Virulence of drug-resistant *Mycobacterium tuberculosis* and activity of drug combinations against drug-resistant and drug-susceptible isolates in *ex-vivo* and *in vitro* models

Emma Rey Jurado

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CERTIFIQUEN

Que el treball de recerca que té com a títol "**Study of drug-resistant *M. tuberculosis* virulence and activity of drug combinations against drug-resistant and drug-susceptible *M. tuberculosis* in *ex-vivo* and *in vitro* models"** ha estat realitzat sobre la seva tutela i reuneix tots els requisits necessaris per la seva tramitació i posterior defensa davant el corresponent tribunal.

Signatura Dr. Julià González Martín

Signatura Dra. Griselda Tudó Vilanova

Barcelona, 2013

A mis padres,

A mi abuela,

A mi hermano

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ABBREVIATIONS

AMK: Amikacin

ATS: American Thoracic Society

AUC: Area Under the Curve

BCG: Bacille Calmette-Guérin

CAP: Capreomycin

CDC: Center for Disease Control and Prevention

CFP-10: 10-kD Culture Filtrate Protein

CIP: Ciprofloxacin

CLZ: Clofazimine

CYC: Cycloserine

DOTs: Directly Observed Treatment Short-course

DST: Drug Susceptibility Testing

EMB: Ethambutol

ESAT-6: 6-kD *Mycobacterium tuberculosis* Early-Secreted Antigenic Target protein

ETH: Ethionamide

FBS: Fetal Bovine Serum

GAT: Gatifloxacin

HIV: Human Immunodeficiency Virus

IFN-γ: Interferon- γ

IGRAs: Interferon-Gamma Release Assays

IL: Interleukin

INH: Isoniazid

IRAK: Interleukin-1 Receptor Associated Kinase

KAN: Kanamycin

LEV: Levofloxacin

LTBI: Latent Tuberculosis Infection

Man-LAM: Mannose-capped Lipoarabinomannan

MAPKs: Mitogen-Activated Protein Kinases

MDR: Multidrug-Resistance

MIC: Minimal Inhibitory Concentration

MOX: Moxifloxacin

NADH: Nicotinamide Adenine Dinucleotide

NF-κB: Nuclear Factor Kappa

NO: Nitric Oxide

OFL: Ofloxacin

PAMPs: Pathogen-Associated Molecular Patterns

PAS: P-Aminosalicylic Acid

PMA: Phorbol Myristate Acetate

PZA: Pyrazinamide

RIB: Rifabutin

RIF: Rifampicin

RIP: Rifapentin

RNI: Reactive Nitrogen Intermediates

ROI: Reactive Oxygen Intermediates

STR: Streptomycin

TB: Tuberculosis

TLR: Toll-Like Receptors

TNF-α: Tumour Necrosis Factor-α

TRAF6: Tumour Necrosis Factor Receptor-Associated Factor 6.

TST: Tuberculin Skin Test

WHO: World Health Organisation

XDR: Extensively Drug-Resistance

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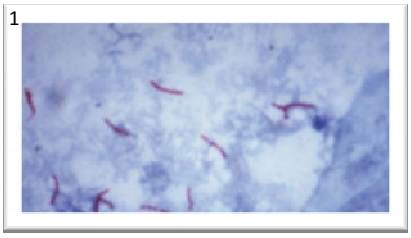
Investigar es enfrentarte a un puzzle apasionante que nunca acabas de resolver del todo, pero que cada día te proporciona una satisfacción, aunque te equivoques, porque cada error elimina la posibilidad de cometer otro.

Sidney Altman

INTRODUCTION

1. GENERAL FEATURES OF TUBERCULOSIS

1.1 Genus of *Mycobacterium tuberculosis*



M. tuberculosis was identified as the causative agent of TB by Robert Koch in 1882 (Koch, 1882), belongs to the *Mycobacteriacea*e family and is the most clinically important member of the *M. tuberculosis* complex (Figure 1).

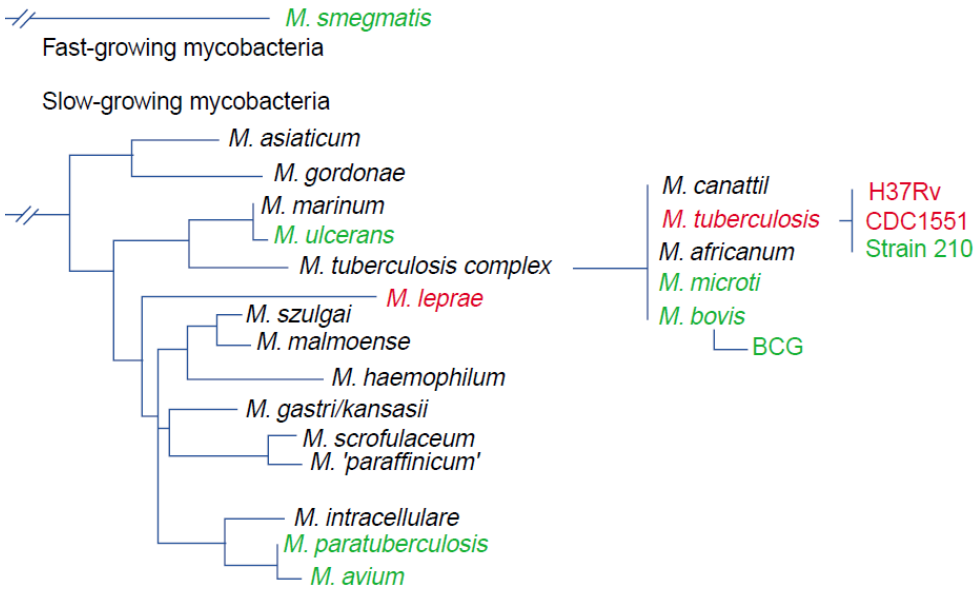


Figure 1. Phylogenetic tree of selected mycobacteria, based on 16S rRNA sequences, including those species whose genome sequences are in progress (green) or completed (red) (Brosch *et al.*, 2001).

The members of this complex include *Mycobacterium tuberculosis* (the human tubercle bacillus), *Mycobacterium bovis* (the bovine tubercle bacillus), *M. bovis* bacillus Calmette-Guérin (BCG vaccine), *Mycobacterium africanum* (heterogeneous group of strains mostly found in equatorial Africa), *Mycobacterium canetti* (human pathogen) and *Mycobacterium microti* (pathogen of voles and small mammals). *Mycobacterium* genus members are aerobic, non motile bacteria, acid-alcohol-fast and with a high percentage of C+G (62-72%). Such bacteria are classified as Gram-positive since they retain crystal violet staining. All *Mycobacterium* species share a characteristic cell wall, which differs from other bacteria by its

richness in lipids, particularly mycolic acids (Figure 2). The cell wall consists of an inner layer of peptidoglycan as well as another layer of hydrophobic mycolic acids and a third layer of arabinogalactan which links the previous two layers which are topped by non-covalent linked outer lipids and carbohydrates.

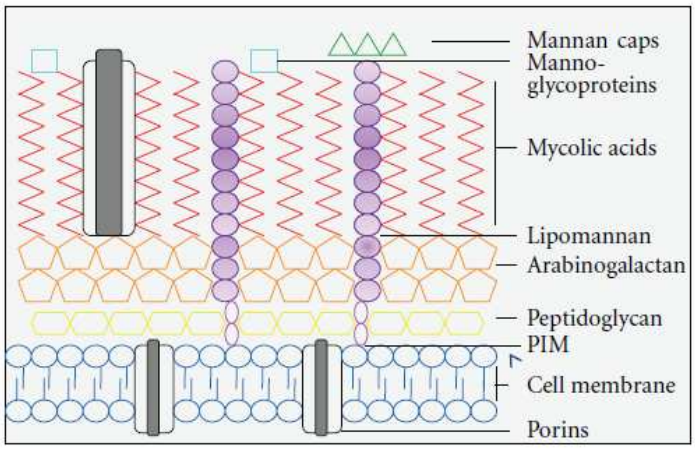


Figure 2. The structure of the mycobacterial cell wall (Kleinnijenhuis 2011).

M. tuberculosis cells are straight or slightly twisted cords from 0.2 to 0.5 µm in width and from 1 to 4 µm in length. Such bacteria have a slow growth with a doubling time of 15 to 18 hours and requiring two to three weeks of incubation to detect visible growth at 37ºC (Murray *et al.*, 2007).

1.2 Current situation of Tuberculosis

Tuberculosis remains a global threat worldwide with nearly nine million incident cases of TB being estimated in 2010 according to the WHO (2011). Furthermore, 1.1 and 0.35 million deaths from TB HIV-negative and HIV-associated TB were reported respectively. Around 80% of TB cases are reported in 22 countries, with India and China reporting the highest burden (Figure 3). Nevertheless, global efforts to control TB have led to a reduction in the absolute numbers as well as the incidence of TB (WHO, 2011).

The WHO has classified the clinical history of TB treatment history into two categories: new cases or previously treated cases (WHO, 2009). A new case is defined as a newly registered episode of TB which has not received any previous TB treatment, thereby providing recent transmission information. By contrast, a previously treated case is a newly registered episode of TB with a clinical history of prior TB treatment. According to the WHO, the proportion of resistance to at least one antituberculous drug was from 0% in two Western

European countries to 56.3% in Baju City, Azerbaijan (WHO, 2008). The emergence of MDR-TB (defined as TB resistant to at least INH and RIF drugs), XDR-TB (defined as MDR plus resistance to one fluoroquinolone and one of the three injectable second-line drugs, AMK, KAN and CAP) and HIV pandemic represents additional obstacles to the control of TB.

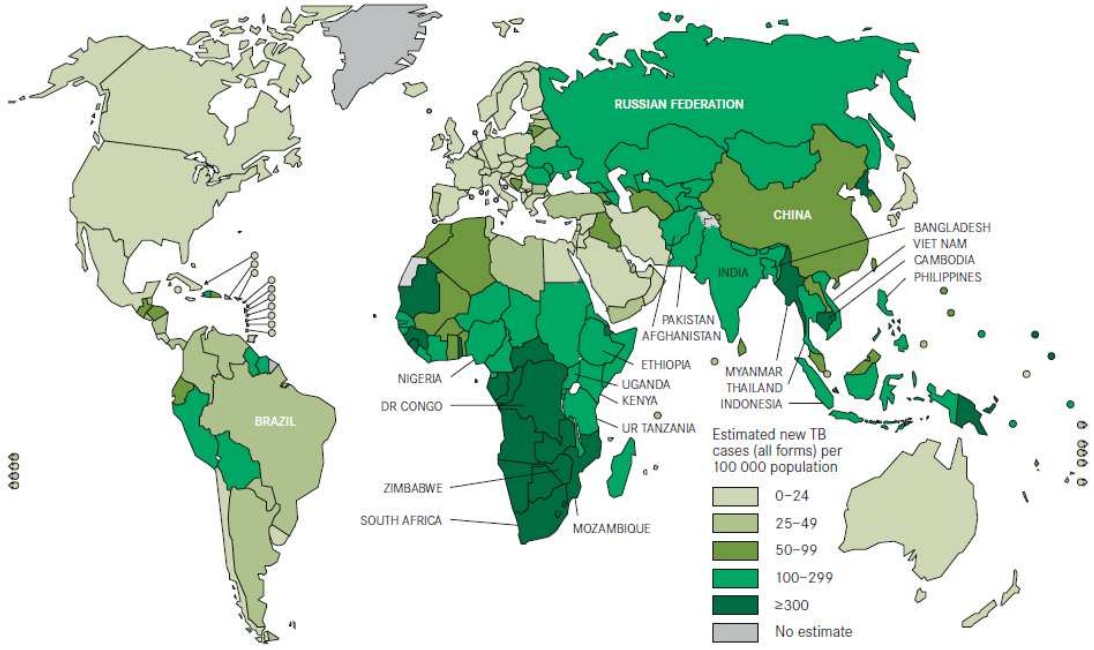


Figure 3. Estimated TB incidence, 2010 (WHO 2011).

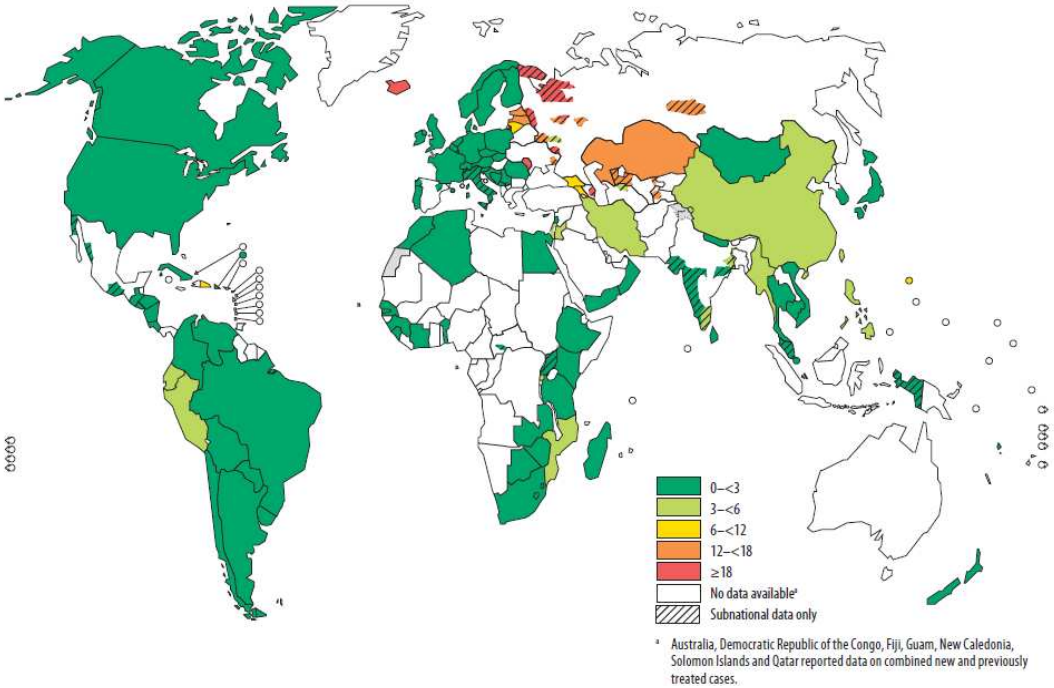


Figure 4. Distribution of the proportion of MDR-TB among new TB cases 1994-2009 (WHO 2010).

Among the 12 million prevalent cases of TB reported worldwide in 2010, the WHO estimated 650,000 cases to be MDR-TB (Figure 4). The proportion of countries with no data available is noteworthy, and thus the rates of incidence are underestimated. However, with the data reported, MDR-TB among new TB cases ranged from 0% to 28.3%. Countries from Eastern Europe and Central Asia such as Azerbaijan or Estonia have described a proportion of MDR-TB exceeding 12% among new cases. Nonetheless, the highest estimated number of cases was reported in China and India with 100,000 cases each (WHO, 2010). Indeed, to date, 58 countries have reported at least one case of XDR-TB (Figure 5).

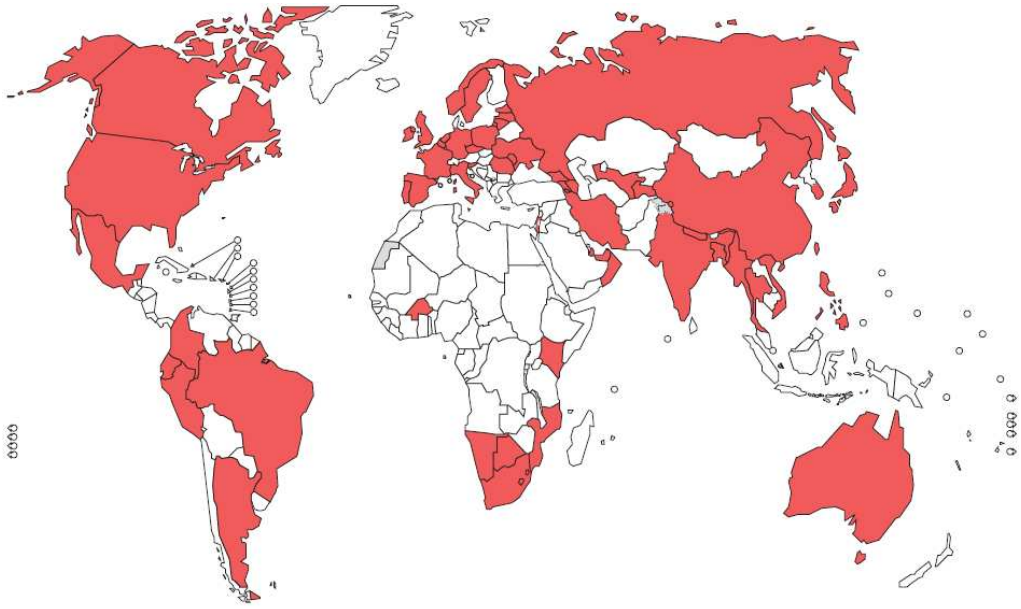
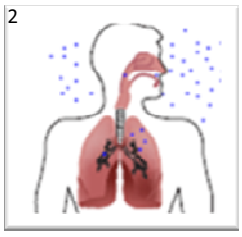


Figure 5. Distribution and countries or territories reporting at least one case of XDR-TB as of January 2010 (WHO 2010).

2. PATHOGENESIS OF TUBERCULOSIS



Aerosol transmission is the route of TB transmission. The aerosol droplet nuclei can be transmitted by coughing, talking or singing from a person with active disease. Pulmonary TB affects the lung parenchyma, the most common site of infection, although extrapulmonary TB may affect other less frequent sites such as the pleura, lymph nodes, abdomen, genitourinary tract, skin, meninges, joints and bones.

After inhalation of infected aerosols into the lungs of the host, the first cells to respond to the bacilli are alveolar macrophages and tissue dendritic cells. In contrast to other pathogens, *M. tuberculosis* survives and replicates inside macrophages. After phagocytosis, an influx of lymphocytes and activated macrophages are driven to the lesion resulting in granuloma formation. This granuloma structure prevents the spreading of the bacilli, while also providing housing for *M. tuberculosis* during a long period of time. At this time the bacilli can be latent and is known as Latent TB infection. In 5-10% of the population these latent bacilli can be released at any time during the lifetime leading to disease reactivation due to host immune factors. These factors include HIV infection, immunosuppression or patients treated with TNF-α (Figure 6).

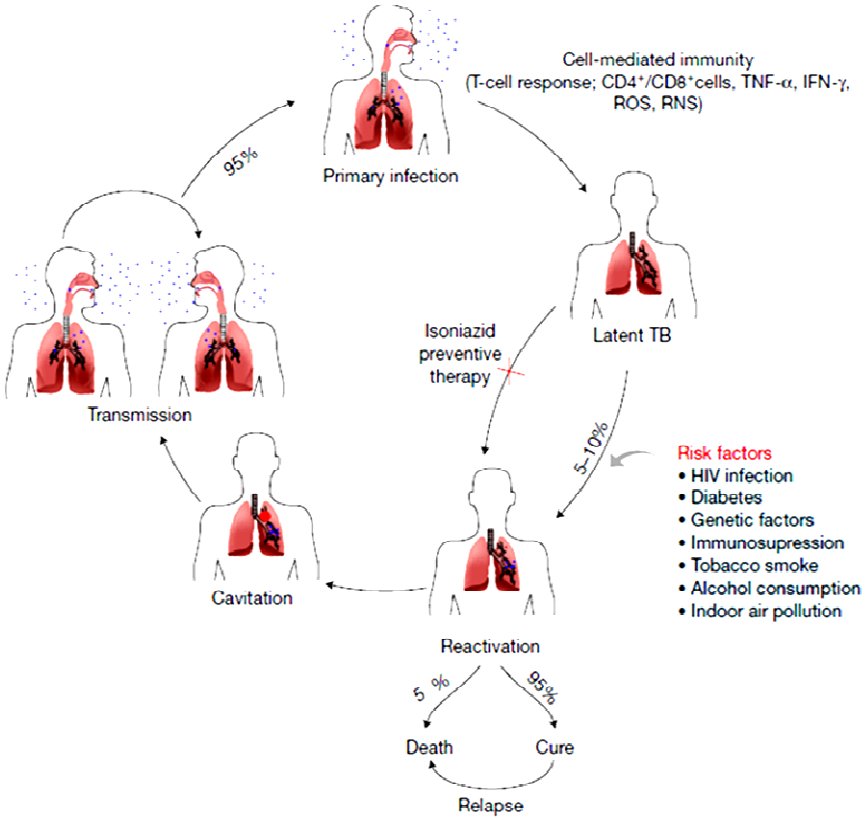


Figure 6. Virulence life cycle of *M. tuberculosis* and progression of TB (Kumar *et al.*, 2011).

2.1 Recognition of *M. tuberculosis*

Innate immune response, the first line of defence against invading pathogens, starts with pattern recognition of microbial structures called pathogen-associated molecular patterns. Some of the components of the cell wall of *M. tuberculosis* have these PAMPs (Figure 2). Man-LAM, one of the most abundant glycolipids of the cell wall of *M. tuberculosis*, is one of the patterns immune cells recognize and has been described as a virulence factor (Kleinnijenhuis *et al.*, 2011).

