4-8 μg/ml (60). *M. xenopi* had already been shown to have a polymorphism at amino acid 63 in the c subunit of the ATP synthase (107). Upon sequencing, the same polymorphism (Ala63Met) was seen for *M. shimoidei* and *M. novocastrense* (**Figure 2 Paper III**). Mutations in amino acid 63 had already been implicated in the resistance of *in vitro*-selected *M. tuberculosis* mutants (Ala63Pro) (18, 35, 107). As it has already been suggested, the bulkier amino acid, methionine might interfere with R207910's ability to reach its postulated binding pocket at amino acid 61 (glutamate), thus preventing the compound's effect on these species (35). It is interesting to see that human mitochondrial ATP synthase, also known to be unaffected by R207910, have the same methionine at amino acid 63 (54). It would appear that the amino acid 63 plays an important role in R207910's ability to reach its target, and thus influences cells' and organism's susceptibility to the compound.

In conclusion, R207910's strong anti-Mycobacterial spectrum has further been established, showing the compound's strong activity against DS and MDR *M. tuberculosis*, MAC isolates as well as other clinically important NTMs. This spectrum of activity further correlates with the polymorphisms seen in the *atpE* gene of the susceptible and resistant Mycobacteria. The findings of this study not only supported the further clinical development of the compound for the treatment of

DS- and MDR-TB but also supported its potential to be used in the treatment of NTM-infections.

3.4 PAPER IV

In vitro Resistance-Development to R207910

Paper IV presents an in depth characterisation of *M. tuberculosis in vitro* resistance-development to R207910. We determined the *in vitro* mutation rate to the compound, the level of resistance, mechanisms of resistance and finally the level of fitness cost associated with resistance. Knowledge of these aspects of R207910-resistance will be key in assisting to set treatment guidelines not only to assure patients' cure but also to prevent the appearance of clinical resistance.

Applying the *Luria et Delbrück* fluctuation assay (84), we selected spontaneous independent R207910-resistant mutants *in vitro* from three DS and four MDR *M. tuberculosis* clinical isolates. Mutants were also selected from the *M. tuberculosis* H37Rv reference strain. For each isolate, 12 low-density non-selective liquid cultures were prepared and after 4 weeks, dilutions of each were plated on selective media and mutant colonies enumerated. Selection was conducted at three different concentrations, 0.3, 0.9 and 3 μg/ml (10x, 30x, 100x the MIC of R207910-susceptible strains). In total, 84 independent cultures were prepared (7 isolates x 12), each of which was exposed to the three selective concentrations. A more precise description can be found in the **Material and Methods** in **Paper IV**.

We observed a concentration-dependent threshold for resistance development, with no mutant colonies appearing at the highest selective concentrations (100xMIC; please refer to **Figure 1** in **Paper IV**). The mutation rate for the two lowest concentrations was comparable to those previously reported for RIF and INH (10^{-8} mutations per cell generation) (34, 148). At the lowest concentration (10xMIC), the mean mutation rate was $1.8x10^{-7}$ mut/Cgen and ranged between $8.9x10^{-9}$ and $4.7x10^{-7}$ mut/Cgen for the different isolates (from here on named parent groups). At 30xMIC, the mean rate was one log lower ($1.3x10^{-8}$ mut/Cgen) with a range between $2.4x10^{-9}$ and $3.9x10^{-8}$ mut/Cgen. No difference was seen between mutation rates of the DS and MDR parent strains. Furthermore, all mutants were confirmed to be resistant to R207910 ($0.12 \geq MIC \leq 3.84$ μg/ml), with no difference seen between mutants derived from DS and MDR parent isolates (**Figure 2** & **Table 1** in **Paper IV**).

Our results indicated that *M. tuberculosis'* spontaneous rate of mutation to R207910 relatively low. The measurements can be translated to reflect that in a population of 10^7 to 10^9 bacilli, the probability is that *one* bacterium will be resistant to the compound. Knowing that TB-treatment will always be combinatorial, if we consider that R207910 might be given with RIF and INH, the probability of attaining bacteria resistant to all *three* drugs is extremely low. In every 10^{23} (10^7 x 10^8 x 10^8) bacilli, one will be resistant to all three drugs, a reflection of the need for three spontaneous mutations.