Recognition of PAMPs is performed by receptors expressed mainly in immune cells known as pattern recognition receptors (Figure 7). Then, exposure of the receptor molecules at the macrophage surface plays an important role in binding of the bacilli to the phagocytes. Pattern recognition receptors include complement receptors, the mannose receptor, immunoglobulin fragment carrying the constant region of the heavy chain, scavenger receptors and TLR. TLR2, TLR4 and TLR9 are described in TB (Abel *et al.*, 2002; Bafica *et al.*, 2005). The adaptor molecule Myd88 links TLRs to IRAK, a kinase. All these interactions result in downstream signalling cascades leading to the activation of a transcription factor, NF-κB, and kinases such as MAPKs which control the upregulation of cytokines and chemokines as well as other molecules essential for T-cell activation (Kawai *et al.*, 2007).

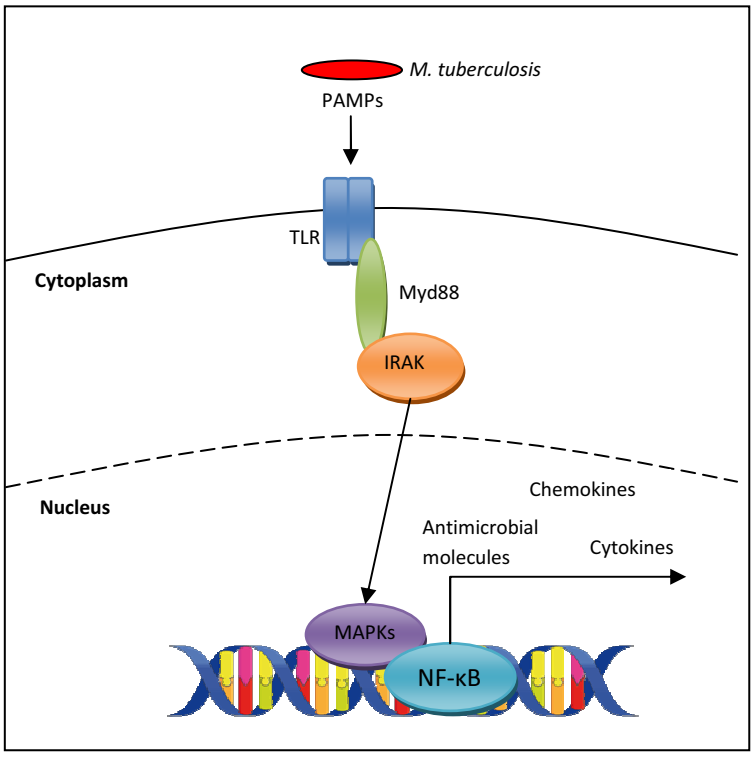


Figure 7. Recognition of *M. tuberculosis* and cascade signalling.

2.2 Adaptive response

Infected cells induce innate cytokines followed by exposure to *M. tuberculosis*, causing an adaptive immune response (Figure 8). Macrophages and dendritic cells produce IL-12 and IL-18 which lead to production of IFN-γ by NK and T-cells (Korbel *et al.*, 2008). In this way, the synergy of IL-12 and IL-18 bring about a T helper 1 cell (Th1) profile characterised by IFN-γ production. IFN-γ activates macrophages leading to production of some microbiocidal substances and cytokines such as TNF-α and IL-6. By contrast, cytokines such as IL-10 or TGF-β provide an antiinflammatory response to control the immune response thereby avoiding the pathology, although IL-10 may also play a protective role.

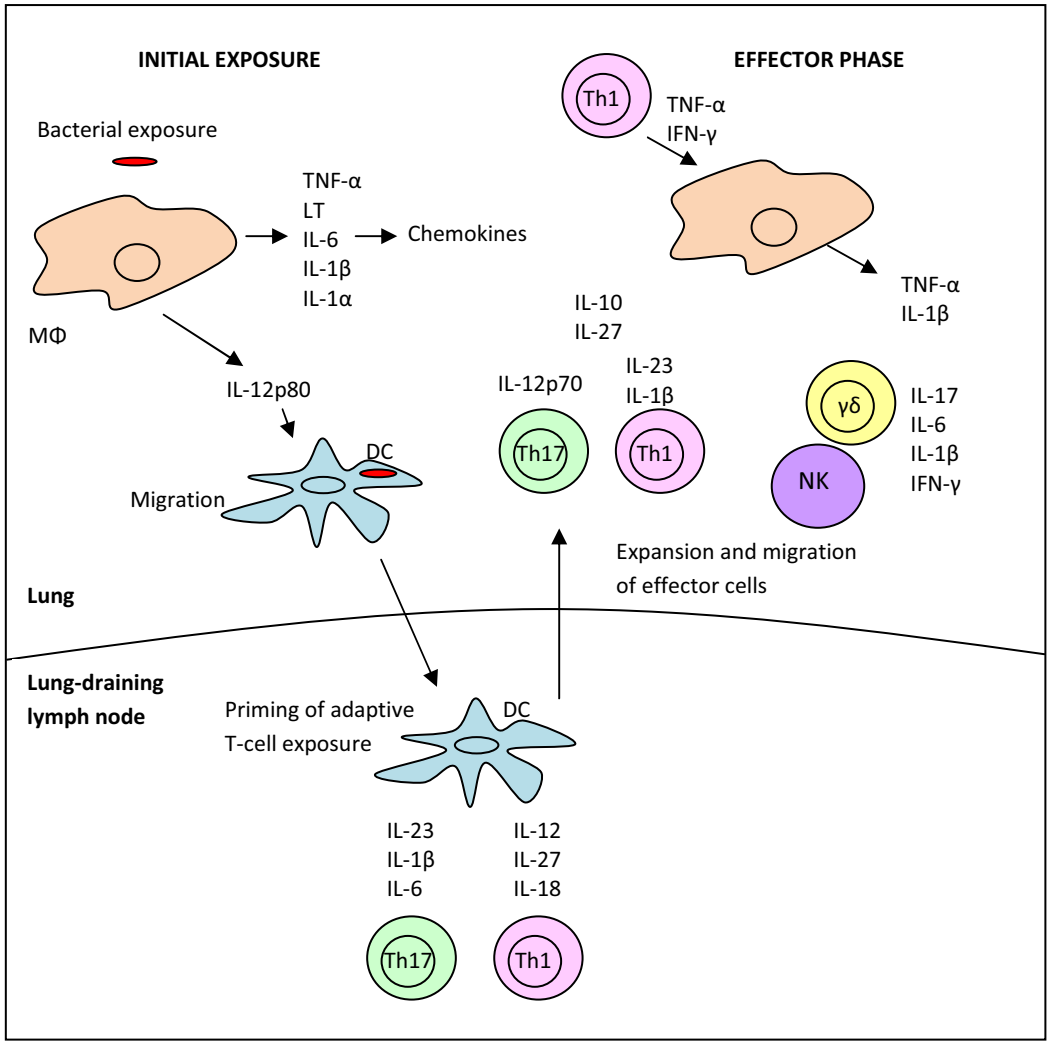


Figure 8. Role of cytokines in mycobacterial infection. MΦ: Macrophage; DC: dendritic cell (Adaptation from Cooper *et al.*, 2011).

2.3 Granuloma formation

TNF-α has been shown to be important in granuloma formation since TNF-α-deficient mice are hypersusceptible to TB. This tuberculous granuloma is a compact, organised aggregate of immune cells (Figure 9). Macrophages can fuse with giant cells, differentiate to foam cells, which are characterized by lipid accumulation or become epitheloid cells. The regions of necrosis, a characteristic feature of tuberculous granuloma, are participating cells which die. These regions are also histologically known as *caseum*. Many other cell types form the granuloma such as T-cells, B-cells, neutrophils and NK cells.

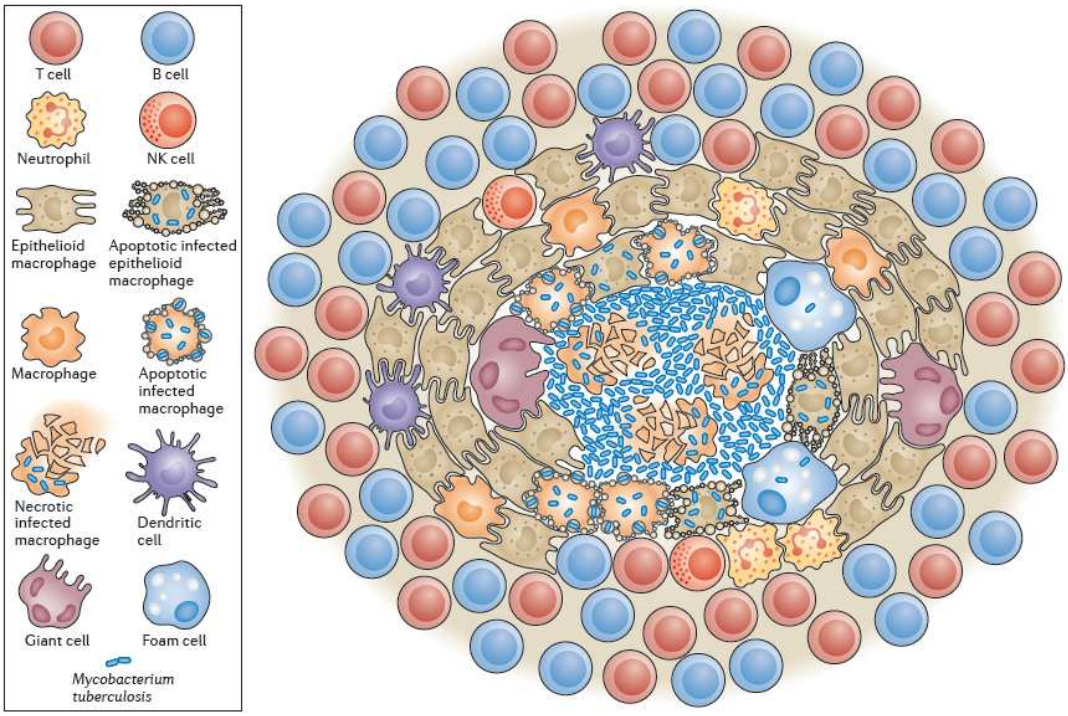


Figure 9. Structure and cellular constituents of the tuberculous granuloma (Ramakrishnan, 2012).

2.4 Antimicrobial defence mechanisms

After phagocytosis, *M. tuberculosis* resides in phagosomes at a pH of around 6.2 (Figure 10). Activation with IFN-γ results in phagosome maturation and phagosome-lysosome fusion reaching pH of 4.5-5.0. This exposes *M. tuberculosis* to host-derived stress including protons from the vacuolar ATPasa, RNI and ROI, free ubiquitin-derived peptides and lysosomal hydrolases.

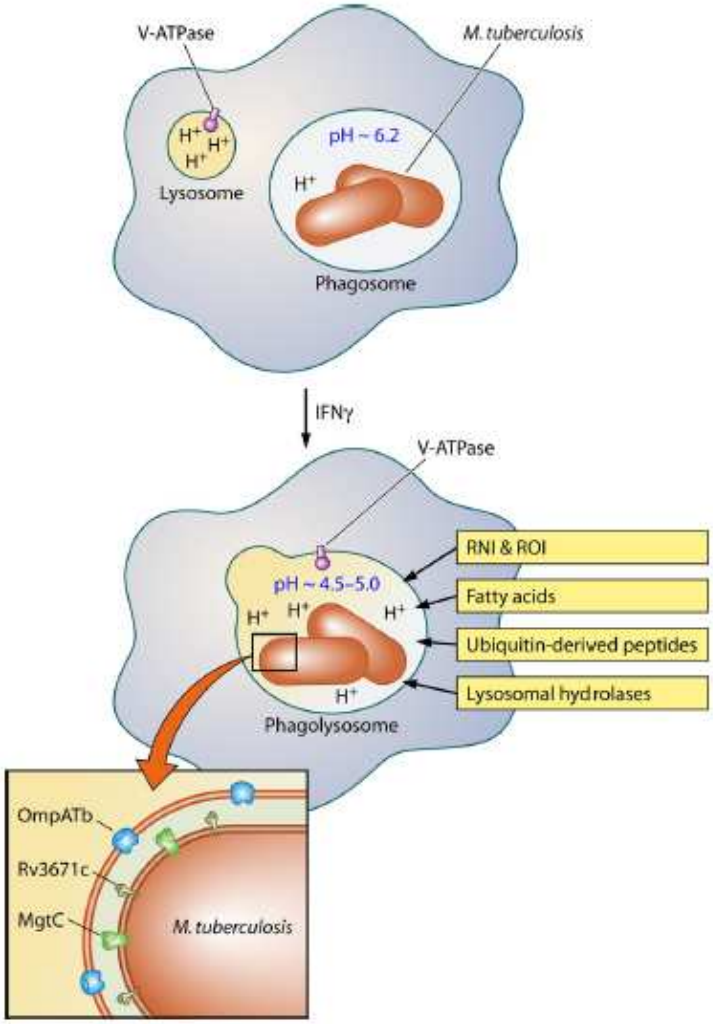


Figure 10. *M. tuberculosis* inside a macrophage (Vandal *et al.*, 2009).

The actions of ROI and RNI such as NO released by innate cells, including macrophages and dendritic cells, is considered a powerful antimicrobial defence mechanism by DNA inactivation of the microorganism (Nathan *et al.*, 2000). Although the role of NO in the killing of *M. tuberculosis* has been described in murine model, there is some controversy as to its implication in human TB. Nevertheless, it has been postulated that the absence of detection of NO in human TB may be due to limitations in detection assays in *in vitro* cultures. Bertholet *et al*. (1999) demonstrated that when expressing the iNOS transgene, human monocytic U937 cells were capable of elevated NO production on supplementation with a cofactor, tetrahydrobiopterin. *In vivo*, tetrahydrobiopterin may be obtained from cells other than human monocytes/macrophages such as lymphocytes and endothelial cells. Furthermore, different levels of NO expression have been found depending on the clinical lineage of the *M. tuberculosis* strain (Koo *et al.*, 2012).

Apoptosis is another mechanism to defend the host from *M. tuberculosis*, which causes DNA cleavage and apoptotic vesicles leading to containment of the bacilli. Nevertheless, after the death of apoptotic cells the bacilli are released and secondary granulomas may be formed, leading to the spread of the disease.

2.5 Evasion of *M. tuberculosis* from the immune system

Survival of *M. tuberculosis* within the human macrophage depends on its interference with cellular processes. Evasion of phagosomal maturation allows *M. tuberculosis* to persist within the macrophages by increasing the pH thereby altering the ATPase pump, secreting ESAT-6/CFP-10 or by an autophagic mechanism (Cardona *et al.*, 2011). *M. tuberculosis* resists acidification with the help of the Rv3671c-encoded membrane-bound serine protease, the putative magnesium transporter MgtC and the pore-forming *M. tuberculosis* outer membrane protein, $OmpA_{Tb}$ (Figure 9). Other ways described to avoid the killing of *M. tuberculosis* are the inhibition of antigen presentation, activation of apoptotic pathways (Lee *et al.*, 2011; Toossi *et al.*, 2012) and the generation of pro-inflammatory cytokines. Kumar *et al*. (2004) described the network of interaction between the host and the pathogen as a "bow tie" framework (Figure 11) in which all the interactions having a role in the final outcome of mycobacterial growth are emphasised.

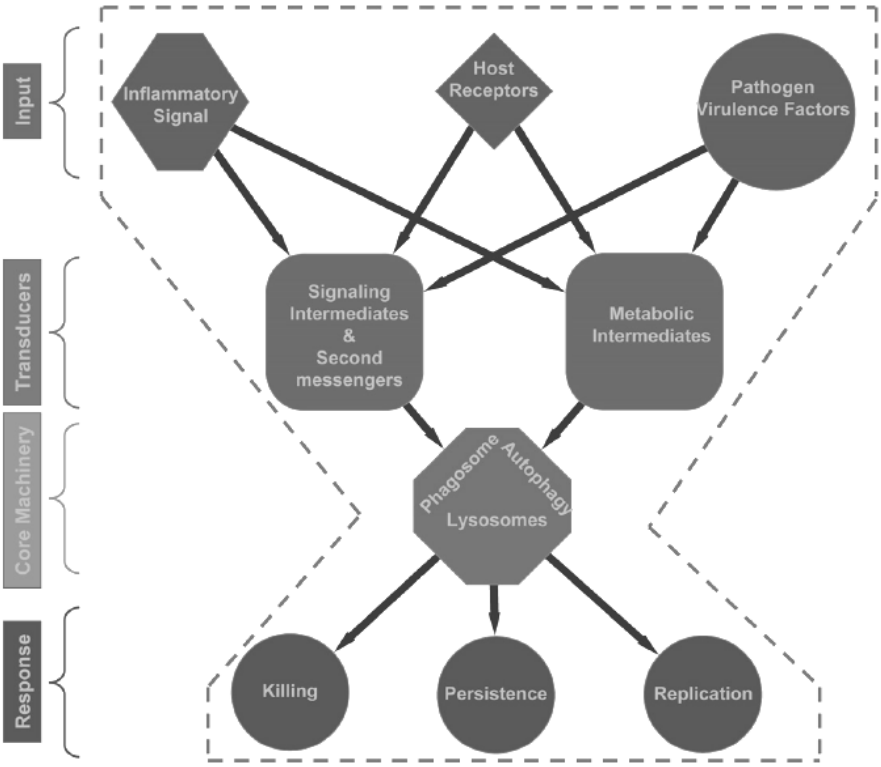
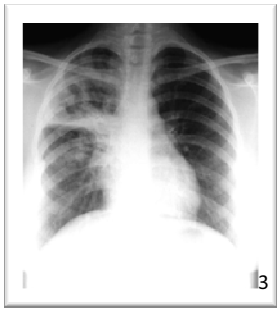


Figure 11. Framework of the network of molecular interactions between the host and the pathogen (Kumar *et al.*, 2011).

3. DIAGNOSIS OF TB



The symptoms of pulmonary TB include fever, persistent cough, breathlessness, weight loss, lack of appetite and extreme tiredness. Moreover, infiltrates or consolidations and/or cavities can be observed by chest-X ray in pulmonary TB.

3.1 Immunodiagnosis of Infection

The detection of TB infection relies on immunological markers of immune response. Two methods are currently used, the TST and methods based on IFN-γ response. The TST has been used to diagnose TB infection for the last hundred years. According to different suppliers and TB control programmes, the TST is performed by injecting 0.1 ml of tuberculin purified protein derivative, PPD, into the inner surface of the forearm, producing when positive, a pale elevation of the skin 6 to 10 mm in diameter. The TST positive reaction is interpreted as a person infected with TB with a risk of progression of TB disease (CDC, 2003). More than 200 antigens that are mostly shared with mycobacteria other than *M.tuberculosis*, including the vaccinal strain of BCG, are included in the PPD and may cause false-positive reactions in previously vaccinated people. The low sensitivity of the TST in certain groups of patients such as those who are immunosuppressed, are young children or have extrapulmonary TB is another limitation of the method. These limitations together with the need for trained personnel and subjective result readings, make TST a weak tool for the diagnosis of TB.

Newer immunodiagnostic methods have emerged as a reliable alternative to the previously mentioned TST. These methods are based on the release of IFN-γ by sensitized T-cells stimulated with specific *M. tuberculosis* antigens (Santín-Cerezalesa *et al.*, 2011). The two main antigens used are the ESAT-6 and the CFP-10, encoded in the region of difference 1 (Santín-Cerezalesa *et al.*, 2011). The most important advance is that these two antigens are present in *M. tuberculosis* but not in *M. bovis* BCG or in the other mycobacteria from the *M. tuberculosis* complex. The two commercially techniques most commonly used to date are the Interferon-Gamma Release Assays: QuantiFERON-TB Gold assays (QFT-G) (Cellestis Limited, Carnegie, Victoria, Australia) and TB-SPOT (Oxford Immunotec, Oxford, UK). Both tests are currently recommended by the CDC for the diagnosis of LTBI.

Some of the drawbacks of the IGRAs is their greater cost (MAG Mutual Healthcare Solutions, 2009) and the need for blood samples. Several studies have been performed to evaluate the implementation of these techniques, however, more studies should be carried out in risk groups such as immunosuppressed patients to assess their efficiency.

3.2 Phenotypic detection

Acid-fast staining remains the simplest, cheapest and fastest test to diagnose TB. However, an expertise microbiologist is required for result evaluation. The staining is done from patient samples, which are most often involving sputum, but may also include pus, cerebrospinal fluid or biopsied tissue. In contrast to other bacteria, the cell wall of *M. tuberculosis* contains large chain lipids known as mycolic acids, giving the property of acid-alcohol resistance. The most frequently used methods to detect acid fast bacilli are the Ziehl-Neelsen and the Auramine-Rhodamine stains (Figure 12). This latter method is recommended for screening in centres with a large amount of samples.

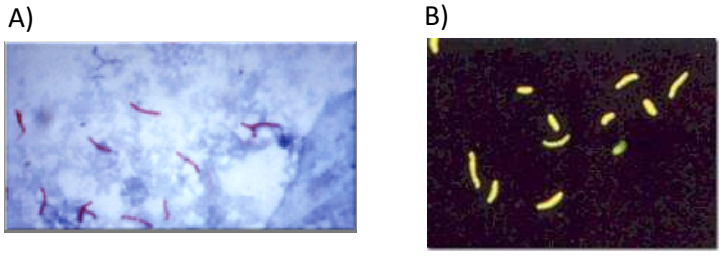


Figure 12. Staining of *M. tuberculosis*. A) Ziehl-Neelsen (CDC), B) Auramine-Rodhamine (http://www.lung.ca/tb/abouttb/what/causes_tb.html).

Definitive diagnosis of TB disease can be made by culturing *M. tuberculosis* from a specimen taken from the patient and is the reference method due to its higher sensitivity than acid-fast staining and the fact that the isolated mycobacteria is required to undergo further analysis such as resistance studies or epidemiological typing. Lowenstein-Jensen solid egg-based medium is used to detect the growth of *M. tuberculosis* complex isolates. Middlebrook 7H9 broth is also used, with supplements such as OADC (oleic acid, albumin, dextrose and catalase) and a combination of antimicrobial agents to avoid contamination. Indeed, the used of new automatic and semi-automatic systems such as BACTEC™ Mycobacteria Growth Indicator Tub (MGIT) 960 (BD Diagnostics Systems, Sparks, USA), Bactec 460TB (BD), MB/Bact ALERT (bioMérieux, Marcy-l'Etoile, France), and VersaTREK (Trek Diagnostic System, Westlake, USA) has improved the quality and efficiency of the culture method (Alcaide *et al.*, 2011). MGIT tubes contain supplemented Middlebrook 7H9 and an oxygen-quenched fluorochrome

embedded in silicone at the bottom of the tube. The MGIT 960 instrument is used to read MGIT tubes by reading fluorescence under ultraviolet light. By contrast, MB/Bact ALERT is a colorimetric method with a carbon dioxide sensor in each bottle and VersaTREK detects the rate of oxygen consumption within the headspace of the culture bottle. Finally, the Bactec 460 TB system uses liquid medium with 14C-labelled palmitic acid as a carbon source, thus, the count of $^{14}CO_2$ and the rate of the increase of gas production are directly proportional to the growth of the mycobacteria (Parsons *et al.*, 2011). Other phenotypic methods to distinguish *M. tuberculosis* from other non tuberculosis *Mycobacterium* are the growth rate or morphology of the colonies.

3.3 Genotypic detection

One of the obstacles to effectively control TB is the delay in achieving the diagnosis. Thus, a wide range of molecular methods have been developed in the last years. These methods are based on amplifying specific sequences of nucleic acids and require a post-amplification analysis by the observation of an amplified fragment or hybridization or sequencing. A variety of genetic targets of *M. tuberculosis* such as IS*6110*, *rpo*B, *hsp*65, 16S rRNA or MBP64 has been described. Both the Amplicor *Mycobacterium tuberculosis* test (Roche Diagnostic System Inc., Basel, Switzerland) and the Amplified M. tuberculosis Direct Test (AMTD; Gen-Probe Inc., San Diego, CA, USA) are commercial tests based on 16S rRNA which have a high smear-positive and low smear-negative sensitivity for respiratory samples (Palomino, 2009). By contrast, new hybridization assays such as the Genotype Mycobacterium Direct assay (Hain Lifescience, Hehren, Germany) and the INNO-LIPA Rif, TB kit (Innogenetics, Gen, Belgium) which can detect *M. tuberculosis* complex from clinical samples, may improve the sensitivity of the method (Alcaide *et al.*, 2011).

3.4 Diagnosis of drug-resistant TB

3.4.1 Phenotypic methods

Phenotypic DST relies on detection of the effect of the drugs on bacterial multiplication or metabolism compared to controls not exposed to the drug.

Conventional DST by fixed proportions, absolute concentration or resistance ratio methods is based on the growth characteristics in liquid or solid culture medium in the presence of the study drug (Canetti *et al.*, 1963). The resistance ratio compares the MIC of the strain studied with the reference strain. By contrast, the absolute concentration is defined as the comparison

of the number of colonies with the presence of drug *versus* the number with the absence of the drug. Nevertheless, the fixed proportions method is most often used, analysing the proportion of colonies able to grow in the presence of the drug in comparison to free-drug medium. The new generation of DST has been derived from the fixed proportions method. The medium used is 7H9 Middlebrook and the strain studied is considered resistant to the drug if more than 1% of the population is able to grow in the presence of the drug. The solid mediums used are Middlebrook 7H10 and 7H11, both of which are based on 7H9 medium. Due to the long period of incubation of solid mediums that takes up to four weeks, liquid mediums are widely used, reducing this time to 5-12 days. Indeed, the standardisation of the innoculum is another essential point to ensure an accurate DST due to the property of *M. tuberculosis* to generate clumps. The rapid phenotypic methods recognized by the WHO are the manual and automated BACTEC^{TM} MGIT960 (BD) with which extreme sensitivity has been achieved. However, other methods have recently been evaluated for routine use (Espasa *et al.*, 2011). It is important to mention the DST of PZA since its metabolite pyrazinoic acid needs an acidic pH of 5.5 to act, therefore requiring specific medium. Nevertheless, since most *M. tuberculosis* strains are unable to grow at this pH, these methods have adapted to a pH of 6 (Lorian, 2005).

Other phenotypic methods have been described to detect resistance to antituberculous drugs. The microscopy observation broth-drug susceptibility assay, slide DST and the microcolony method are rapid and low-cost methods based on visualization of bacilli by microscopy. The colorimetric redox indicator and the nitrate reductase assay rely on the ability of the bacilli to oxidate and reduce metabolites. Mycobacteriophage is another DST method which evaluates lytic phages has been used to detect RIF resistance, however, this technique has high contamination rates as well as indeterminate results (Van Deun *et al.*, 2010).

3.4.2 Genotypic methods

Molecular or genotypic DST depends on identification of resistance-conferring mutations of the bacillary genome, some of which may vary geographically. The main drawback is that these mutations have not been characterized for some drugs, and thus, would fail to detect some phenotypic resistant strains. These methods have a high risk of cross-contamination, requiring accurate definition of the areas of the different steps. Extraction and amplification of the target gene from the sample is performed with all genotypic techniques. In contrast to phenotypic DST methods, which take several weeks to become positive, molecular tests can take from a few hours to a few days. The rapid diagnosis of drug-resistant TB facilitates an appropriate therapy and avoids new drug-resistances (Van Deun *et al.*, 2010). Different

methods to detect drug-resistance TB have been developed in the last years, from home-made (Espasa *et al.*, 2005; Aragón *et al.*, 2006) to commercially developed methods (Figure 12 and Table 1).

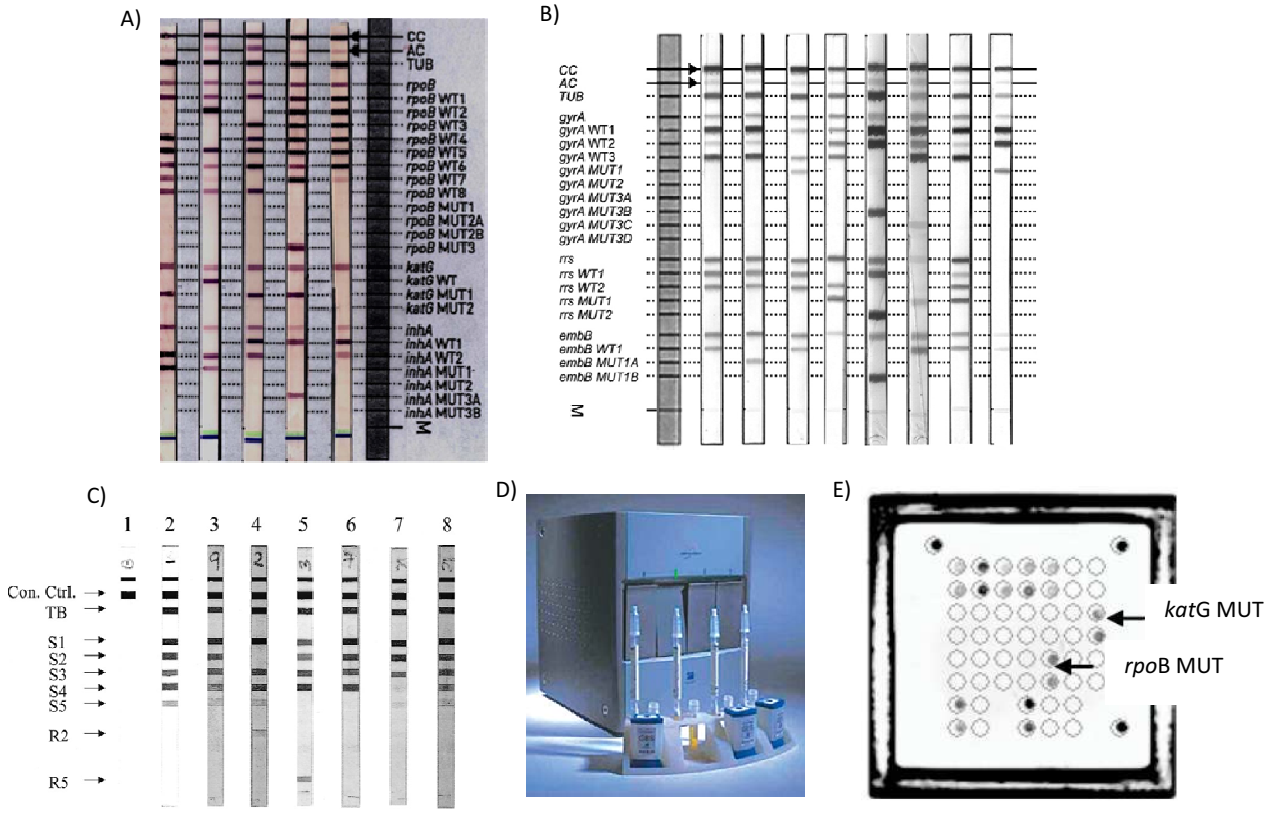


Figure 12 . The main genotypic methods to detect drug-resistant *M. tuberculosis*. A) Genotype MTBDR plus, B) Genotype MTBDRsl, C) INNO-LiPA Rif-TB, D) GeneXpert MTB/RIF, E) Microarray (Ahmad *et al.*, 2000; Aragón *et al.*, 2006; Tsongalis *et al.*, 2006; Huang *et al.*, 2009; Lacoma *et al.*, 2012).

DNA sequencing is one the most accurate genotypic techniques, visualizing the complete nucleotide sequence of the amplified target DNA. Some years ago the line-probe assays appeared, being methods based on reversed hybridisation of the amplification products from the sample DNA to a series of DNA probes immobilised on a strip. Probes bound to the sample DNA are then visualized as bands on the strip. Line-probe assays include INNO-LiPA Rif-TB (Innogenetics, Ghent, Belgium) which detect the most frequent mutations in the *rpo*B gene and Genotype MTBDR plus kit (Hain Life-Science, Nehren, Germany) which also covers the *kat*G and *inh*A genes. Both methods have shown a detection of 91% of MDR-TB, but only 56-70% for monoresistant INH/RIF. Recently, a new commercial test which detects MDR and resistance to EMB as well as second-line TB drugs known as Genotype MTBDRsl have been

launched. Real-time PCR is also a useful method to detect INH, RIF and fluroquinolone resistance.

DNA microarrays are another tool to detect resistance. These methods are based on binding the amplified fluorescence labelled target DNA from the sample to a large set of oligonucleotide probes immobilised on a polyacrylamide gel pad or glass carrier. The fluorescence emitted depends on the degree of homology between probe and sample DNA, thereby allowing identification of the mutations. GeneXpert MTB/RIF (Cepheid, Sunnyvae, CA, USA and FIND Diagnostics, Geneva, Switzerland) has recently been introduced as a rapid method for identification of MDR-TB (Lawn *et al.*, 2011). This is a novel, single-tube, molecular beacon-based real-time PCR assay for the detection of RIF-resistant *M. tuberculosis* even in clinical sputum samples. As most RIF-resistant isolates are also resistant to INH, RIF resistance can be used as a marker of MDR-TB. The greatest advantage of this new method is that it uses a single sample-processing cartridge, thereby being a hands-free operation as well as having a low risk of cross-contamination. GeneXpert has shown a specificity of 100% and a sensitivity from 71% to 100%, and the results are available in just 2 hours (Helb *et al.*, 2010).

Table 1. Characteristics of the main genotypic methods to detect drug-resistant *M. tuberculosis*.

Assay	Amplification method	Detection	Mutations detected	Drug-resistance detected
Homemade DNA sequencing	PCR	Fluorimetric	*	*
INNO-LiPA Rif-TB	Nested-PCR	Colorimetric	*rpo*B	RIF
Genotype MTBDR plus kit	Nucleic acid sequence-based amplification	Colorimetric	*Kat*G, *inh*A and *rpo*B	INH, RIF
Genotype MTBRsl	Nucleic acid sequence-based amplification	Colorimetric	*emb*B, *rrs*, *gyr*A	EMB, aminoglycosides, fluoroquinolones
Homemade DNA microarray	PCR	Fluorimetric	*	*
GeneXpert MTB/Rif	Real-time PCR	Fluorimetric	*rpo*B	RIF

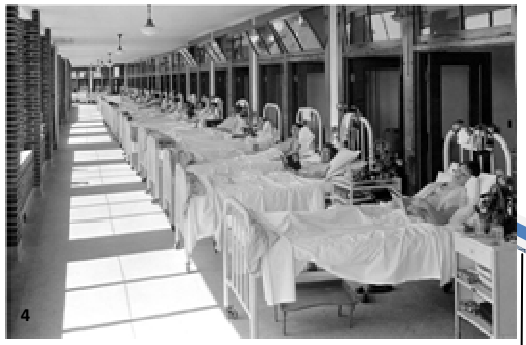
*the researcher designed the panel of mutations to study.

4. TREATMENT OF TUBERCULOSIS

4.1 History

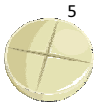
Different approaches have previously been implemented to fight against TB such as the use of sanocrysin, sulphones and vitamin D (Figure 13). Nevertheless, the treatment was based on patient bed rest in a sanatorium and surgery of the affected portion of the lung. STR was introduced as the first drug in the treatment of TB in 1946. Due to the emergence of isolates of *M. tuberculosis* resistant to STR, PAS was added a few years after. In 1952, INH appeared as a new promising drug against TB due to its low toxicity and MIC. A few years later, combined treatment consisting of INH, STR and PAS became an alternative to prevent drug-resistance. A new regimen based on 3 months of STR, PAS and INH, and 9 months of PAS and INH was implemented from the 1960s to 1986. During this period, the implementation of DOTs, a strategy promoted by the WHO greatly improved the control of TB after verification of therapeutic compliance by a healthcare worker. Research studies with PZA in the 1950-60s in which the killing of persistent bacilli after treatment was observed, led this drug to be considered as a good alternative to treat TB. Together with later experiments with RIF, which showed acceleration of the killing, new clinical trials launched included RIF and PZA to reduce the rate of relapse. When VIH infection led to toxicity to thioacetazone, EMB was given in its place (Nunn *et al.*, 1991). A shorter therapy was then proposed with an 8-month regimen consisting in 2 months of INH, RIF, PZA and EMB and 6 months of EMB and INH. RIF was often not used in most of the low-income countries because of its high cost in comparison to the other drugs and due to the recommendation of reserving this drug for cases requiring retreatment. Moreover, during these years a 9-month therapy was used in high-income countries, consisting in 2 months of INH, RIF and EMB and 7 months of INH and RIF. A 6-month regimen of 2 months of INH, RIF, PZA and EMB and 4 months of INH and RIF later demonstrated to be more effective than the 8-month regimen (Jindani *et al.*, 2004). Thereafter, the current short-course treatment was developed (Mitchison *et al.*, 2012).

1925-1946
Sanocrysin,
Sulphones,
Vitamin D



1946
STR. First drug against TB

1950s-1960s
Studies with PZA and RIF



1952
INH. First oral-drug against TB

1960s
First therapy combination with INH, PAS and STR during 12 months.

Implementation of DOTs

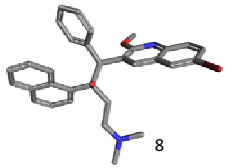
1980
Short-course therapy: 6, 8 or 9 months.

OFL. First fluoroquinolone to treat drug resistance.

1992
LEV. New generation of fluoroquinolones

1998
WHO policy on INH preventive therapy

2000
PA-824, TMC-207, OPC-67683. Potential new drugs



2004
Regimens of 6-months show better efficacy than others.

2010
Potential new regimens

Figure 13 . Timeline of TB treatment.

4.2 Treatment of latent Tuberculosis

Treatment of latent TB is a challenge for the management of TB. The aim of treating latent TB is to avoid progression to active TB. The current standard preventive therapy used is 9-month INH or 6-month INH with efficacies of 90-93% and 69%, respectively (Comstock, 1999). Due to the low treatment compliance rates and toxicities of these therapies, other regimens such as INH plus RIP have also been recommended (CDC, 2011). Trials with INH and PZA in the treatment of latent TB have reported severe side effects, thus discouraging their use in these cases (CDC *et al.*, 2003). According to the CDC, preventive therapy is recommended in persons with a diagnosis of LTBI and some of the following risk factors: HIV infection, recent contact with a TB case, fibrotic changes on chest X-ray consistent with previous TB, organ transplantation, immunosuppression for other reasons (such as taking TNF-α antagonists), residing and working in high risk congregate settings (for example homeless shelters or hospitals), working in mycobacteriology laboratory and age under 4 years of age or exposure of children or adolescents to adults in high-risk categories (Salgado *et al.*, 2011; CDC, 2012; Kall *et al.*, 2012). In some countries, recent immigrants (<5 years) from high prevalence countries and injection drug users are also risk populations in which preventive therapy is recommended.

4.3 Treatment of active Tuberculosis

The regimen currently used to treat drug-susceptible TB consists of 2 months of initial intensive phase of INH, RIF, EMB and PZA followed by 4 months of INH and RIF. The success of the drug therapy in TB relies on the following aspects: the prevention of drug resistance, therapy with several drugs simultaneously and the attack of different physiologic populations by different drug targets. Resistance against antimycobacterial agents arises due to spontaneous bacteria chromosomal mutations, thus with the use of 2 or 3 drugs the probability of drug-resistant strains appearing is nearly inexistent. Mitchison described the special population hypothesis in the 60s, which explained how the following populations could coexist in patients: extracellular and active bacilli, bacilli in replication, non-replicative and intracellular bacilli in acidic environment or totally dormant population. In this way, INH, RIF and STR are active mostly against the dividing bacilli, in the metabolic and replication populations. By contrast, PZA is active against the non-replicative population residing in acidified vacuoles present in macrophages. Finally, the totally dormant population would not be susceptible to drugs (Mitchison, 2000). The WHO classifies antituberculous drugs into 5 groups (Table 2). These drugs may also be classified as first or second-line drugs according to

their clinical choice, in which the first-line drugs include INH, RIF, EMB, PZA and STR and all the other drugs are second-line.

Table 2. Groups of antituberculous drugs according to the WHO.

Group and description	Drugs
Group 1: First oral agents	INH, RIF, EMB, PZA, RIB
Group 2: Injectable agents	KAN, AMK, CAP, STR
Group 3: Fluoroquinolones	MOX, LEV, GAT, OFL
Group 4: Oral second line agents	ETH, Protionamide, CYC, Terizidone, PAS
Group 5: Agents with unclear efficacy	CLZ, LNZ, Amoxicillin/Clavulanate, Thioacetazone, Clarithromycin, Carbapenems

INH, isonazid; RIF, rifampicin; EMB, ethambutol; PZA, pyrazinamide; RIB, rifabutine; KAN, kanamycin; AMK, amikacin; CAP, capreomycin; STR, streptomycin; MOX, moxifloxacin; LEV, levofloxacin; GAT, gatifloxacin; OFL, ofloxacin; ETH, ethionamide; CYC, cycloserine; PAS, p-aminosalicylic acid; CLZ, clofazimine; LNZ, linezolid.

4.4 Treatment of drug-resistant Tuberculosis

Genetic resistance to antituberculous drugs is due to spontaneous mutations in the chromosome at a frequency of 10^{-6}-10^{-8}. Inadequate treatment, such as in monotherapy or poor adherence to treatment results in the appearance of clinically drug-resistant TB, defined as acquired drug resistance (Figure 14). Primary drug resistance is due to the transmission of a drug-resistant TB.

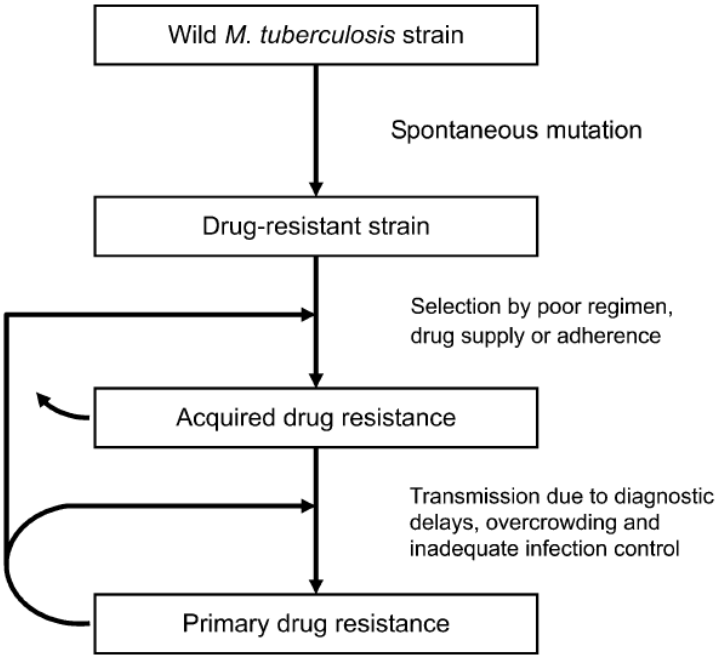


Figure 14. Concepts in the development of drug-resistant TB (Zhang *et al.*, 2009).