The lack of resistant mutants at the highest selective concentration indicates that there is a Mutant Preventive Concentration (MPC) of R207910; i.e. a concentration at which

the least susceptible bacilli in a population 10^{10} bacterial cells cannot survive (39, 40). Although only 10^{8} cfu were plated at each selective concentration, a total of 84 independent cultures were plated on 100xMIC, representing $8.4x10^{9}$ bacteria. In a total of $8.4x10^{9}$ bacteria, not a single mutant colony was observed, suggesting that the MPC of R207910 is between 0.9 µg/ml (30xMIC) and 3 µg/ml (100xMIC). More precise MPC-determinations, plating 10^{10} cfu, would be needed to confirm this statement.

The ideal dose for any given antibiotic is the one that achieves a concentration above the MPC at the site of infection, thereby targeting both the susceptible and resistant population. When R207910 was given at 400 mg/ day for 7 days, maximum serum concentrations reached 5.5 µg/ml at day seven, without causing adverse effects (117). This would suggest that growth of resistant bacilli within a patient would be inhibited to grow at least as long as this maximal serum concentration is maintained. Although the exact levels of the compound within the lungs are not known, R207910 has shown good tissue distribution (18), suggesting good drug levels would be present at the site of infection.

To characterise the mechanism of resistance among the mutants, we sequenced the *atpE* gene of 53 mutant clones, from both 10x and 30xMIC selection assays. Five different point mutations causing amino acid substitutions within the ATP synthase c subunit (shown in **Figure 6** and listed in **Table 1** of **Paper IV)** were identified in 15 mutants. Three of these, Asp28Val, Ala63Pro and Ile66Met, had previously been reported in *in vitro*-selected resistant mutants and two, Asp28Pro and Glu61Asp, were not described previously (18, 60, 107). As discussed earlier, structural analysis has suggested that R207910 has a binding pocket within subunit c of the ATP synthase, more specifically to the Glutamate at position 61. The substitutions Asp28Val, Ala63Pro and Ile66Met are all thought to interfere with the compound's interaction in this binding pocket (35, 107). The same mechanism of resistance would probably apply for the two newly identified substitutions.

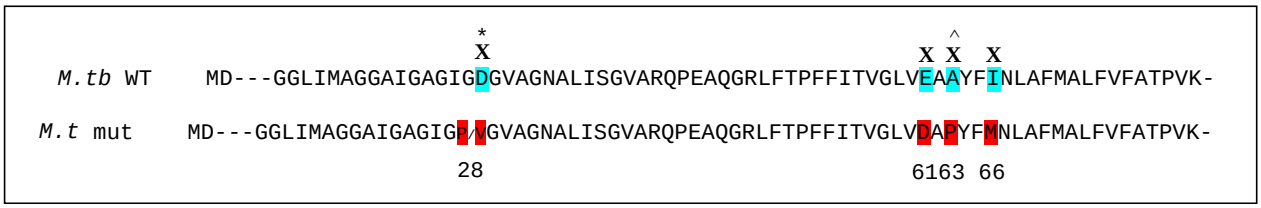


Figure 6: Sequence alignment of atpE protein sequences from R207910-suseptible and R207910-resistant *M. tuberculosis*. (X) Amino acid in which a substitution was been identified. (*) Corresponding amino acid substitution previsouly seen in an *M. smegmatis* resistant mutant. (^) Corresponding amino acid in which naturally resistant NTM and mammalian *atpE* proteins have polymorphism (met). Fig adopted from *Huitric et al 2007* (60).