The appearance of drug-resistant TB and particularly, MDR and XDR-TB, has become an obstacle to efficient treatment. To face this challenge, the WHO has developed guidelines for the management of drug-resistant TB (ATS *et al.*, 2003). The following drugs are recommended in MDR-TB: PZA or EMB, one fluoroquinolone (better if last generation) and CYC or PAS if CYC cannot be used (Figure 15). The proposed duration of the regimen is 8 months of the intensive phase with a total treatment duration of 20 months, modified according to the patient response to therapy. Indeed, depending on the resistance to these drugs, other group 5 drugs (Table 2) such as LNZ, imipenem and clarithromycin have been proposed to treat some cases of MDR and XDR-TB. All these second-line drugs to treat MDR tuberculosis have high cost-effectiveness and present more toxicity than first-line drugs, presenting obstacles for patient compliance to treatment. Therefore, it is important to optimize the therapeutic regimens for drug-resistant TB.

The length of treatment may influence patient compliance, thus several trials are currently ongoing with the aim of shortening TB treatment. MOX, a new fluoroquinolone, has appeared as a promising new drug to reduce the length of treatment (Conde *et al.*, 2009), although antagonism with RIF has been described (Nijland *et al.*, 2007). Higher doses of RIF (Ruslami *et al.*, 2007) and the use of RIP have also been proposed to shorten treatment time. According to the WHO there are research gaps with respect to the lack of a optimum treatment regimen in patients with INH resistance, polydrug resistance, MDR-TB, XDR-TB and chemoprophylaxis for MDR-TB contacts are research gaps (WHO, 2011).

Figure 15. WHO recommendations for the treatment of MDR-TB.

4.5 Antituberculous drugs

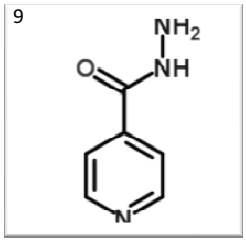
The pharmacokinetic characteristics of the main antituberculous drugs are represented in Table 3.

Table 3. Pharmacokinetic characteristics of the main antituberculous drugs.

Drug	Dose	C_{max} (µg/ml)	Half-life (h)	AUC (µg·h/ml)	Protein binding (%)	MIC for drug-susceptible *M.tuberculosis* (µg/ml)
INH	5[a]	5.4-7.1	0.5-4	19-48.2*	20-30	0.05-2
RIF	10[a]	8-20	2-5	14.91	85	0.5
RIP	10[a]	15.05	13.19	319.54	97	0.125-0.25
RIB	5[a]	0.2-0.5	16-69	5.8-9.2	71	0.008-0.064
PZA	20-25[a]	38.7±5.9	9-10	502±101	10-20	20
EMB	15-20[a]	2-5	4-6	24.9	20-30	1-5
STR	15[a]	25-50	2-3	NF	35	2
KAN	15[a]	14-29	2-4	NF	0-5	0.5-4
AMK	15[a]	16-38	2-2.5	NF	<10	0.25-1
CAP	15[a]	30	2	NF	NF	1-4
LEV	500-1000[b]	6	6	45-46	24-38	0.12-1
OFL	200mg[a]	0.5-2.5	5.8	29.2	8-30	0.5-2.5
CIP	500[c]	1.5-2.9	5-6	5-6	22	0.25-3
GAT	400[b]	3-5	6-7	30	20	0.007-0.12
MOX	400[b]	2-4	9	39-40	50	0.031-0.12
ETH	250-500[c]	2-20	2-4	7.67	30	0.6-2.5
PAS	8-12[a]	9-35	0.75-1	NF	50-60	1
CYC	10-15[a]	25-30	10	NF	<20	5-20
CLZ	50-100[b]	0.145	1680	1.45	NF	0.06-2
LNZ	300-600[b]	12.7-12.9	4.26-4.4	80.2-91.4	31	0.25-0.5

Units for doses: [a]mg/kg/day; [b]mg/day; [c]mg/12hour; [*]Depending on fast/slow acetylator; NF: not found. INH, isonazid; RIF, rifampicin; EMB, ethambutol; PZA, pyrazinamide; RIB, rifabutine; RIP, rifapentine; KAN, kanamycin; AMK, amikacin; CAP, capreomycin; STR, streptomycin; MOX, moxifloxacin; LEV, levofloxacin; GAT, gatifloxacin; OFL, ofloxacin; CIP, ciprofloxacin; ETH, ethionamide; CYC, cycloserine; PAS, p-aminosalicylic acid; CLZ, clofazimine; LNZ, linezolid. (Lee *et al.*, 1977; Blaschke *et al.*, 1996; Woo *et al.*, 1996; Alvirez-Freites *et al.*, 2002; ATS *et al.*, 2003; Lorian, 2005; Global Alliance for TB drug Development., 2008; Juréen *et al.*, 2010; Mensa *et al.*, 2011; Schön *et al.*, 2011).

4.5.1 Isoniazid



INH is a specific drug against mycobacteria, and has an MIC against drug-susceptible *M. tuberculosis* ranging from 0.05 to 2µg/ml. This drug has good bioavailability, with low protein binding and good tissue distribution (Global Alliance for TB drug Development., 2008). Acetylation of INH, which is done in the liver, varies within the population according to the genetic background. Thus, pharmacokinetics such as the half-life or the AUC depend on the ability of the drug to acetyl and thereby distinguish between fast and slow acetylators. INH carries an uncommon potential for liver toxicity, although its risk is increased with alcohol abuse. Other adverse reactions have been reported such as gastrointestinal, haematological and hypersensitivity effects.

INH is a pro-drug which must be activated by a catalase-peroxidase enzyme, katG (Figure 15). This enzyme oxidizes INH, which is enhanced by the presence of manganese ions. After this oxidation, this compound generates a range of radicals such as NO which is also important in antimycobacterial action (Kim *et al.*, 2012). The oxidized INH forms a covalent link to the carbon at position 4 of the nicotinamide of NAD(H). The resulting entities are known as INH-NAD(H) adducts which inhibit InhA, a NADH-specific enoyl-acyl carrier protein reductase of the fatty acid synthase type II system (FASII) involved in fatty acid synthesis. This process inhibits mycolic acid, which is required in the cell wall of the mycobacteria, thereby leading to cell death.

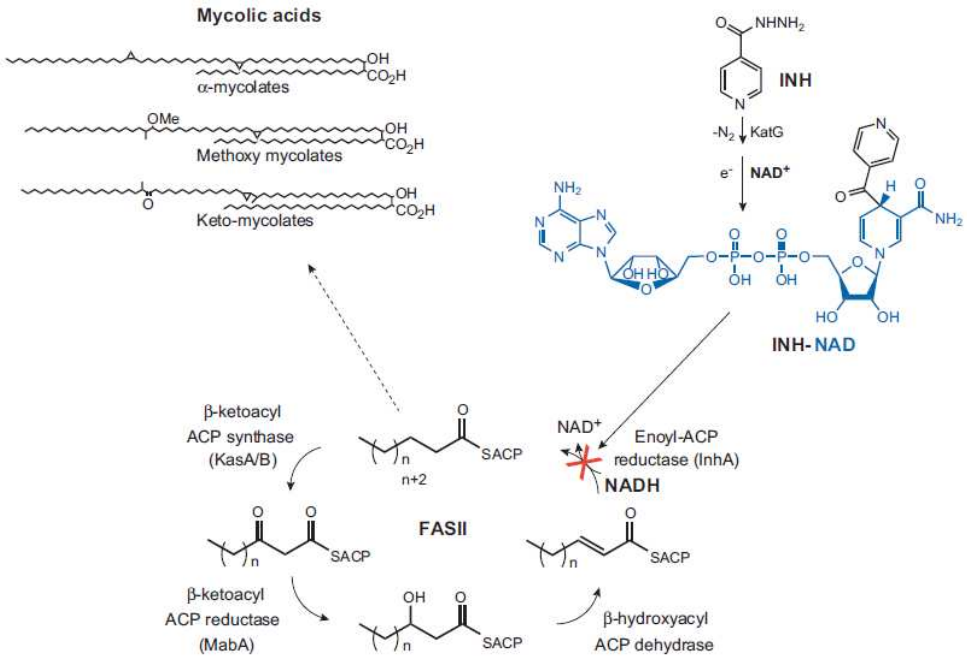


Figure 15. Mechanism of action of INH in *M. tuberculosis* (Vilchèze *et al.*, 2007).

The first association of INH-resistant clinical isolates of *M. tuberculosis* was the changes in the catalase-peroxidase system of these organisms (Zhang *et al.*, 1992), and later the characterization of the *kat*G gene (Heym *et al.*, 1993). Two molecular mechanisms have been shown to be the main cause for isoniazid resistance, mutations in the *kat*G gene and mutations in the promoter region of the *inh*A gene (Table 4). Among the cases resistant to INH a mutation in codon 315 of the *kat*G gene is the most frequent, with reports from 41.6% having been described in Syria (Madania *et al.*, 2012) to 98.4% in Kazakhstan (Kozhamkulov *et al.*, 2011). The other most frequent mutation is that in the promoter region of the *inh*A gene, being reported from 16.9% in Bangladesh (Rahim *et al.*, 2012) to 32% in Syria (Madania *et al.*, 2012). By contrast, Korea has shown a higher proportion of mutations in the *inh*A promoter region (40.4% versus 23.1% in the S315T-*kat*G gene). The distribution of the two mutations in Barcelona is 55% and 32% for the *kat*G gene and the promoter region *mab*A-*inh*A, respectively (Coll *et al.*, 2005). Moreover, high levels (MICs≥1µg/ml) and low levels of resistance (MICs<1µg/ml) have been associated with mutations in the *kat*G gene and *inh*A gene, respectively (Dantes *et al.*, 2012). Resistance to INH is considered if the MIC is greater than or equal to 0.1µg/ml. Clinical isolates resistant to INH range from 0.1 to 256µg/ml (Almeida Da Silva *et al.*, 2011).

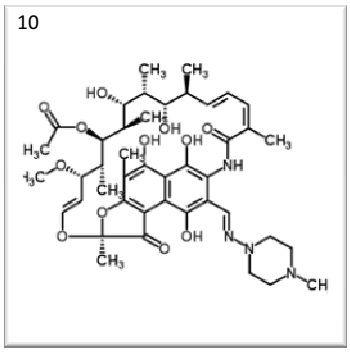
Although most of the mutations associated with INH resistance are found in the *kat*G gene and the promoter region of the *inh*A gene, mutations in other positions have been described (Table 4). Mutations and overexpression of the *inh*A gene as well as mutations in positions other than S315T-*kat*G gene have also been reported. Mutations in the *kas*A gene and the *ndh* gene encoding NADH dehydrogenase (Lee *et al.*, 2001) and the *glf* genes have also been linked to resistance to INH. However, the *kas*A mutation has been found in drug-susceptible isolates suggesting a polymorphism (Lee *et al.*, 1999; Cardoso *et al.*, 2004). Efflux pumps of *M. tuberculosis* which can transport INH out of the cell have also been described (Machado *et al.*, 2012). Neither mutations nor efflux pumps have been found in some clinical resistant isolates of *M. tuberculosis*, suggesting other unknown mutations or mechanism of resistance to INH may be involved. Using proteomic assays, Argyrou *et al*. (2006) proposed other INH targets such as SahH, Rv2623, Rv2971 and Rv1187 which could be essential for mycobacteria growth. The clinical significance of all these changes remains unclear.

Table 4. Mechanisms of isoniazid resistance of *M. tuberculosis*.

Genetic mechanism	**Cellular target/effect**
Mutations in *kat*G: D63E, H108Q, T262R, T275P, S315T/R, A350S, G629S	Loss of catalase-peroxidase activity Inhibition of mycolic acids
Mutation in *inh*A: S94A, I16T, I21V, I47T, I95P Mutation in the promoter region: C(-15)T Overexpression of *inh*A	Reduce affinity for its cofactor NADH, finally inhibition of mycolic acids
Mutation upstream from *aph*C: C(-81)T, G(-74)A, C(-54)T, G(-51)T, G(-48)T	Compensates for the loss of catalase-peroxidase
Mutation in *ndh*: R268H	Causes accumulation of NADH, which acts as a competitive inhibitor for binding *inh*A

The *kat*G gene is also involved in the susceptibility of *M. tuberculosis* to oxidative damage, however, the effect of the mutation on this function is not clear. As a consequence of the mutation in the *kat*G gene, organisms have developed compensatory mutations such as in the regulatory region of the *ahp*C gene, an alkyl hydroperoxide reductase. One of first studies in this field showed how clinical isolates resistant to INH reduced catalase activity and had a relative lack of virulence in guinea-pigs (Zhang *et al.*, 1992). Nevertheless, these studies were performed before the mapping of the genome of *M. tuberculosis*.

4.5.2 Rifampicin



RIF belongs to the rifamycins which inhibit the prokaryotic DNA-dependent RNA polymerases. The target of RIF is the *rpo*B gene product β-subunit of DNA-dependent RNA polymerase activity, binding to the DNA/RNA channel (Campbell *et al.*, 2001). Apart from *M. tuberculosis*, this drug inhibits the growth of other mycobacteria and Gram positive

and Gram negative bacteria such as *Staphylococcus aureus*, *Neisseria meningitidis* or *Haemophilus influenza*. RIF is a highly bactericidal against *M.tuberculosis*, with an MIC of 0.5 µg/ml in drug-susceptible isolates. Due to its low solubility, RIF has a variable bioavailability (Jayaram *et al.*, 2003; Bhise *et al.*, 2010). The metabolization of RIF by the hepatic cytochrome P450 enzymes can lead to adverse reactions such as hepatotoxicity, hypotension, shortness of breath, gastrointestinal effects when it is combined with other drugs (Girling *et al.*, 1979).

More than the 95% of the isolates resistant to RIF are associated with mutations in the RIF-Resistant Determining Region (RRDR) composed of a cluster of 81 bp in the *rpo*B gene, and this mutation usually results in a single amino acid change in the protein sequence (Figure 16). The most frequent changes are found in codons 531 and 526 of the β-subunit which has been reported 55-73% and 13-23% of the cases of RIF resistance, respectively (Clemente *et al.*, 2008; Bolotin *et al.*, 2009; Wang *et al.*, 2009; Minh *et al.*, 2012). Mutations in codon 516 of the *rpo*B gene have not been linked to cross-resistance with other rifamycins such as RIB, whereas other positions have been reported (Williams *et al.*, 1998; Tan *et al.*, 2012). The critical concentration for resistance to RIF is 1µg/ml and the MICs of the resistance isolate range from 1 to 256µg/ml (Hwang *et al.*, 2003).

Compensatory mutations in the *rpo*A and *rpo*C genes have recently been described (Comas *et al.*, 2011; Brandis *et al.*, 2012). Most of the isolates resistant to RIF are also resistant to INH, although geographical differences have been observed (Kurbatova *et al.*, 2012; Smith *et al.*, 2012).

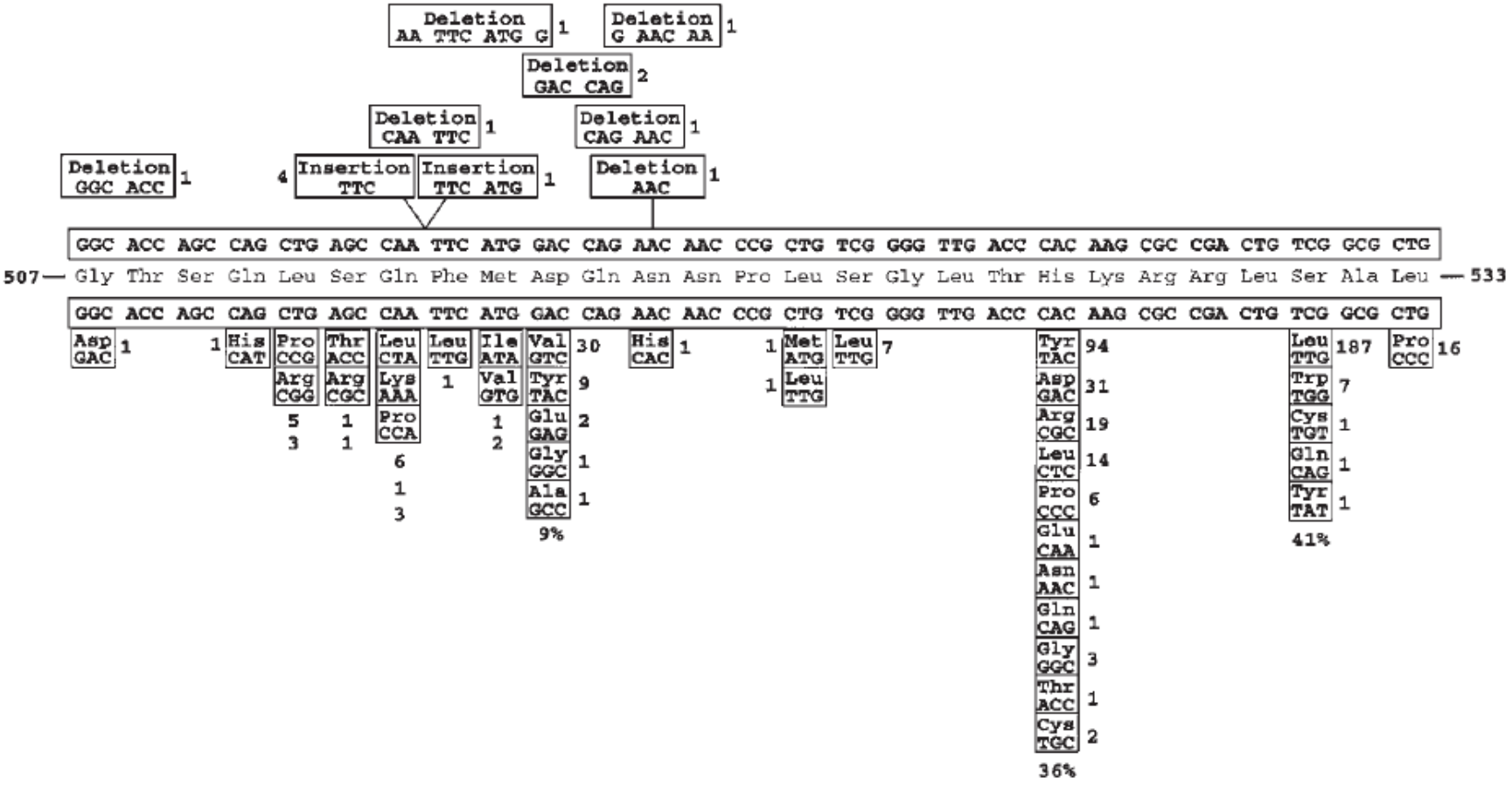
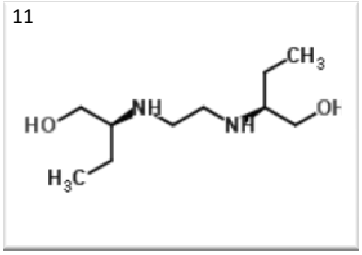


Figure 16. Clustering mutations in the *rpo*B gene found in 478 RIF-resistant isolates of *M. tuberculosis* (Ramaswamy *et al.*, 1998).

4.5.3 Ethambutol



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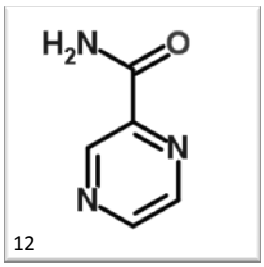
EMB inhibits a set of arabinosyl trasnferase enzymes, called EmbA, EmbB and EmbC, which are critical for synthesis of arabinan, an essential polysaccharide of the mycobacteria cell wall (Alderwick *et al.*, 2011). This drug is active against *M. tuberculosis,* and other species of mycobacteria, without activity against other microorganisms have been found. It is of note there is a clear separation between the MIC in drug-susceptible strains and the critical concentration breakpoint in INH and RIF. However, the breakpoint established for EMB is 5µg/ml and the MIC for drug-susceptible isolates of *M.tuberculosis* ranges from 1 to 5µg/ml (Scho *et al.*, 2009). Poor and variable absorption of EMB has been reported (Tappero *et al.*, 2005; Jönsson *et al.*, 2011), however, it is used to avoid resistance of INH and/or RIF. The most common toxic effect of EMB is optic neuropathy which is generally reversible. Other reported side effects include pruritus, joint pain, gastrointestinal upset, abdominal pain, malaise, headache, dizziness, mental confusion, disorientation, and possible hallucinations (Global Alliance for TB drug Development., 2008).

Resistance to EMB results from a first step of overexpression of the Emb proteins and a second step of mutation in the conserved operon *Emb*B. Mutations in the *Emb*B gene, particularly in codon 306 (Plinke *et al.*, 2011), have been found in resistant clinical isolates and are associated with high-levels of resistance (Alcaide *et al.*, 1997). Moreover, mutations in codon 406 have shown to be more prevalent than those of codon 306 in the area of Catalonia (Moure *et al.*, 2008). Nevertheless, around 75% of the isolates with phenotypic resistance to EMB have not reported any associated mutation (Tessema *et al.*, 2012).

4.5.4 Pyrazinamide



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PZA is a pro-drug that requires the amidase of *M. tuberculosis* and an acidic pH to convert to pyrazinoic acid, the active moiety of PZA. The bacilli absorb PZA by passive diffusion, and pyrazinoic acid is excreted by an inefficient efflux pump that requires energy. As a result, pyrazinoic acid is accumulated inside the bacilli (Zhang *et al.*, 2003). This conversion has been shown to be catalysed by human enzymes, thereby suggesting that the activation of PZA occurs within the acidic environment of the caseous lesion and phagolysosome. The mentioned requirements have led to limitations in *in vitro* studies of the antituberculous activity of this drug. The absorption of PZA may vary

(Wilkins *et al.*, 2006) and the half-life of 9-10 hours is quite high compared with other antituberculous drugs. Similar to INH and RIF, PZA may cause hepatoxicity associated with the dose. Other side effects include arthralgias, anorexia, nausea and vomiting, dysuria, malaise and fever, sideroblastic anaemia and hypersensitivity.

Susceptibility to PZA is considered with an MIC of up to 20µg/ml. Since PZA needs acidic medium to be active, DST has been adapted to liquid medium at pH of 6. Acquired resistance to PZA in *M. tuberculosis* is usually found in the converting enzyme PZase, conferred by a mutation in the *pnc*A gene or its promoter (Feuerriegel *et al.*, 2012; Kim *et al.*, 2012; Simons *et al.*, 2012). A wide variety of changes without any hotspot region has been found in PZA-resistant isolates such as Thr47Ala, Ala146Glu, Gly162Asp, Leu172Pro, Asp8Ala, Phe13Leu, Tyr64Ser, Glu107stop, Ala143Pro and Leu172Arg (Feuerriegel *et al.*, 2012; Stoffels *et al.*, 2012). None of these mutations is predominant. Moreover, efflux of pyrazinoic acid by the bacilli has been described as another mechanism of resistance (Zimic *et al.*, 2012).

4.5.5 Fluoroquinolones

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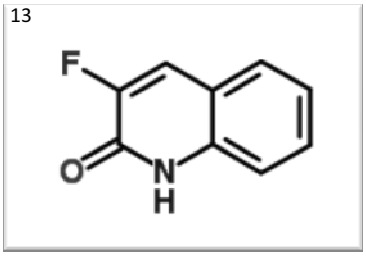


Fluoroquinolones inhibit the ATP-dependent enzymes topoisomerase II (DNA gyrase) and topoisomerase IV, which relax and recoil DNA during transcription, replication, and recombination. As a result, the DNA synthesis of the bacteria is damaged. Fluoroquinolones are effective against a wide range of bacteria due to their mechanism of action and activity against Gram negative, Gram positive and mycobacteria has been described in the last decade. CIP, OFL, LEV, MOX and GAT fluoroquinolones have been reported to be bactericidal against *M.tuberculosis* (Figure 17). The MICs of older fluoroquinolones, such as OFL and CIP, range from 0.5 to 2.5 whereas the newer generation has shown greater effects with MICs from 0.25 to 1. Indeed, the newer fluoroquinolones such as LEV (OFL modified) and MOX and GAT (CIP modified) provide more favourable pharmacokinetic parameters (AUC and C_{max}) than those of older generation drugs (Lubasch *et al.*, 2000). The low percentage of protein binding of most fluoroquinolones, the low ionization and the high solubility facilitate their transport to the tissues and even to cerebrospinal fluid (Thwaites *et al.*, 2011). Moreover, fluoroquinolones have been found to modulate both cellular and humoral immunity. Lower production of pro-inflammatory cytokines has been reported when the fluoroquinolone is added *in vitro*, providing protection from damage due to excessive inflammatory response (Dalhoff, 2005; Shalit *et al.*, 2006; Hara *et al.*, 2011). GAT and MOX are still in phase III of

clinical development and are considered promising drugs to shorten treatment (Dorman *et al.*, 2009; Lienhardt *et al.*, 2012). These new fluoroquinolones are metabolized by glucuronidation and sulfation rather than CYP450-mediated like other fluoroquinolones and, therefore, do not interfere with the metabolism of antiretroviral drugs. Prolongation of the QT interval has been reported in treatment with fluoroquinolones, being a dose-dependent effect. Other side effects include arthropathy and hypoglycaemia. Nonetheless, taking all of the above into account, fluoroquinolones are key for the present and future of treatment of TB.

The DNA gyrase is encoded in two genes, *gyr*A and *gyr*B. The most frequent mutations associated with resistance to quinolones in *M. tuberculosis* are in the Quinolone Resistance-Determining Region (QRDR) of the *gyr*A (Long *et al.*, 2012; Suzuki *et al.*, 2012) and the *gyr*B gens, although the implications of the mutations in the latter gene remain unclear (Pantel *et al.*, 2011). The most frequent mutations in the *gyr*A gene are at positions 89 (Asp89Asn), 90 (Ala90Val) and 94 (Asp94Tyr, Asp94Ala, Asp94Asn, Asp94His) (von Groll *et al.*, 2009; Long *et al.*, 2012). Cross-resistance has been reported in fluoroquinolones (von Groll *et al.*, 2009), however, isolates resistant to old generation fluoroquinolones have been reported to be drug-susceptible to the new generation of these drugs (Suzuki *et al.*, 2012). Efflux pumps conferring resistance to fluoroquinolones have also been described in *M. tuberculosis* (Escribano *et al.*, 2007).

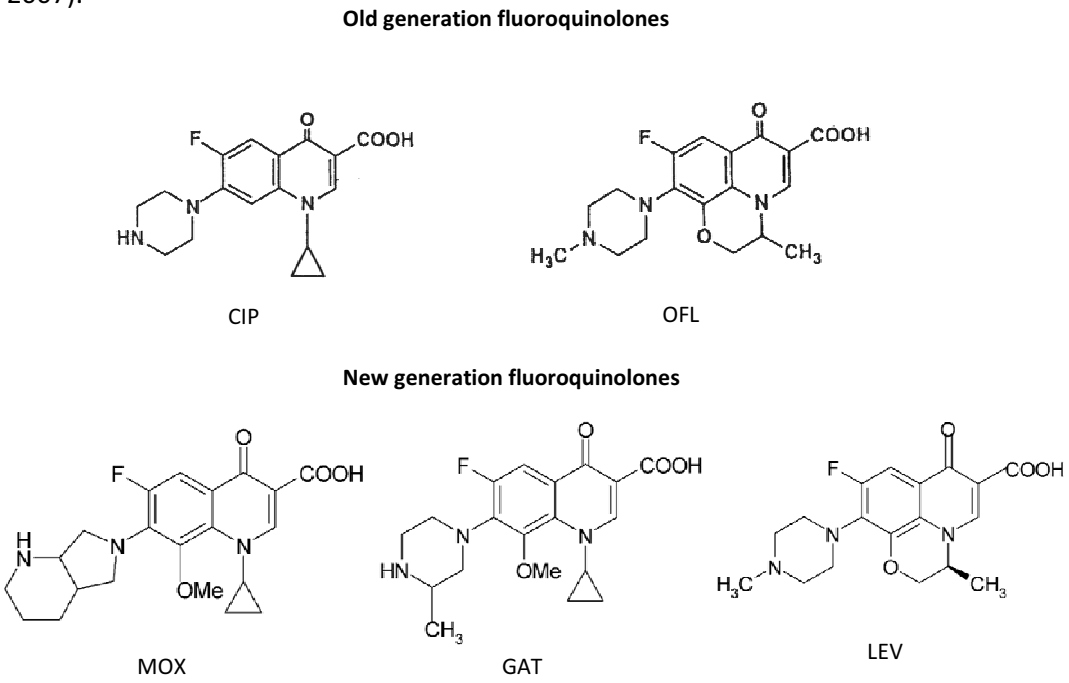


Figure 17. Fluoroquinolones used in TB (Tokura, 1998; Nguyen *et al.*, 2004)

4.5.6 Aminoglycosides

The mechanism of action of aminoglycosides is to inhibit protein synthesis, although it remains unclear how these drugs achieve this inhibition. Indeed, aminoglycosides can cause a range of pleiotropic effects such as membrane damage or ribosomal blockage. Aminoglycosides have activity against some Gram positive, Gram negative and mycobacteria. Due to their high ionization, the absorption of aminoglycosides is extremely low, therefore requiring parenteral administration. Within-cell concentrations are low since the low solubility of these drugs complicates penetration into tissues, especially in cerebrospinal fluid. Aminoglycosides have a narrow therapeutic range, with dose-dependent adverse effects in the kidney and audiovestibular apparatus (Kumana *et al.*, 1994). STR, AMK, KAN and CAP are the drugs used in the treatment of drug-resistant TB (Figure 18), with MICs against *M.tuberculosis* ranging from 0.25µg/ml to 4µg/ml.

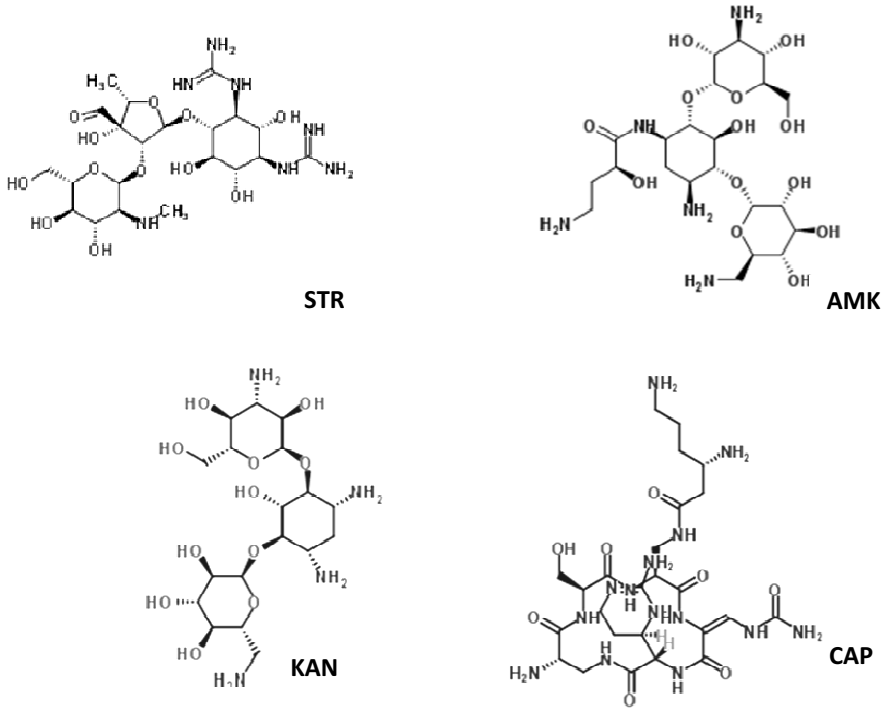


Figure 18. Aminoglycosides used in the treatment of TB (http://www.chemspider.com).

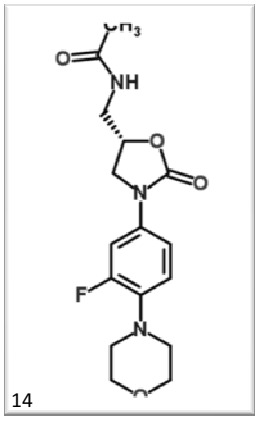
Mutations in the *rpsl* and *rrs* genes have been associated with resistance to STR in *M. tuberculosis*, being found in 37- 85% of clinical isolates resistant to STR (Tudó *et al.*, 2010). The most frequent mutations in the *rpsl* gene are in positions 43 (Lys43Arg) and 88 (Lys88Thr, Lys88Gln), being associated with a high-level of resistance (Tudó *et al.*, 2010). On the other

hand, mutations in the *rrs* gene have been found in the 530 loop. Unlike the mechanism of resistance of the first-line TB drugs, the mutations associated with resistance to the aminoglycosides KAN, AMK and CAP are poorly understood. A recently published systematic review described the rrs A1401GA SNP as a possible sensitive and specific marker of resistance to AMK and the *rrs* gene and *eis* promoter as possible markers of resistance to CAP and KAN (Georghiou *et al.*, 2012). Cross-resistance between aminoglycosides has been reported (Via *et al.*, 2010).

4.5.7 Other second-line drugs

PAS and ETH are active against *M. tuberculosis*, albeit with frequent and/or severe side-effects. Cycloserine and ethionamide inhibit the cell wall of the mycobacteria and PAS inhibits folic acid synthesis and salicyclic acid metabolism.

A series of drugs such as LNZ, CLZ, amoxicillin-clavulanate, clarithromycin and imipenem that have been used to treat other bacteria have appeared as alternatives to treat MDR and XDR-TB. Nevertheless, their specific use in the treatment of TB remains unclear. Although the combined therapy of clavunate with imipenem has cured MDR patients (Chambers *et al.*, 2005), the use of these drugs is recommended in cases of resistance to other second-line drugs. Early bactericidal activity of amoxicillin-clavulanate against *M. tuberculosis* has been reported (Chambers *et al.*, 1998), however, its efficacy in combination with other drugs has been poorly investigated. CLZ is used to treat lepromatous leprosy and has recently appeared as an alternative drug to treat XDR-TB.



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- **Linezolid**

LNZ belongs to the oxazolidinones, which target the ribosome causing an inhibition of protein synthesis. Although LNZ has shown *in vitro* activity against *M. tuberculosis*, to date few articles have described its activity in combination with other antituberculous drugs and its experience treating MDR and XDR-TB. The MIC for a drug-susceptible isolate of *M. tuberculosis* is 0.5 µg/ml. A slow effect has been described on the stationary and logarithmic growth phase of *M.tuberculosis in vitro* (García-Tapia *et al.*, 2004). Recently, two studies have evaluated the treatment of TB with LNZ (Schecter *et al.*, 2010; Lorenzo *et al.*, 2012). Schecter *et al*. (2010) showed how 22 (73%) out of 30 cases of patients infected with MDR-TB had successfully completed the treatment and 3 of these patients had discontinued treatment with LNZ

because of side-effects (Schecter *et al.*, 2010). De Lorenzo *et al*. (2012), also described the good response of LNZ, in 75% of cases of XDR-TB which had sputum conversion (Lorenzo *et al.*, 2012). LNZ is well absorbed and has high bioavailability (Dryden, 2011) and good penetration into tissues (Wiskirchen *et al.*, 2011). The critical breakpoint to consider *M. tuberculosis* resistant to LNZ is 1µg/ml. The *rpl*C T460C mutation has been associated with resistance to LNZ (Beckert *et al.*, 2012).

The mechanisms of resistance of *M. tuberculosis* to the previously described drugs are displayed in table 5.

Table 5. Mechanisms of drug resistance in *M. tuberculosis*.

Agent	MIC (µg/ml)[a]	Mechanism of action	Gene(s) involved in resistance	Frequency of spontaneous mutation
INH	0.1	Loss of catalase-peroxidase Inhibition of mycolic acid synthesis.	*kat*G, *inh*A	10^{-7}
RIF	1	Inhibition of RNA synthesis	*rpo*B	10^{-10}
EMB	2.5-5*	Inhibition of arabinogalactan synthesis	*emb*B	10^{-5}
PZA	20	Unknown	*pnc*A	10^{-5}
STR	2	Inhibition of protein synthesis	*rrs, rps*L	10^{-6}
AMK/KAN	1-5	Inhibition of protein synthesis	*rrs, eis* promoter	10^{-6}-10^{-7}
CAP	2.5-10*	Inhibition of protein synthesis	*tly*A	10^{-6}-10^{-7}
Fluoroquinolones	0.5-2.5	Inhibition of DNA synthesis	*gyr*A, *gyr*B	10^{-6}-10^{-8}
ETH	2.5-10*	Inhibition of mycolic acid synthesis	*eta*A/*eth*A	NF
PAS	1-8	Inhibition of folic acid synthesis and salicylic acid metabolism	*thy*A	10^{-6}
LNZ	1	Inhibition of protein synthesis	*rpl*C	NF

*Differences by Middlebrook 7H10 agar dilution or MGIT960 system. [a] MICs of drug-resistant isolates. NF: Not found. INH, isonazid; RIF, rifampicin; EMB, ethambutol; PZA, pyrazinamide; KAN, kanamycin; AMK, amikacin; CAP, capreomycin; STR, streptomycin; PAS; p-aminosalicylic acid; LNZ, linezolid (Alangaden *et al.*, 1995; Billington *et al.*, 1999; Maus *et al.*, 2005; Okamoto *et al.*, 2007; Bergval *et al.*, 2009; Zhang *et al.*, 2009; van Ingen *et al.*, 2010; Stoffels *et al.*, 2012).

4.5.8 New drugs

During the last 50 years the treatment of TB has scarcely changed. Drug-resistant TB together with HIV co-infection have complicated the management of TB, being urgent the need for research into new antituberculous drugs.

Around 30 drugs and novel regimens for the treatment of TB are currently in the clinical phases of development (Figure 19). New derivatives of LNZ, PNU 100480 and AZD 5847, which target protein synthesis inhibitors, are currently in phase I of clinical development. Wallis *et al*. (2010) showed greater antimycobacterial effects with 1000mg daily doses of PNU 100840 than with LNZ. Other new drugs including nitroimidazoles such as OPC 67683 and PA-824 have been used in an attempt to mimic the host defence strategies with the production of NO and reactive nitrogen intermediates. The same early bactericidal activity as standard treatment was observed in smear positive pulmonary TB patients treated with PA-824, although the patients were only followed over 14 days (Diacon *et al.*, 2012). Another drug in phase II of clinical development is TCM207 or bedaquiline which has appeared as a promising new drug for the treatment of latent TB due to its inhibition of ATP synthase (Andries *et al.*, 2005; Dhillon *et al.*, 2010). These new drugs have shown greater sterilising activities than standard first-line regimens with RIF, PZA and INH in mice (Williams *et al.*, 2012).

4.5.9 New regimens

The use of long-term treatments has led to the search for new, more efficient as well as less toxic regimens to ensure compliance and successful treatment outcomes. Moreover, the currently licensed drugs such as RIP, CLZ or fluoroquinolones are under revaluation to propose new regimens.

RIP has shown 5-fold greater potency and also provides a longer half-life than RIF (Rosenthal *et al.*, 2012). Nevertheless, the Tuberculosis Trials Consortium Study 29 does not support this superiority of RIP (Dorman *et al.*, 2011). In this phase II trial, adults with sputum smear-positive pulmonary TB were randomly assigned to receive either RIP 10 mg/kg or RIF 10 mg/kg combined with INH, PZA and EMB. Despite RIP being well-tolerated and safe, it was not found to be significantly more potent than RIF. CLZ was found to have a sterilising activity which could increase that of TMC207 plus PZA and RIP (Williams *et al.*, 2012). Moreover, a 9-month regimen including GAT, EMB, PZA and CLZ supplemented by KAN, prothionamide and INH achieved less than 1% of failure and close to 90% culture conversion (Van Deun *et al.*,

2010). Both TCM207 and PA-824 in combination with PZA or MOX are currently being tested in the clinical development of novel regimens to shorten TB treatment (Williams *et al.*, 2012).

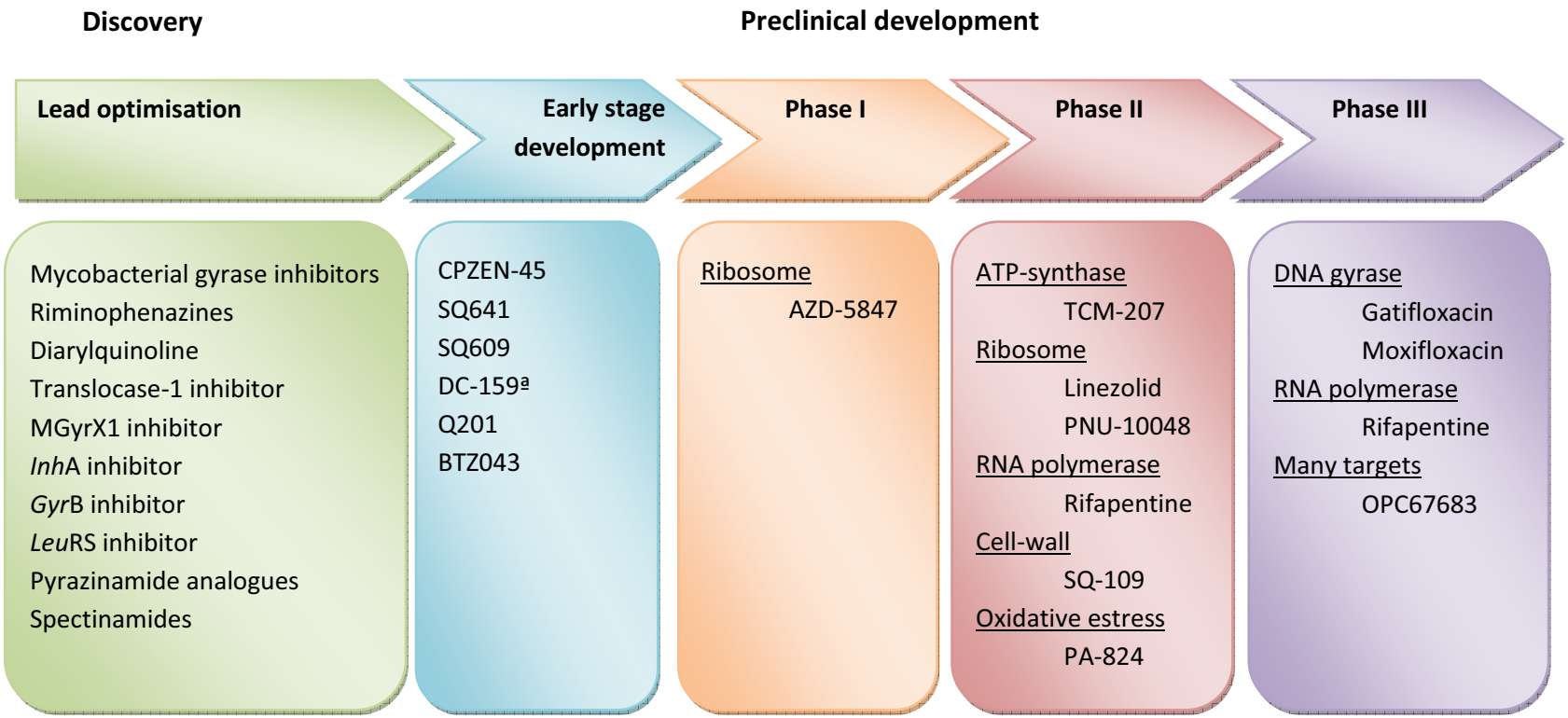


Figure 19. Development of drugs against TB

5. ANTIMICROBIAL COMBINATIONS IN *IN VITRO* APPROACHES

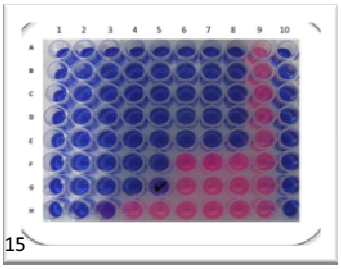
Antimicrobial combinations are used to provide broad-spectrum coverage in patients who are seriously ill or to prevent or delay the emergence of drug-resistant organism. Apart from the importance of these combinations in *M. tuberculosis,* their relevance has also been shown in the treatment of *Acinetobacter baumannii*, *Staphilococcus aureus* in biofilms or *Candida albicans*. DST in *M. tuberculosis* is carried out for each drug individually however, drugs act in combination. Consequently, the clinical outcome may depend on the interactions of the drugs against *M. tuberculosis*.