More interesting was the observation that in 38 mutants, showing high levels of resistance, no changes were observed in the *atpE* gene. Furthermore, no mutation was seen upon sequencing of the F0 operon and entire ATP synthase (F1 and F0 operon) of 11 and 3 mutants, respectively. We also tested a subset of the mutants' susceptibility to second-line drugs and saw no cross-resistance. Our data suggest the existence of a second mechanism of resistance to the ATP synthase inhibitor. One of the possible mechanisms is a mutation in BlaI. BlaI was implied in Mycobacterial resistance to

beta-lactams. Two ATP operons (F0F1) were found to be co-transcribed with *rv1303*, a membrane bound-protein with unknown function that is regulated by the BlaI repressor (119). The authors further considered the observation that in *S. aureus* the cleavage of BlaI by another enzyme, BlaR, might lead to an increased expression of the rv1303-F1F0 transcript upon exposure to ATP synthase inhibitors (119, 155). It was therefore interesting to speculate that a resistance mechanism among our mutants could be the deactivation of BlaI, which would shut off the enzyme's repression of the rv1303-F0F1 operons and increase the expression of the ATP synthase. An increased expression of ATP synthase would result in resistance to R207910. However, no mutation was identified upon sequencing the *blaI* of the mutants (data not shown). It is too speculative to suggest where other resistance mechanisms might exist within the bacilli and we have not been able to test other options. We are now running whole-genome sequencing to see whether resistance mechanisms might be identified.

Mutations conferring resistance are known to affect the bacterial fitness and depending on the gene's function in the bacterial cell, the level of biological cost will vary (17). Fitness assays were therefore performed on a mutant clone of each of the five identified classes of *atpE* mutants as well as one clone of the mutants exhibiting a *wild-type atpE* gene. Specifically, competition assays between each mutant and its R207910-susceptible, isogenic parent strain were conducted in non-selective broth. As shown in **Figure 3** of **Paper IV**, all mutants had a relative fitness equal to 1, defined as the ratio between the mutant's and the susceptible strain's growth in non-selective conditions. This suggests that for the six classes of resistant mutants (the 5 with c subunit substitutions and the class with an unidentified resistance mechanism), resistance to R207910 does not come with a selective disadvantage to the bacilli.

To further confirm the lack of fitness cost seen in the resistant mutants, it would have been interesting to further assess their ATP synthase function. In their target-validation of the ATP synthase, *Koul et al.* did however observe that the ATP production of a mutant *M. smegmatis*, harbouring the Asp28Val mutation, was not affected when exposed to R207910 (72). It is further interesting to consider the three naturally resistant NTM species as well as the human cells' c subunit which have a polymorphism at amino acid 63, with a methionine instead of the *M. tuberculosis* alanine (54, 60, 107). The presence of different amino acids, at least in position 63, does not appear to affect the function of the ATP synthase. This would explain the maintained biological function in our R207910-resistant mutants.

It is concerning that resistance to R207910 does not appear to confer a fitness cost to the bacilli. This would suggest that within the human host, even in the absence of the compound, resistant *M. tuberculosi*s would have the same selective advantage as susceptible bacilli populations. However, this does not imply a negative impact on the clinical use of the compound. Based on our results, the rate of resistance to R207910 is low, concentration-dependent and at a concentration of 3 μg/ml, a level attainable in the human host, resistance-development is prevented. Lastly, the treatment of TB will always be a combination regimen, with drugs that target different key functions of the bacilli, a vital aspect in the strategy to prevent development of resistance. Our results therefore indicate that resistance to R207910 can be prevented in the clinical setting, provided TB-treatment guidelines of combination therapy are followed.

4 CONCLUDING REMARKS & FUTURE PERSPECTIVES

The four papers of this thesis focused on two key aspects within the field of TB-research; drug-resistance development and the characterisation of a new anti-TB drug candidate.

In **Paper I**, the span and resistance-level of *in vitro*-selected RIF-resistance mutations was similar to what is seen in RIF-resistant clinical isolates, with the exception of one amino acid substitution (Ser522Leu). Such *in vitro*-systems, at least for RIF, offer a good model for investigating the resistance-development in clinical isolates, enabling more precise manipulation of strains. For example, to investigate the hypothesis of mutator phenotypes among strains of the Beijing genotype, fluctuation assays could be run on DS Beijing isolates that exhibit such "mutator" polymorphisms.