5.1 Definitions

Antimicrobial interactions *in vitro* can be defined as synergism, antagonism or indifference. Synergism means the combined effect is significantly greater than that of the drugs tested individually. By contrast, antagonism is a negative effect in which the combined effect is significantly less than the action of the drugs tested separately. If the drugs do not interact with one another, the combined effect is expected to be the sum of the effect of drugs tested individually, defined as indifference. Different methods have been described to study antimicrobial combinations *in vitro*. It is noteworthy that all these techniques are thought to study bacteria other than *M. tuberculosis*. The most reliable techniques in the study of antimicrobial combinations against *M. tuberculosis* are checkerboard assays and killing curves.

5.2 *In vitro* techniques

5.2.1 Checkerboard assays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The checkerboard assay is a widely used method. This technique is based on the pattern formed, tubes or microtitration trays, by multiple dilutions of the two antimicrobials tested, in concentrations above, below or equal to their MICs against the organisms tested (Figure 20). One of the advantages of this method is the number of concentration combinations that may be simultaneously tested.

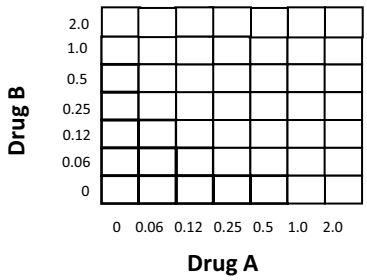


Figure 20 . Example checkerboard testing.

Results of checkerboard assays are interpreted by the pattern on isobolograms which plot the Fractional Inhibitory Concentration (FIC) index on an arithmetic scale (Figure 21). The FIC index is calculated as follows: $A/MIC_A + B/MIC_B = FIC_A + FIC_B$, where, A and B are the MICs in combination with each antibiotic in combination, and MIC_A and MIC_B are the individual MICs. Synergism is interpreted when the FIC index is less than or equal to 0.5, antagonism is considered with a FIC index of more than 4, and indifference values are deemed from 0.5 to 4 (Lorian, 2005). This interpretation is based on combinations of two drugs, where each drug decreases up to two dilutions with respect to its MIC.

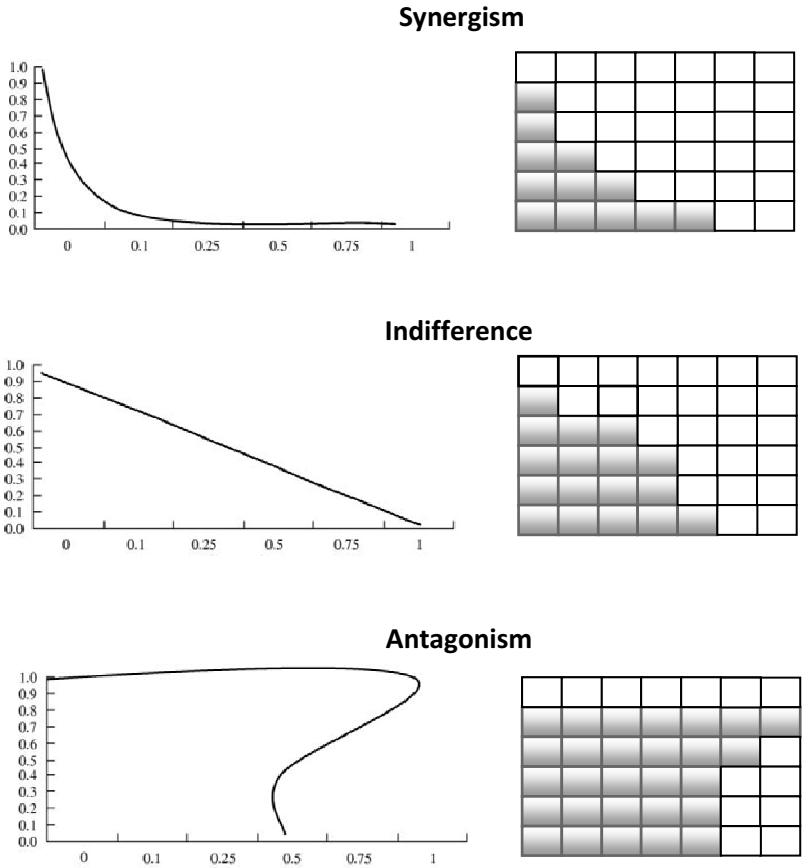


Figure 21. Interpretation of checkerboard assay. Shaded squares are the visible growth of the bacteria (Adaptation from Cuenca-Estrella, 2004).

Most checkerboard studies evaluate combinations of two drugs against bacteria, with interpretation and validation being easier than with combinations of more than two drugs (Dosler *et al.*, 2011). More than two drugs have been tested in checkerboard assays, overall with *M.tuberculosis* and antifungal combinations. Testing more than two drugs has been adapted with three dimensional or n-dimensional approaches and variations of FIC values (Bhusal *et al.*, 2005; Dai *et al.*, 2010). Bushal *et al*. (2005) showed the differences between two three-drug combinations tested against MDR *M. tuberculosis* using a three dimensional isobologram of FIC indices (Figure 21). Moreover, the same authors proposed a FIC index value of 0.75 to determine synergism in three-drug combinations since the three drugs decreased up to two dilutions with respect to their MIC, with a subsequent sum of the formula of 0.75 (Bhusal *et al.*, 2005).

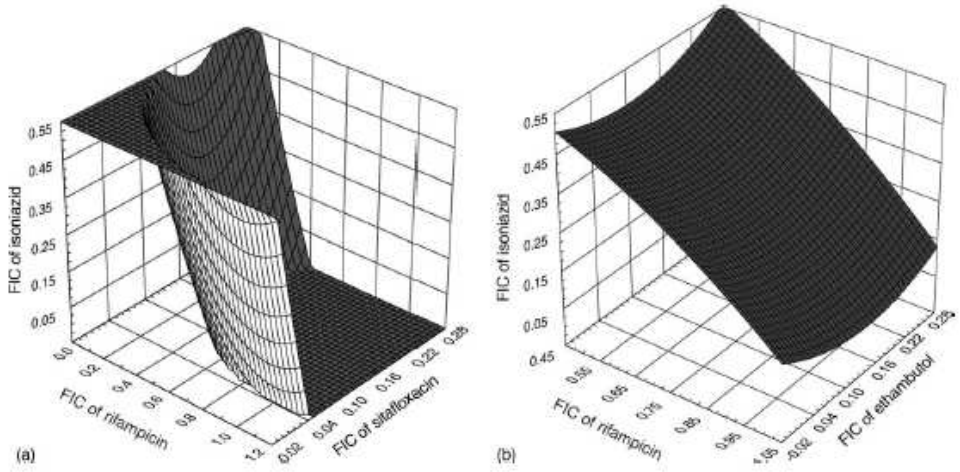
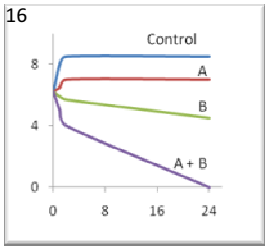


Figure 21. Three dimensional isobologram of FIC indices. a) combination showing the sinking of the isobologram towards the origin indicating synergism, b) combination did not show significant depression indicating indifference (Bhusal *et al*., 2005).

5.2.2 Killing curves



In contrast to checkerboard assays, killing curves provide a dynamic technique and measure the microbicidal activity rather than only inhibitory data (Mueller *et al.*, 2004). Nevertheless, the number of concentrations tested is limited due to the number of tubes required, therefore the concentrations tested need to be chosen with previous knowledge. The technique is based on counting the number of viable organisms over time, testing different combinations and concentrations with a standardized inoculum (Lorian, 2005). The results of antimicrobial interaction in this

technique are interpreted as synergism if the combination demonstrates a 100-fold increase in killing at 24h compared with the most active drug, and antagonism is interpreted with the presence of a 100-fold decrease (Figure 22). Killing curves with *M. tuberculosis* require an adaptation of the inoculum preparation due to the tendency to form clumps. Indeed, the incubation and end times must be modified due to the higher doubling time of *M. tuberculosis* compared with other bacteria.

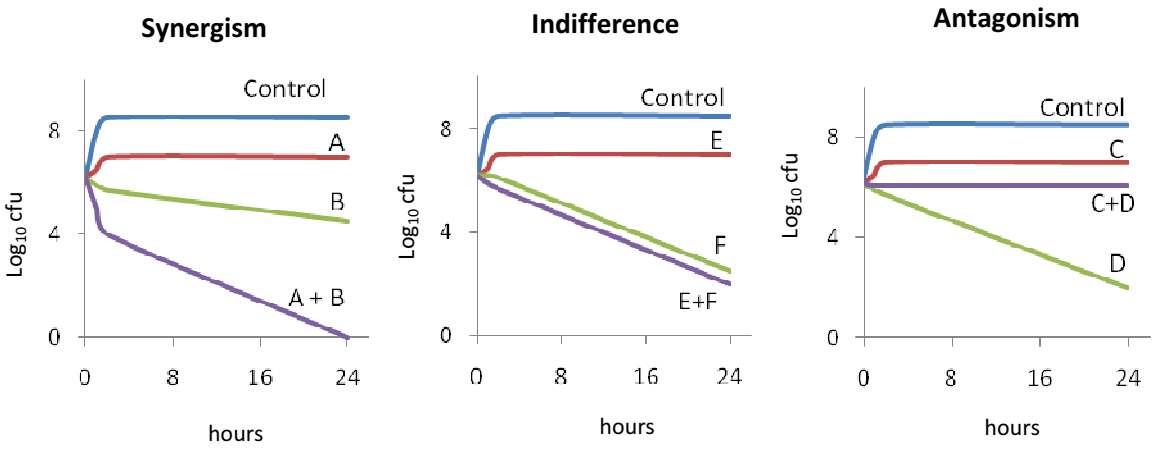


Figure 22. Interpretation of killing curve method.

5.3 Effect of drug combinations against bacteria

Several studies have reported drug combinations resulting in synergism against bacteria. This synergism can be explained by different mechanisms. The sequential inhibition of a common biochemical pathway has been shown when sulfamides and trimethoprim are combined *in vitro* against *E. coli*, *H. Influenza* or *B. Pertussis*. The use of enzyme inhibitors such as β-lactamase inhibitors have been shown to inhibit efflux pumps which contribute to resistance to a broad range of antimicrobials, including *M. tuberculosis* (Flores *et al.*, 2005). ABC transporters have been described in *M. tuberculosis*, which provide resistance to aminoglycosides, fluroquinolones, RIF or INH (Nguyen *et al.*, 2006; Balganesh *et al.*, 2012). Adams *et al*. (2011) showed activation of bacterial efflux pumps in response to membrane and oxidative stress and antimicrobial peptides *in vitro*, all of these conditions being present in macrophages. Moreover, β-lactamases have also been reported to protect susceptible antibiotics from degradation by enzymes. The combination of cell wall-active agents such as

penicillin or glycopeptides with other poorly penetrating antibiotics such as aminoglycosides, can enhance access to the intracellular target resulting in synergism.

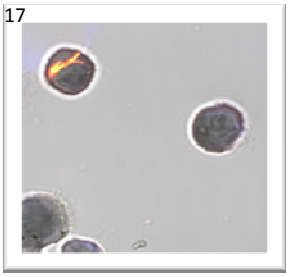
Fluoroquinolones such as GAT have been described to enhance the antimicrobial activity of RIF and INH against *M. tuberculosis* by checkerboard and time killing assays (Bhusal *et al.*, 2005). This latter paper supports the previous work by Rastogui *et al*. (1996) in which the combination of LEV, EMB, RIF and AMK was found to be the most bactericidal. LNZ showed antagonism with vancomycin and a trend to antagonism with CIP against *S.aureus* using time killing curves (Grohs *et al.*, 2003). By contrast, Rodriguez Diaz *et al*. (2003) did not find either synergism or antagonism of fluoroquinolones combined with LNZ in any of the *M. tuberculosis* isolates studied. Recently, Reddy *et al*., (2012) tested the combined effect of the new amtimycobacterial compounds SQ109 and PNU by microdilution chequerboard dilution and time killing assays, showing a non antagonism effect.

6. FITNESS OF DRUG-RESISTANT *M. tuberculosis*

The concept of fitness in infectious pathogens is defined as the ability to survive, reproduce, and be transmitted. Therefore, this definition can express the characteristics of the microorganism within the host to survive the stress conditions inside the host, and the capacity to disseminate and set up in a new host (Cohen *et al.*, 2003). Taking the previously mentioned definition as a reference, the methodologies to perform an approach of the fitness of drug-resistant *M. tuberculosis* can focus on studying survival, dissemination or transmission. These approaches include measurement of growth rates in cell cultures, infectivity in terms of dissemination to tissues in animal models, the ability to respond to stress conditions, and the rates of secondary cases in epidemiological studies.

6.1 Approaches to study the fitness

6.1.1 Cell cultures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Cell culture is the process by which cells are grown under controlled conditions. These conditions can be essential nutrients, growth factors, hormones, gases such as O_2 and CO_2 and physical-chemical environment such as temperature, pH or osmotic pressure. For example, mammalian cells are usually grown at 37ºC and in 5% of CO_2 in a cell incubator. Cells can be obtained from the tissue, then isolated and proliferated until they occupy all of the available substrate, being the primary culture. These cells are subcultured by transfer to a new flask with fresh medium, and then a cell line is obtained. Immortalized cell lines can be obtained with several passages, indefinitely proliferating the same cell without the appearance of mutations or modifications. Several immortalized cell lines are available from commercial companies.

Since *M. tuberculosis* is an intracellular pathogen and macrophages are the first response to infection, these cells are used to study the fitness of *M. tuberculosis* strains. These models provide an approach of the early stage of the infection, when the phagocytosis of *M. tuberculosis* is done by the alveolar macrophages located in the lungs. Indeed, correlation between the intracellular growth of *M. tuberculosis* isolates in macrophage culture models and the transmission dynamics of TB in humans have been reported (Zhang *et al.*, 1999; Theus *et al.*, 2005; Theus *et al.*, 2006). Macrophages can be obtained from mice or humans and can be

primary cultures or immortalized cell lines. Mouse primary cultures can be obtained from bone marrow, lung alveoli or peritoneal exudates. Human monocyte-derived macrophages obtained from healthy volunteer donor blood are also used. Primary cultures may be more representative of the *in vivo* setting, however, variability between donors is high and in the case of humans, sample availability may be low. Due to the limitations mentioned, a range of cell lines are available in order to mimic macrophage conditions to carry out studies of host-pathogen interactions, activity of antituberculous drugs or survival and replication of bacilli inside macrophages (Zhang *et al.*, 1999; Kuehnel *et al.*, 2001; Dhillon *et al.*, 2010; Zhang *et al.*, 2005).

The widely used cell lines available include mouse, J774 and MH-S, and human, THP-1 and U937 (Table 6). These cell lines have shown to be good models in terms of receptor expression, bacterial uptake, survival and replication of *M. tuberculosis*.

Table 6 . Cell lines used in *M.tuberculosis* research.

Cell line	Cell type	Biological source	Specie
J774	Macrophage	Solid tumour	Mouse
MH-S	Macrophage	SV40 transformation of alveolar macrophages	Mouse
THP-1	Monocytes*	Peripheral blood from acute monocytic leukaemia patient	Human
U937	Monocytes*	Malignant cells from patient with histiocytic lymphoma	Human
A549	Type II pneumocyte	Cancerous lung tissue	Human

*Monocytes are differentiated to macrophages by the addition of activators such as PMA.

J774 cells have showed to bind more bacilli to macrophages than human monocyte-derived macrophages, 13% *versus* 9% (Mehta *et al.*, 1996). By contrast, THP-1 has shown similar binding rates to human monocyte-derived macrophages (Stokes *et al.*, 1999). MH-S (Melo *et al.*, 2000) and THP-1 (Stokes *et al.*, 1999) display similar receptor expression and binding particles to alveolar murine and human monocyte-derived macrophages, respectively, except for binding to zymosan (a glucan which binds to TLR2). U937 cells, which need PMA as

THP-1 to be activated, bind to 57±3% of *M.tuberculosis* and express HLA class I but not CD1 (Passmore *et al.*, 2001). Recently, dendritic cells (Wolf *et al.*, 2007) and alveolar pneumocytes (Fine *et al.*, 2012) have also been proposed as a cell culture model, due to their implication in early stage immune response to infection such as what is observed with macrophages. Fine *et al.* (2012), showed how *M.tuberculosis* grows inside A549 cells, a human type II pnemocyte cell line, and unlike macrophages, led to late endosomes. Nevertheless, the differences between cell types used, in terms of methodology such as the bacilli/cell rate or incubation times, make it difficult to compare all these studies and draw conclusions thereof.

6.1.2 Animal models


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Cell cultures are reliable and easy to work with, however, to study not only the early stage of the infection various animal models have been proposed (Table 7). Indeed, animal models allow evaluating the dissemination of the pathogen to tissues other than those of the lung. Mice are the most widely used model, however, other animals such as guinea pigs and rabbits are also used to study *M. tuberculosis* infection (Gupta *et al.*, 2005). Apart from studying virulence of different strains of TB, these animal models are useful to study TB transmission, immunopathogenesis, TB response, antimicrobial efficacy or vaccines. Infected mice, guinea pigs and rabbits are the main animal model used because: 1) the infection can occur by inhalation, 2) they manifest innate and acquired immune response, 3) bacillary growth in the lung of these animals is often initially controlled, and 4) they eventually succumb to the disease (Dharmadhikari *et al.*, 2008).

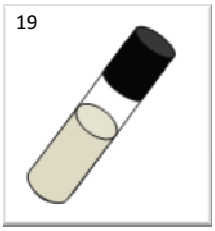
Table 7. Comparing features of common animal models of tuberculosis.

Model	Histopathology			Relative susceptibility to *M. tuberculosis*	Immunologic reagents available	Laboratory space requirements and cost	Similar to human latent TB infection	Most common experimental uses
	Necrosis	Caseation	Cavitation					
Mouse	Minimal[a]	Usually not	No	Low	Extensive	Relatively small	No[c]	Tuberculosis immunology; drug efficacy
Rabbit	Yes	Yes	Yes	Very low[b]	Moderate	Relatively large	No	Tuberculosis pathogenesis
Guinea pig	Yes	Yes	Infrequent	Very high	Relatively few	Moderate	No	Vaccine efficacy; airborne transmission; drug efficacy
Nonhuman primate	Yes	Yes	Yes	High	Extensive	Large	Yes	Tuberculosis pathogenesis; tuberculosis and retroviral immunodeficiency

[a]can depend on immune status, [b]*Mycobacterium bovis* typically used, [c]Cornell model may, inducing latency by timely administration of steroids. Adaptation from Dharmadhikari, 2008.

The low cost, availability of reagents and well-studied genetics of the mice are the main advantages, although they do not cause granuloma formation as in humans. By contrast, guinea pigs and rabbits have a more similar progression of stages but the cost-effectiveness is higher than that of mice. Due to the ability of mice to tolerate large numbers of bacilli, it is useful to evaluate the effects of chronic exposure to TB. Similar to humans, cell surfaces in mice use TLR to recognize mycobacteria. Rabbits have a histopathology very similar to humans, thus providing a good tool to study granuloma formation; however, infection in rabbits is usually induced with *M.bovis* instead of *M. tuberculosis* due to its high susceptibility. The high susceptibility to TB in guinea pigs, allows mimicking of the conditions of TB in children or in immunosuppressed hosts. Other animals such as *Cynomolgus macaque* have been proposed because they manifest latent and reactivation TB like humans. However, these animals are only used in the final pre-clinical stages of vaccine or drug development because of the high cost and space requirements needed in BSL3 facilities.

6.1.3 Competitive assays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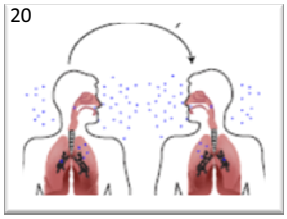
Competitive fitness assays are used to measure the "relative fitness" of bacteria. These assays are based on the ability of the strain to compete for limited resources, and therefore, evaluate the fitness from an evolutionary point of view. Two strains of interest are mixed together in equal proportions and compete for limited resources in a common environment. In these experiments, the fitness is expressed as the number of generations of drug-resistant compared with drug-susceptible isolates (Gagneux *et al.*, 2006; Borrell *et al.*, 2009; Bhatter *et al.*, 2012). To determine the effect of *rpo*B mutations in the fitness of *M.tuberculosis* Gagneux *et al*. (2006) competed spontaneous RIF-resistant mutants against their RIF-susceptible ancestor *in vitro*. Competitive assays are a good approach to the impact of the mutation on the fitness of the bacteria. Nonetheless, their use has limitations in terms of virulence, pathogenesis or transmission. Indeed, compensatory mutations or other modifications in clinical isolates during infection or transmission may not be present in the mutated strains from the laboratory.

6.1.4 Epidemiological studies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During the last years, conventional together with the molecular epidemiology have increased the knowledge of TB transmission. In epidemiology, an index case is defined as the initial patient

with TB who transmits the disease to other people, the secondary cases. Another term in epidemiology is the cluster, which is defined as a group of patients with a genetic background similar to *M. tuberculosis* strains in a determined population or geographical area over a period of time.

The number of secondary cases is a measure described to evaluate the fitness of the bacteria (Burgos *et al.*, 2003). In this way, the comparison of secondary cases of drug-resistant with drug-susceptible isolates of *M. tuberculosis* may be a parameter to know if drug-resistance has a cost in the transmission of the disease. Nevertheless, the number of secondary cases can be influenced by factors other than those of the fitness of the bacteria, such as the TB control program of the setting, the host and the conditions in the population. Thus, this is the result of the characteristics of the *M.tuberculosis* strain, host response and the facility to be spread in the community. Furthermore, other measures of the transmissibility of the strains are the comparison of frequency and size of clusters of cases of drug-resistant and drug-sensitive *M.tuberculosis* (Borrell *et al.*, 2009).

6.2 Effect of drug-resistance on *M.tuberculosis* fitness

With the detection of the first patients infected with *M.tuberculosis* resistant to some antituberculous drugs, the question of the virulence of these strains emerged. Before the mapping of the genome of *M. tuberculosis* different studies were carried out to study the behaviour of these microorganisms resistant to drugs. Middlebrook and Cohn reported that isolates resistant to INH had less pathogenicity in comparison with drug-susceptible isolates in a guinea pig model (Middlrebrook *et al.*, 1953). Nevertheless, similar to later work by Ordway *et al* (1995), the resistant clinical strains showed a wide range of virulence. The different mechanism of resistant of the antituberculous drugs with different associated mutations could explain the diversity observed by these initial studies.

Mutations in the *kat*G gene may alter or eliminate mycobacterial catalase-peroxidase activity and may, therefore, involve a loss of virulence or fitness. In an early study, Li *et al* (1998) showed how a deletion of the *katG* gene results in less growth of *M.tuberculosis* in mice. Nevertheless, clinical isolates of *M.tuberculosis* frequently have single point mutations in the *kat*G gene which may only cause a partially loss of this catalase-peroxidase activity. The point is how important this activity is in the fitness of the bacteria. Moreover, compensatory mutations to compensate this loss of activity have been described. Mutations in the promoter of the *aph*C gene, which have been reported as compensatory mutations, may protect bacilli from the toxic effects of organic peroxides by causing an overexpression of this gene encoding

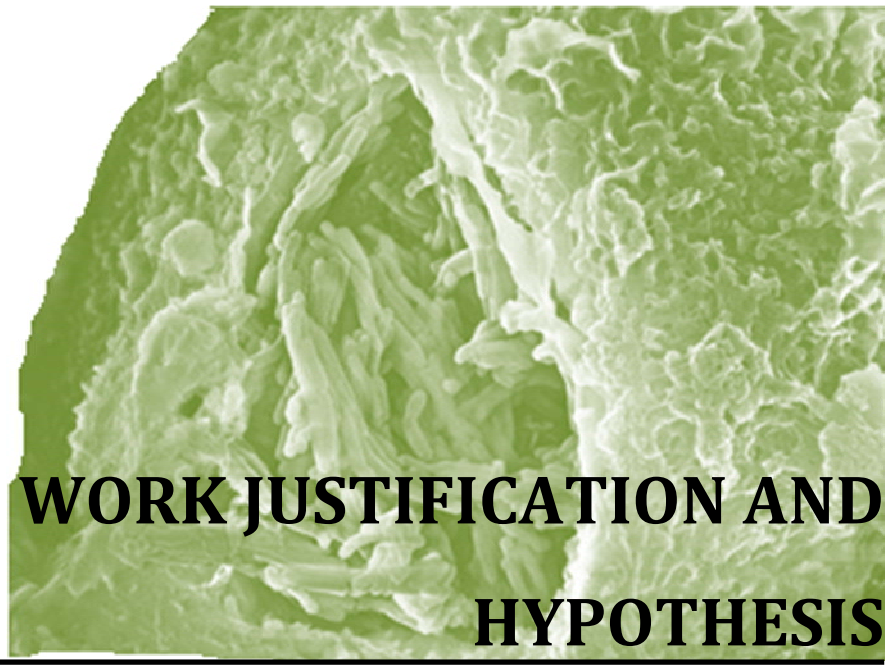
alkyl hydroperoxide reductase (Sherman *et al.*, 1996; Kelley *et al.*, 1997). On the other hand, there are studies on virulence or transmission without having the genetic background, thereby only reporting the phenotypic resistance. More studies have been carried out to evaluate the fitness of drug-resistant *M. tuberculosis*. Burgos *et al*. (2003) reported fewer secondary cases of INH-resistant than drug-susceptible TB cases, with no secondary cases caused by MDR-TB in an epidemiological study in San Francisco with 1800 patients. By contrast, van Doorn *et al*. (2006) showed that isolates with the mutation in position 315 of *kat*G gene were clustered as frequently as INH-susceptible isolates. Similarly, Gagneux *et al*. (2006) reported how isolates with a *kat*G 315 as well as an *inh*A mutation were effectively propagated in the population whereas *kat*G mutations other than S315T were not in any cluster. Nevertheless, this later work did not compare these mutations with drug-susceptible isolates.

Due to the complexity of MDR and XDR-TB, it is important to study if these isolates have a loss of fitness or are transmitted to the population in the same way as drug-susceptible TB. MDR isolates frequently have mutations in both *kat*G and *rpo*B genes, among other genes, therefore the fitness of these isolates could be affected by these mutations independently or may present mutation accumulation. Billington *et al*. (1999) studied the fitness inducing RIF resistance in *M. tuberculosis* H37Rv by competitive assays. Most of the RIF resistance isolates showed less fitness than their parent drug-susceptible strains, although the relative fitness varied widely. Similar findings were described by Gagneux *et al*. (2006). Indeed, this kind of studies has the limitation of not using clinical isolates and therefore, compensatory mutations may not be detected. Compensatory mutations for RIF resistance have been identified in the *rpo*A, *rpo*B and *rpo*C genes which encode the different subunits of the RNA polymerase (Brandis *et al.*, 2012). Thus, loss of fitness of isolates harbouring these compensatory mutations may be restored (Comas *et al.*, 2011).

Strain lineages of *M.tuberculosis* are associated with particular geographic regions and have shown different immunologic responses as well as virulence in animal models (Krishnan *et al.*, 2011; Portevin *et al.*, 2011). Particularly, the Beijing family, which has been associated with MDR, has a reportedly high virulence, albeit some studies show diversity within this group (Aguilar *et al.*, 2010). Indeed, a glycolipid has been described in strains belonging to the Beijing family which could cause hyperlethality in mouse models (Reed *et al.*, 2004).

Men love to wonder, and that is the seed of the science.

Ralph Waldo Emerson



WORK JUSTIFICATION AND HYPOTHESIS

The emergence of *M. tuberculosis* resistance to antituberculous drugs has led to the need for careful TB surveillance and control. Further knowledge of drug-resistant *M. tuberculosis* plays an important role in avoiding TB transmission as well as designing more efficient schedules of TB treatment.

I. Drug-resistance in *M. tuberculosis* is caused by spontaneous mutations in the chromosome. Since drugs target metabolic elements which are essential for bacterial growth, such mutations may have effects on physiological processes in microorganisms. These targets, which include the *kat*G, *inh*A or *rpo*B genes, are important for cell wall synthesis, protein synthesis and to protect bacilli from the toxic effects of organic peroxides. Mutations in these genes may make it difficult for bacteria to grow, to cause the disease or be transmitted to the population. However, this fitness disadvantage may be restored by compensatory mutations observed *in vitro* and *in vivo*.

II. Treatment of drug-resistant TB requires an individualized regimen depending on drug resistance, interactions and toxicity to the patient. Indeed, few drugs are available to treat drug-resistant TB, and especially MDR-TB. The length of the treatment together with drug toxicity makes compliance to drug-resistant treatment difficult. Although drug susceptibility testing is done individually, the different drugs used in TB treatment act in combination. Nonetheless, few studies have been undertaken to study the efficacy and synergy of drug combinations against *M. tuberculosis*. Thus,

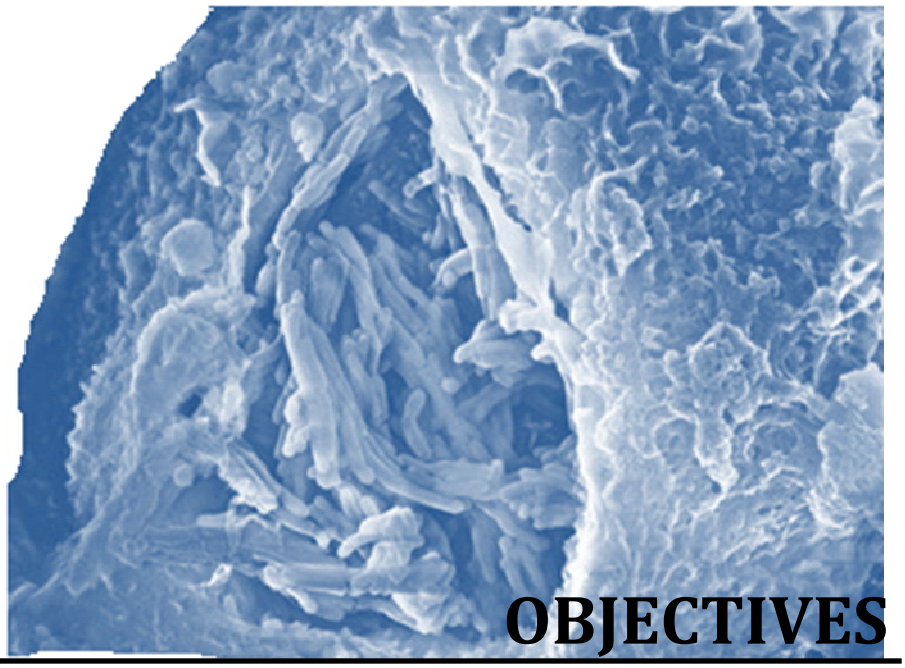
    a. First and second-line antituberculous drugs may have synergistic activity as well as display varying degrees of efficacy.

    b. Checkerboard assay adaptation to three drugs may be useful to study synergy in drug combinations.

    c. The macrophage model may be another approach to test the efficacy of drug combinations against *M. tuberculosis* since this microorganism multiplies inside macrophages. This model allows evaluation of the penetration of the drug inside macrophages and consequently the intracellular efficacy of drugs individually and in combination.

The chess-board is the world; the pieces are the phenomena of the universe; the rules of the game are what we call the laws of Nature. The player on the other side is hidden from us. We know that his play is always fair, and patient. But also we know, to our cost, that he never overlooks a mistake, or makes the smallest allowance for ignorance

Thomas Henry Huxley



OBJECTIVES

I. To analyse the ability of clinical isolates of *Mycobacterium tuberculosis* resistant to antituberculous drugs to penetrate and grow within murine macrophages compared with drug-susceptible isolates.

II.a To evaluate the ability of isolates of *M. tuberculosis* with different associated mutations to penetrate inside macrophages compared with drug-susceptible isolates.

II.b To evaluate the growth of isolates of *M. tuberculosis* with different associated mutations inside macrophages compared with drug-susceptible isolates.

II. To determine the *in vitro* synergistic activity of the following combinations against clinical isolates of *M. tuberculosis* resistant to INH compared with drug-susceptible isolates.

a. INH + RIF + EMB
b. OFL + RIF + EMB

III. To determine the *in vitro* synergistic activity of the following combinations against clinical isolates of multi-drug resistant *M. tuberculosis* compared with drug-susceptible isolates.

a. LEV + LNZ + AMK
b. LEV + LNZ + EMB
c. LEV + AMK + EMB

IV. To determine the antimicrobial and the synergistic activity of drug combinations of objective III against multidrug-resistant and drug-susceptible clinical isolates of *M. tuberculosis* in a cell culture model of human macrophages from the THP-1 cell line.

V.a To determine the individual antimicrobial activity of EMB, OFL, AMK and LNZ against *M. tuberculosis* infected-human macrophages.

V.b To determine the antimicrobial activity of the combinations described in objective IV against *M. tuberculosis*-infected human macrophages.

V.c To determine the concentration of the drugs alone and in combination inside *M. tuberculosis*-infected human macrophages.

What is a scientist after all? It is a curious man looking through a keyhole, the keyhole of nature, trying to know what's going on.

Jacques Cousteau

METHODOLOGY AND RESULTS

Objective I

Paper I. Impaired fitness of *Mycobacterium tuberculosis* resistant isolates in a cell culture model of murine macrophages.

Rey-Jurado, E., Tudó, G., Borrell, S., Alcaide, F., Coll, P., Español, M., Martín-Casabona, N., Mick, V., Montemayor, M., Moure, R., Salvadó, M., Vicente, E., González-Martín, J. Impaired fitness of *Mycobacterium tuberculosis* resistant isolates in a cell culture model of murine macrophages. J Antimicrob Chemother 2011; **66**: 2277-2280.

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Impaired fitness of *Mycobacterium tuberculosis* resistant isolates in a cell culture model of murine macrophages

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Objectives: We analysed the ability of *Mycobacterium tuberculosis* clinical isolates to penetrate and grow inside murine macrophages as a surrogate of fitness.

Methods: Thirty-five drug-resistant and 10 drug-susceptible *M. tuberculosis* isolates were studied in a murine macrophage model from the J774.2 cell line in a 6 day protocol, performing semi-quantitative counts in Middlebrook 7H11 medium. The mycobacterial penetration index (MPI) after infection and the mycobacterial growth ratio (MGR) inside the macrophages were determined to evaluate the fitness of isolates.

Results: Isolates with the *katG* S315T mutation and multidrug-resistant (MDR) isolates had a significantly lower MGR compared with drug-susceptible isolates. The MPI of the isolates with the *katG* S315T mutation showed a significant decrease compared with the MPI of those without this mutation. A trend to significantly lower values was also observed on comparing the MPI of the MDR isolates with that of the drug-susceptible isolates and the isolates resistant to isoniazid.

Conclusions: The isoniazid-resistant and MDR isolates with mutations in the *katG* gene showed decreased multiplication inside murine macrophages, suggesting a lower fitness of *M. tuberculosis* with these resistance patterns.

Keywords: *M. tuberculosis*, *katG* mutation, *inhA* mutation, *rpoB* mutation, macrophage cultures

Introduction

In *Mycobacterium tuberculosis*, the agent of tuberculosis (TB), a loss of strain fitness due to changes produced by acquired drug resistance mutations has been described.[1] Initial studies on the virulence of these strains in animal models revealed lesser growth of the resistant strains compared with that of susceptible strains.[2] A turning point in fitness studies occurred when *katG* mutation at position 315 was associated with isoniazid resistance. This gene encodes catalase–peroxidase activity, which is key in preventing oxidative stress, and, thus, a mutation in this gene could affect the fitness of the mycobacteria. Isoniazid resistance mutations are observed mainly in the *katG* gene at position 315 and to a lesser extent in the intergenic zone *mabA-inhA*.[3] *In vivo* and *in vitro* models, such as epidemiological studies, have analysed the behaviour of resistant and susceptible strains, determining whether the acquisition of mutations in these strains has led to a cost in fitness,[4,5] albeit with no clear conclusions being drawn.

In the present study, we analysed the ability of *M. tuberculosis* clinical isolates to penetrate and grow inside murine macrophages as a surrogate of fitness.

Materials and methods

Isolates, MICs and molecular resistance analysis

Forty-five non-clustered *M. tuberculosis* clinical isolates (35 drug resistant to isoniazid and/or rifampicin, and 10 drug susceptible) from the

collection of the Mycobacterial Research Group of Barcelona (1995–2005) were selected for study.

The MIC of isoniazid for each isolate was determined using the proportions method in Middlebrook 7H11 medium incubated for 21 days at serial 2-fold concentrations ranging from 0.1 to 102.4 mg/L.

The ribosome binding site (RBS) region of the *inhA* gene (GenBank U02492.1), the entire *katG* gene (GenBank X68081.1) and the rifampicin resistance-determining region of the *rpoB* gene (GenBank L27989.1) were sequenced using primers described previously.

Macrophage culture and M. tuberculosis inoculum preparation

J774.2 cell line murine macrophages (Sigma 85011428) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine, 4.5 g/L glucose (Lonza, Switzerland), 10% heat-inactivated fetal calf serum (Lonza), and a 100 U/mL penicillin and streptomycin mixture (Sigma), at 37°C in 5% CO_2 until exponential growth was achieved.

The isolates were grown in 7H9 MGIT medium (Becton Dickinson, MD, USA), supplemented with 0.25% Tween 80 (Merck, Germany) to avoid clump formation. When the MGIT was positive, the sample was centrifuged at 1174 **g** for 20 min, discarding the supernatant. Then, after adding 5 mm glass beads, the tube was shaken for 45 s and sonicated for 1 min. Lastly, clumps were disaggregated by 14 passages through a syringe needle (20G 1″ 0.9×25 mm) and 4 passages through an insulin needle (27G $\frac{1}{2}$″ 0.4×12 mm). The inoculum was adjusted to a turbidity equivalent to that of a 0.5 McFarland standard, making dilutions up to ~100 000 cfu/mL. Fifty μL of this inoculum was plated on Middlebrook 7H11 medium and incubated at 37°C in 5% CO_2 for 21 days. Quantitative counts were made thereafter.

Infection protocol

The ability of infection of the different *M. tuberculosis* clinical isolates studied was determined using the mycobacterial penetration index (MPI) and the mycobacterial growth ratio (MGR). The MPI was defined as the percentage of mycobacterial cells penetrating inside the macrophages, and the MGR was defined as the intracellular growth index of the mycobacterial cells during the 6 day protocol.

Determination of MPI

Volumes of 1 mL of supplemented antibiotic-free DMEM containing 50 000 murine macrophages were seeded in 24-well plates and incubated at 37°C in 5% CO_2. Each isolate studied was tested in duplicate. The macrophages were incubated for 3 h with *M. tuberculosis* at a multiplicity of infection of 1:1. This point was considered day 0. The macrophages were washed three times with DMEM and lysis was performed by adding 0.5% NP40 detergent (Roche, Switzerland). After two washes, the cell lysate was seeded onto Middlebrook 7H11 plates and incubated at 37°C in 5% CO_2 for 3 weeks. Quantitative counts were made and the MPI was calculated as the proportion of cfu isolated in cell lysates on day 0 with respect to the cfu of the inoculum used.

Determination of MGR

A duplicate set of infected cells remained in incubation during the 6 day protocol. The medium was renewed after 1, 4 and 5 days of infection. The MGR was calculated as the ratio between the cfu of cell lysates on day 6 and the cfu of cell lysates on day 0.

Statistical analysis

The MPI and MGR of the different isolate groups were analysed using the Kruskal–Wallis test for more than two group comparisons. If the Kruskal–Wallis test showed significance, the Mann–Whitney *U*-test for two group comparisons was performed. Bonferroni adjustments for multiple comparisons among groups were used in this case. Statistical analyses were performed using SPSS 16, v02.

Results

Table 1 shows the molecular mechanisms of resistance and the MIC of isoniazid for the 35 drug-resistant isolates studied. Neither

Table 1. Characteristics of the 35 drug-resistant *M. tuberculosis* isolates

Gene mutation	Type and location of mutations	Type of resistance	INH MIC (mg/L)	No. of isolates
katG				13
	S315T	INH	<1	1
	S315T	INH	1–9	3
	S315T	INH	≥10	2
	S315T	INH	ND	4
	S315T	INH, STR	1–9	1
	S315T	INH, RIF[a]	≥10	2
inhA				5
	C−15T	INH	<1	3
	C−15T	INH	1–9	1
	C−15T	INH, STR	1–9	1
rpoB				5
	S531L	RIF	NA	1
	S531L	INH, RIF[b]	1–10	1
	S531L	INH, RIF[b]	≥10	1
	S531L	INH, RIF, EMB[b]	<1	1
	S531L	RIF, STR	NA	1
katG and *rpoB*				8
	S315T and H526A	INH, RIF, STR, PZA	1–9	1
	S315T and H526A	INH, RIF, STR, PZA	≥10	1
	S315T and S531L	INH, RIF	1–9	4
	S315T and S531L	INH, RIF	<1	1
	S315T and S531L	INH, RIF, STR	≥10	1
Wild-type				4
	—	INH, STR	≥10	1
	—	INH	<1	2
	—	INH	≥10	1

ND, not done; NA, not applicable; INH, isoniazid; STR, streptomycin; RIF, rifampicin; PZA, pyrazinamide; EMB, ethambutol; S315T, serine→threonine change in the *katG* gene at position 315; C−15T, nucleotide change in the *inhA* gene; S531L, serine→leucine change in the *rpoB* gene at position 531; H526A, histidine→adenine change in the *rpoB* gene at position 526.