Paper II, **III** and **IV** presented and discussed the characterisation of R207910, a new anti-TB drug candidate that has since entered Phase II clinical trials. Targeting a new site within the bacilli, the ATP synthase, this Diarylquinoline's strong effect on DS, DR, MDR and XDR *M. tuberculosis* and its ability to potentiate the first-line drugs, indicate that R207910 is a promising new TB-drug with the potential to decrease the time of TB-treatment, prolong dosing-intervals during treatment and most importantly, be used for the treatment of DS-, MDR- and XDR-TB. These three factors are essential in the challenge to eliminate TB globally. Simplifying and shortening treatment will improve patients' quality of life and make it substantially easier to assure compliance to treatment. This will in itself minimise the risk for resistance-development and subsequently prevent the further spread of resistant strains. Lastly, effective treatment against MDR and XDR-TB is essential to effectively control and stop the transmission of MDR and XDR-strains.

Although R207910-resistant mutants did not show an abrogated fitness level, the concentration-dependent and relatively slow mutation rate, suggests that resistance to the compound can be prevented, especially considering treatment doses above the *mutant preventive concentration* deem attainable. It is important to underline that such a prediction is based on the notion that the compound should always be used in combination with other active drugs and that treatment guidelines are strictly followed. Resistance to the existing first-line drugs is also preventable, yet due to their ill use, drug-resistance has emerged and successfully spread globally. The characterisation of R207910-resistance in *M. tuberculosis* before the compound reaches clinical use does present a head start. Treatment doses and guidelines can be set that are directed at not only curing TB-patients but also at prolonging and hopefully preventing the emergence of clinical R207910-resistance.

5 ACKNOWLEDGEMENTS

These past years of work would never have been possible without the guidance, help, support and encouragement from my supervisors, colleagues, friends and family.

Sven, you always gave me the freedom to explore the world of science. Thank you for always encouraging me to attend conferences, work in other areas of TB and participate in courses – be they in Sweden or overseas. I really appreciate the trust you put in me, giving me the independence to plan my work and make sure I get it done on time. Thank you for all your positive support and encouragement during these last few crazy months.

Dan, är du i stan?! ☺ Thank you for guiding me through the frustrations of fluctuation assays and fitness assays. We at the SMI miss the fun discussions during the "fika breaks" and you delicious semlor at Mardi-Gras!

Koen, working with you has been wonderful! You have always found the time to answer my longest of emails, enthusiastically coming with input, opinions and new ideas. Thank you so much for the discussions we've had over the years and for all the encouragement and genuine interest you have shown. Hartelijk dank! ☺

Working in the P3-lab would have been unbearable without everyone in the "TB-gang"! **Pontus**, the labs Strindberg look-alike and Linnaeus fanatic! Your help has been invaluable, not only during this race to the end, but always. You can have 100-things to do (incl preparing you excel sheets☺), but you always have the time to answer a question, help in the lab, or just simply grab a cup of coffee. You are a superstar! **Jimpan**, you are our very own singing-bee! I would have never survived those P3 marathons without your smile, good humour, nor, I have to admit, Amanda Lear playing in the background. Jag kommer att sakna alla våra pratstunder ☺. Thank you: **Lisbeth**, our "lab mum", for always making sure we were well, thank you for all your help in the lab and the good laughs at "fika time" ☺; **Melles**, my "life-line superstar"; **Solomon**, for all the talks of good Czech beer, **Andrzej**, for teaching me acid-fast staining ☺; **Gunilla**, for the lovely talks about Portugal; **Alex**, "labbets klippa", for your encouragement during crazy times in the lab; **Ramona**, the labs early bird – no matter how early I got into work, you were always there!; **Senia**, always a ray of sunshine; **Maria** and **Anna**, for the new inspiration you have brought to the lab; **Benon**, for your warm smile – it was wonderful to see you in Keystone!; **Fred**, for the good laughs over coffee; **Sofia**, tambem pelas conversas sobre um copo de café – te desejo boa sorte com tudo!; **Lech**, for making me laugh when my brain was too tired to think; **Jola**, my "computer & statistics program star", for the good talks and laughs in the corridor, coffee room and P3 ☺; **Nasrin**, welcome to the TB-gang! ☺; **Tuija**, my mentor, for always making sure we were safe in the P3; **Juan Carlos**, aka "JCT", the "inventor/gadget expert" of the lab; and **Kristian**, aka Krille, the philosopher of the lab.

Thank you to everyone at **Bacteriology**; for the years' of fun on "Bakt days", Christmas celebrations and the everyday "fika breaks". A special thank you to you **Britt-Marie**, the star of the corridor. Where would we be without you?! You always

have our well-being at heart and always make sure everyone feels welcome and happy ☺.

Tack **Sven B.,** för den underbara resan till Ghana; den var en betydelsefull och lärorik resa på många plan. Tack för det intresse och stöd du alltid visar! ☺

Markus Maeurer, **Sanna**, **Sayma** and **Lalit**, it was great fun to collaborate with you and learn more about TB-immunology. **Sayma**, it was great fun working with you in the P3, you always have a smile on your face! ☺

Thank you to the entire **Markus Maeurer** group for the fun talks, P3-lab work, fun at conferences and the Journal Club. **Markus S**, the journal clubs have been invaluable!☺ **Isabelle**, on y est presque! Merci pour le bon temps à Keystone et pour tout ton encouragement ces derniers temps. Et, oui! Il faut qu'on aille finalement prendre une (ou deux) bierres! ☺

Everyone at the "**Koen Andries Lab**" in Beerse, thank you for welcoming me to your lab. **Anil**, thank you for the good discussions and input on the project and **Nacer**, merci pour les bonnes conversations pendant mon temps à Beerse et pour tout ton aide avec mon application!; **Peter**, thank you for the good collaboration on the papers! ☺ **Luc**, **Brenda** and **Karen**, it was great working with you in the lab, having good laughs, learning a bit of Flemish (I'm afraid I've forgotten it all) and eating frietje. ☺

And now to my "second work-home", the **MTC**:
The "**Klas Kärre Corridor**" – through which I've walked several times in a single day! It's always wonderful to hear the laughter, loud music, loud voices and singing whilst walking past the different labs and offices. **André**, é sempre fantastico rire contigo! And I'm *not* being sarcastic ☺ Obrigada por todas as conversas e risos!

Everyone in **Chiodi Group,** thanks for all the great espresso breaks (which often included some yummy chocolate)! **Francesca,** thank you for always welcoming us for coffee; **Monsieur,** pour tes sourires; **Nancy,** for the good laughs and chitchats! We're both nearly done! ☺; **Simone,** grazie mille per sempre me imprestare la tua tazza – è per il bene dell'ambient!; **Alberto,** the greatest collector of beer caps! Thanks or all the chats & laughs; **Stefano,** sempre generoso e gentile – grazie per il tuo olio d'oliva! ☺**, Malgocha, Lihn, Tang** and members of the coffee breaks, **Wendy** and **Polya –** you are all always smiling ☺!

The Muppet Crew – what would PhD-ing have been without our monthly pubs! **Kermit** and **Miss Piggy**, I only had to attend one pub before becoming a Muppet myself (a true honour ☺)! The best was preparing them – finding outfits we could look *gorgeous* (**Kermit**, your Morticia style was the best!) and cooking late into the night, with a cold bottle of beer and wonderful laughter! The **Pubmobile** and its fantastic driver, **Mauro!** And the honorary Muppets – **DJ Thoughtful**, you never seized to amaze us with you hair-dos and your eccentric dancing; **Lech** (Sminkio?), you too are quite the dancer, only with a slightly different style! Already after your first pub we knew there was a Muppet in you! **Greger** and **Sandor**, thanks for always helping out, and more importantly, for the wonderful Langos! ☺ Good luck to the **NEW** crew! ☺

And of course, **Gerry**! ☺ The master of Trivial Pursuit – I heard you're hosting the next tournament! Going for beers with you and **Mauro** is always great fun – I still can't believe you guys didn't dare finish off that bottle of Tequila! ☺

Jo and the whole "**Thursday group**", thank you for all your support and for making me dare ask all my questions! And for all the wonderful conversations over a pint (or two) of good old Sam Adams! ☺

To the Uppsala girls – **Sofia**, **Anna**, **Disa** and **Anna-Karin**! Thanks for the fun singstar nights! Sorry for missing the last few – lets plan for the next one soon ☺

Piiha-Lotta, tack för de underbara tiderna i Linköping och de underbara pratstunderna dem senaste tiderna!

The biggest Superman-fanatic I've ever known… Well ok, the *only* one I've known… **Joshua D**, you made me think critically, ask questions and seek the answers myself, I will always cherish that. Thank you Josh for the great times riding Sara, eating delicious food and laughing ☺.

My dearest friend, **Branca**, we've known each other since kindergarten! Although weeks can go by between our skype calls, it always feels like it was only yesterday. ☺ Branca, you are a star!

Nicolas, mon frère français, t'est le meilleur des meilleur! N'importe le jour, t'as toujours un sourire a me prêter et un "hug" a me donner. "Life is *truly* wonderful"! ☺ Merci pour ces derniers temps quand t'est venu me faire compagnie devant la télé avec des pâtes "à la pesto". Je te promets, la prochaine fois je ferai un *vrai* repas – même que ca sois du pesto faite-maison! …Et après on sortira dancer!

Danika, solo tre anni fa… Sensa te e la insalata con pomodorini, *nozzardlla* e aceto balsamico, la vita sarebbe…BORING! ☺ And now there are two of you and soon you will be three ☺. **Danika** e **Mauro**, voi due soni meravigliosi. Si se tratta di una giornata *rosa* o *blu*, mi fate sempre sorridere e ridere. Mi fate sempre sentire en casa - mi hanno anche il mio proprio camera! Ma promesso che Massimiliano Ruden può la prendere in prestito, anche quando fatto il babysitting ☺.

I am truly a lucky girl who is surrounded by a loving and supporting family.
Elisa och **Gustaf**, det finns inga ord som kan beskriva allt vad ni har gjort för mig – mitt kroppspråk har alltid varit bättre än mina ord. Utan ert stöd, tålamod och kärlek hade jag aldrig nått där jag är idag. Ni är underbara! Och nu har jag äntligen lyckats skriva klart min avhandling, som jag hade aldrig lyckats börja utan er inspiration den kvällen i Cervinia ☺.

Shaggy, även kallad **Médé 6**, även kallad **Elvis**, även kallad **Andreas** ☺. Du är underbar! Ditt leende och dina kramar är de "goaste" som finns! Jag längtar till att dansa disco med dig i Spanien!

Maria, du är en klippa! Du har alltid hundra saker på gång, och hinner med allt! Vi har båda haft mycket på gång men förhoppningsvis blir det lite lugnare snart. Jag längtar till vår middag med teater! ☺

Elisa, Gustaf, Anna, **Lasse**, **Elisa**, **Gabriel**, **Johannes**, **Gabriel**, **Sofia**, **Mikael**, **Benjamin**, **Jakob, Maria och Shaggy.** Det är alltid underbart att komma hem till söndagsmiddag med ett bord dukat för allt mellan 14-17 personer, med springande barn och glada leenden – ett riktigt organiserat kaos ☺!

Papa. Et oui, "ta grande petite dernière" va presque devenir Docteur ☺. Tu m'as envoyer en Suède et t'as voulu me laisser rester pour finir mes etudes ici, merci papa. Je suis tellement contente que tu viens pour mon "grand jour"; because… I love you! ☺

Viv's and **Daves**, in kilometres we have always been far from each other, yet you have and are always close by. You've both been such great support all throughout the years, not the least now in these crazy times – your phone calls of encouragement have made all the difference. I love you.

And lastly, my two ladies, Mama **Mim's** and **Astoush**. Mim's, you've been the greatest! Ever since I was a little kid, you have always been there for me, watching out for your little sister. I don't know what I would have done without your patience, support and encouragement these last months – indeed I owe you many massages and glasses of wine in front of some *good* TV! Astoush, thank you for all your hugs and kisses whilst I've been writing my "sickness book", and for making everyday a sunny day ☺.

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