[a]Isolate resistant to rifampicin without a known *rpoB* mutation.

[b]Isolates resistant to isoniazid without a known *katG* or *inh*A mutation.

the MPI nor the MGR significantly differed according to the MIC ranges for the isolates studied.

MPI according to the resistance pattern

No significant difference was observed on comparing the MPI of the isolates, according to four categories: susceptible; isoniazid resistant; rifampicin resistant; and multidrug resistant (MDR). A trend to significantly lower values was observed on comparing the MPI of the MDR isolates with that of the drug-susceptible and the isoniazid-resistant isolates ($P=0.042$ and $P=0.036$, respectively).

MPI according to the mutations associated with resistance

A lower MPI was found in the resistant isolates with a mutation in the *katG* gene compared with those resistant without this mutation ($P=0.009$), although no significant differences were observed on comparison with drug-susceptible isolates (Figure 1a).

MGR according to the resistance pattern

On comparing the above-mentioned categories, the MGR was significantly lower in the MDR isolates than in the drug-susceptible isolates ($P=0.012$). The isoniazid-resistant isolates showed a trend to having a significantly lower MGR than drug-susceptible isolates ($P=0.074$). Isolates resistant to only rifampicin did not significantly differ from drug-susceptible isolates.

MGR according to the mutations associated with resistance

A significantly lower MGR was found in the 21 isolates with a *katG* gene mutation compared with drug-susceptible isolates ($P=0.013$). No significant difference was found when drug-susceptible isolates were compared with those without a *katG* gene mutation (Figure 1b). Furthermore, no significant difference was observed on comparing the 13 isolates with only a *katG* gene mutation and 8 isolates with mutations in the *katG* and *rpoB* genes with the drug-susceptible isolates ($P=0.030$ and $P=0.041$, respectively).

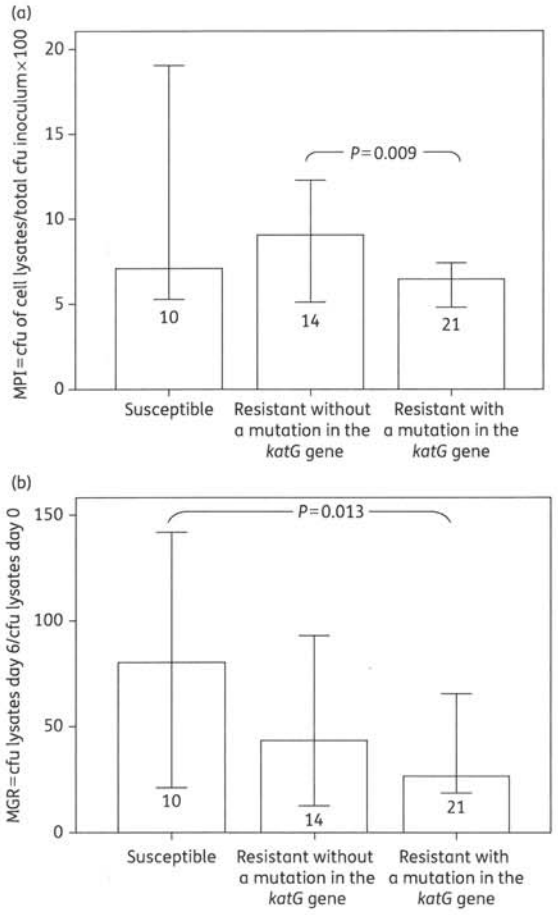

Figure 1. MPI (a) and MGR (b) values of isoniazid-susceptible and -resistant isolates according to the presence or absence of a mutation in the *katG* gene. Data are represented as median values ±95% confidence interval of the different isolates of each group. The number of each group is shown inside each bar.

Discussion

The most important result of the present study is that isoniazid-resistant isolates with a *katG* gene mutation at position 315 and MDR isolates showed significantly lower growth in a murine macrophage model compared with drug-susceptible isolates.

The study of the molecular epidemiology of *M. tuberculosis* isolates is an excellent method to analyse TB transmission patterns by analysis of clusters and secondary cases and to indirectly study the fitness and virulence of clinical isolates. In this context, a significant number of isolates reported in different studies are worthy of comment. Burgos *et al.* detected fewer secondary cases from resistant isolates (MDR and only isoniazid resistant) than from susceptible isolates. In contrast, van Doorn *et al.* found that isolates with a *katG* gene mutation at position 315 were clustered as frequently as those susceptible to isoniazid. Likewise, Gagneux *et al.* indicated that isolates with the same mutation had as many secondary cases as those with a mutation in the *inhA* gene and suggested the same hypothesis as van Doorn *et al.*, despite not comparing these isolates with drug-susceptible isolates.

Although *in vitro* models of macrophages from cell cultures provide an excellent opportunity to directly study the fitness of *M. tuberculosis* isolates, the literature is limited, making it difficult to draw clear conclusions. To our knowledge, the present study has analysed the capacity of penetration and multiplication of the largest number of susceptible and drug-resistant clinical isolates (10 and 35, respectively) according to their resistance pattern as well as their associated mutations reported to

date. Similar to the previously mentioned epidemiology studies,[7] we observed that the fitness of MDR isolates decreased compared with that of drug-susceptible isolates.

We also observed an alteration in the fitness of the isoniazid-resistant isolates with mutation in the *katG* gene at position 315, in contrast to the previously reported results.[7,8] This fitness alteration in isoniazid-resistant isolates with a mutation in the *katG* gene could be explained by some loss of catalase–peroxidase function. Discordance with epidemiological studies may be explained by the greater difficulty in detecting impaired fitness with indirect methods. In this sense, some aspects of molecular epidemiological analysis could contribute to increase the specificity of further studies on the epidemiological links among isolates from clusters, such as the use of secondary markers and contact tracing to determine whether patients belong to the same cluster or are not linked at all.

Recently, it has been reported that the dynamics of TB transmission may also be affected by the bacterial genetic background of *M. tuberculosis*, which may influence the clinical localization, diagnostic aspects and the virulence of the isolates.[10] Thus, future studies on the fitness of *M. tuberculosis* should consider these aspects.

In conclusion, isoniazid-resistant and MDR isolates with a *katG* gene mutation showed a decrease in their multiplication inside murine macrophages, suggesting a lower fitness of TB with these resistance patterns.

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Transparency declarations

None to declare.

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Objective II

Paper II. Synergistic effect of two combinations of antituberculous drugs against *Mycobacterium tuberculosis*.

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DRUG DISCOVERY AND RESISTANCE

Synergistic effect of two combinations of antituberculous drugs against *Mycobacterium tuberculosis*

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SUMMARY

Fluoroquinolones such as ofloxacin are promising drugs to treat drug-resistant tuberculosis (TB) and have been proposed for shortening the treatment of TB. The objectives were to study the synergistic effect of the combinations of three drugs and to evaluate the *in vitro* interactions of the following combinations against *Mycobacterium tuberculosis*: A) isoniazid, rifampicin, and ethambutol and B) ofloxacin, rifampicin, and ethambutol using an adaptation of the two-dimensional chequerboard assay. A total of 12 isolates resistant to isoniazid or to isoniazid-streptomycin and 11 drug-susceptible isolates were tested. The fractional inhibitory concentration (FICI) was calculated as follows: $FICI = FIC_A + FICB + FIC_C = A/MIC_A + B/MIC_B + C/MIC_C$ where A, B and C were the MICs of each antibiotic in combination and MIC_A, MIC_B and MIC_C were the individual MICs. The FICI was interpreted as synergism when the value was ≤ 0.75. In combination A, 11 drug-susceptible isolates decreased the individual MIC one to three dilutions, showing indifferent activity in 81.8% (FICI = 0.88–1.6) and synergistic activity in 18.1% (FICI = 0.6). In combination B, 21 out of the 23 isolates studied (91.3%) showed synergism (FICI = 0.31–0.62). In conclusion, adaptation of the two-dimensional chequerboard assay is a reliable method to study *in vitro* three-drug combinations. Both three-drug combinations tested may be useful against drug-resistant isolates, although the combination including ofloxacin showed better efficacy, being of potential use in drug-susceptible and isoniazid-resistant isolates.

1. Introduction

Tuberculosis (TB) continues to be one of the infectious diseases of greatest incidence in the world, with around 9,000,000 new cases being declared annually.[1] According to WHO recommendations, treatment of tuberculosis is based on a six-month regimen using isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA) to prevent the development of drug resistance due to selection of mutants to one or more drugs.[1] Among all the incident cases of TB worldwide, 3.6% are estimated to be multidrug resistant (MDR).[1] Treatment of drug-resistant TB requires an individualized regimen depending on drug resistance, interactions and toxicity to the patient. However, there are few antituberculous drugs which may be used as alternatives against MDR isolates. In recent years, fluoroquinolones such as ofloxacin (OFX) or moxifloxacin (MXF) have appeared as a promising new therapy and are frequently used in the treatment of drug-resistant cases.[2] Moreover, clinical trials have found that fluoroquinolones may also be useful in the treatment of drug-susceptible *Mycobacterium tuberculosis* in order to shorten the treatment.[3] Nonetheless, although the different drugs used act in combination, drug-susceptibility testing in *M. tuberculosis* is done individually for each drug and therefore, there is no *in vitro* marker of this combined effect. From this point of view, this effect could be studied using the chequerboard dilution and/or the time-kill method. Nevertheless, there are some difficulties using these methodologies with three or more drugs, since the number of crossed concentrations to be cultured grows exponentially. Consequently, few studies have been undertaken on the efficacy and synergy of drugs administered in combination against *M. tuberculosis*,[4–7] in spite of the importance of this severe disease and the long period of treatment needed.

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The objectives of the present study were: first, to study the synergistic effect of the combinations of three drugs and second, to analyze the following two combinations, INH, RIF, EMB and OFX, RIF and EMB against INH-resistant and drug-susceptible isolates.

2. Material and methods

Twelve INH-resistant clinical isolates and 11 drug-susceptible (10 clinical isolates and the H37Rv reference strain) were studied. All the experiments were determined in duplicate with the proportions method in Middlebrook 7H11 solid medium supplemented with 10% OADC, incubating the plate 21 days at 37 °C in 5% CO_2. The isolates studied were grown in Lowenstein-Jensen medium slants. Colonies were suspended with sterile distilled water containing 5 mm glass beads (Afora, Spain) and vortexed during 45 s. The supernatant was harvested and adjusted to 0.5 McFarland with a nephelometer (Becton–Dickinson, MD, USA). As a control 50 μl of a 1/100 inoculum was seeded on an antibiotic-free Middelebrook 7H11 plate.

The individual MICs for EMB, RIF, OFX and INH were determined for each isolate using the following ranges of drug concentrations: 0.31 μg/mL to 5 μg/mL for EMB; 0.125 μg/mL to 2 μg/mL for RIF; 0.125 μg/mL to 2 μg/mL for OFX; 0.025 μg/mL to 102.4 μg/mL for INH-resistant isolates and from 0.0125 μg/mL to 1 μg/mL for INH-susceptible isolates.

The MIC in combination with three drugs was studied crossing five concentrations for each drug: the individual MIC found, one MIC above and three below, giving 625 possible combinations. In order to avoid seeding this number of plates simultaneously an alternative methodology was developed in two consecutive steps. First, three concentrations of each drug were tested, including the MIC and the immediately lower and upper concentrations. In the second step, the three concentrations lower than the MIC were included. The Fractional Inhibitory Concentration Index (FICI) was calculated as follows: $FICI = FIC_A + FICB + FIC_C = A/MIC_A + B/MIC_B + C/MIC_C$ where A, B and C were the MIC of each antibiotic in combination and MIC_A, MIC_B and MIC_C were the MIC of each antibiotic alone. The results of the FICI were analysed according to the chequerboard method[8] adapted to three drugs in combination. As described by Bhusal et al.,[7] a decrease of two dilutions under the individual MIC was interpreted as synergistic with a FICI <0.75; indifference was determined from 0.75 to 4 and a FICI >4 was considered as antagonistic activity.

3. Results

The MIC in combination of the INH, RIF and EMB combination (Table 1) against the 11 drug-susceptible isolates decreased the individual MIC from one to three dilutions, showing indifferent activity in 81.8% (FICI = 0.88–1.6) and synergistic activity in 18.1% (FICI = 0.6). The MIC in combination in the INH-resistant isolates decreased the individual MICs three dilutions, leading to synergistic activity in all (FICI = 0.31–0.38). The differences between INH-resistant and drug-susceptible isolates were significant ($p < 0.001$). The decreases observed in the MIC in combination in the OFX, EMB and RIF combination were similar in all the isolates studied (Table 2), being mainly of two or three dilutions with respect to the individual MIC. Twenty-one out of the 23 isolates studied (91.3%) showed synergism (FICI = 0.31–0.62). No difference was observed between INH-resistant and drug-susceptible isolates compared with this combination.

The OFX, RIF and EMB combination showed significantly more synergism than that of the INH, RIF and EMB combination ($p = 0.007$) for drug-susceptible isolates.

4. Discussion

The study of the combined effect of the different drugs used in the treatment of TB could be the first step for predicting the efficacy of drug combinations. To date, most *in vitro* studies on drug combinations in the treatment of TB have involved two-drug combinations.[4–6] Thus, to our knowledge, this is the second manuscript describing the study of synergism among combinations of three drugs against *M. tuberculosis*.[7] Moreover, studies of drug combinations with three drugs are probably a more realistic approach since most treatment regimens involve combinations of at least three antituberculous drugs. The present study shows that this methodology is feasible as previously reported.[7]

Table 1
MICs and FICI of the INH, RIF and EMB combination of the isolates studied.

Isolate	Susceptibility pattern	MIC alone (μg/mL)			MIC in combination (μg/mL)			FIC			FICI
		INH	RIF	EMB	INH	RIF	EMB	INH	RIF	EMB	
18098	INH	51.2	0.5	2.5	6.4	0.06	0.31	0.12	0.12	0.12	0.38
14533	INH	0.8	0.5	2.5	0.1	0.06	0.31	0.12	0.12	0.12	0.38
12765	INH	1.6	1	2.5	0.2	0.12	0.31	0.12	0.12	0.12	0.38
63752	INH	0.8	0.5	2.5	0.1	0.06	0.31	0.12	0.12	0.12	0.38
49493	INH	3.2	1	2.5	0.2	0.12	0.31	0.06	0.12	0.12	0.31
39984	INH	51.2	1	2.5	6.4	0.12	0.31	0.12	0.12	0.12	0.38
44016	INH	51.2	1	2.5	6.4	0.12	0.31	0.12	0.12	0.12	0.38
14531	INH	0.8	0.5	2.5	0.1	0.06	0.31	0.12	0.12	0.12	0.38
63734	INH	0.8	0.5	2.5	0.1	0.06	0.31	0.12	0.12	0.12	0.38
63762	INH	0.8	1	2.5	0.1	0.12	0.31	0.12	0.12	0.12	0.38
63753	INH	0.8	1	2.5	0.1	0.12	0.31	0.12	0.12	0.12	0.38
12776	INH	0.8	0.5	2.5	0.1	0.06	0.31	0.12	0.12	0.12	0.38
1S	*	0.02	0.5	2.5	0.01	0.12	0.31	0.5	0.25	0.12	0.88
2S	*	0.02	0.5	2.5	0.01	0.12	0.31	0.5	0.25	0.12	0.88
3S	*	0.02	0.12	2.5	0.01	0.12	0.31	0.5	1	0.12	1.6
4S	*	0.05	0.25	2.5	0.01	0.12	0.31	0.25	0.5	0.12	0.88
5S	*	0.05	0.25	2.5	0.01	0.12	0.31	0.25	0.5	0.12	0.88
6S	*	0.05	0.25	2.5	0.01	0.12	0.31	0.25	0.5	0.12	0.88
7S	*	0.05	0.5	2.5	0.01	0.12	0.31	0.25	0.25	0.12	0.6
8S	*	0.05	0.5	2.5	0.01	0.12	0.31	0.25	0.25	0.12	0.6
9S	*	0.05	0.25	2.5	0.01	0.12	0.31	0.25	0.5	0.12	0.88
10S	*	0.02	0.5	2.5	0.01	0.12	0.31	0.5	0.25	0.12	0.88
H37Rv	*	0.05	0.25	2.5	0.01	0.12	0.31	0.25	0.5	0.12	0.88

MIC: Minimal Inhibitory Concentration; FIC: Fractional Inhibitory Concentration; FICI: Fractional Inhibitory Concentration Index; INH: Isoniazid; RIF: Rifampicin; EMB: Ethambutol. * Drug-susceptible isolates.

Table 2
MICs and FICI of the OFX, EMB and RIF combination of the isolates studied.

Isolate	Susceptibility pattern	MIC alone (μg/mL)			MIC in combination (μg/mL)			FIC			FICI
		OFX	EMB	RIF	OFX	EMB	RIF	OFX	EMB	RIF	
18098	INH	1	2.5	0.5	0.25	0.31	0.06	0.25	0.12	0.12	0.5
14533	INH	1	2.5	0.5	0.25	0.31	0.06	0.25	0.12	0.12	0.5
12765	INH	1	2.5	1	0.25	0.31	0.12	0.25	0.12	0.12	0.5
63752	INH	1	2.5	0.5	0.25	0.31	0.06	0.25	0.12	0.12	0.5
49493	INH	1	2.5	1	0.25	0.31	0.5	0.25	0.12	0.5	0.87
39984	INH	1	2.5	1	0.25	0.31	0.12	0.25	0.12	0.12	0.5
44016	INH	1	2.5	0.5	0.25	0.31	0.06	0.25	0.12	0.12	0.5
14531	INH	1	2.5	1	0.25	0.31	0.12	0.25	0.12	0.12	0.5
63734	INH	1	2.5	0.5	0.25	0.31	0.06	0.25	0.12	0.12	0.5
63762	INH	1	2.5	0.5	0.25	0.31	0.06	0.25	0.12	0.12	0.5
63753	INH	1	2.5	1	0.25	0.31	0.12	0.25	0.12	0.12	0.5
12776	INH	1	2.5	1	0.25	0.31	0.5	0.25	0.12	0.5	0.87
1S	*	0.5	2.5	0.5	0.06	0.31	0.03	0.12	0.12	0.06	0.31
2S	*	0.5	2.5	0.5	0.06	0.31	0.03	0.12	0.12	0.06	0.31
3S	*	0.25	2.5	0.12	0.06	0.31	0.03	0.12	0.12	0.25	0.62
4S	*	0.5	2.5	0.25	0.06	0.31	0.03	0.12	0.12	0.12	0.4
5S	*	0.5	2.5	0.25	0.06	0.31	0.03	0.12	0.12	0.12	0.4
6S	*	0.5	2.5	0.25	0.06	0.31	0.03	0.12	0.25	0.12	0.5
7S	*	0.5	2.5	0.5	0.06	0.31	0.03	0.12	0.12	0.06	0.31
8S	*	0.5	2.5	0.5	0.06	0.31	0.03	0.12	0.12	0.06	0.31
9S	*	0.5	2.5	0.5	0.06	0.31	0.03	0.12	0.12	0.06	0.31
10S	*	0.5	2.5	0.5	0.06	0.31	0.03	0.12	0.12	0.06	0.31
H37Rv	*	0.5	2.5	0.25	0.06	0.31	0.03	0.12	0.12	0.12	0.4

MIC: Minimal Inhibitory Concentration; FIC: Fractional Inhibitory Concentration; FICI: Fractional Inhibitory Concentration Index; OFX: Ofloxacin; EMB: Ethambutol; RIF: Rifampicin; INH: Isoniazid. * Drug-susceptible isolates.

The INH, RIF and EMB combination showed synergism in the INH-resistant isolates but not in those which were drug-susceptible. Most of the MICs in combination in the INH-resistant isolates decreased to concentrations approaching susceptible rates suggesting that the action of the combination could overcome the resistance in the INH-resistant isolates. This has been previously described in mice infected with low level INH-resistant isolates (MICs $\leq$ 5 μg/mL) and treated with INH in which reductions in spleen and lung CFU counts were observed.[9] In human TB cases resistance of isolates with low INH-resistance is not always detected, thereby explaining the cure of patients infected with these isolates with standard TB treatment.[10]

With the appearance of fluoroquinolones, new therapeutic regimens to treat patients infected with *M. tuberculosis* resistant became possible. Our results demonstrate this potential of fluoroquinolones,[3] since interestingly, the OFX, RIF and EMB combination showed synergism against both resistant as well as drug-susceptible isolates. Indeed, testing of this combination with other fluoroquinolones more powerful than OFX may demonstrate even greater synergism.[6,11] The fact that the only difference between the two combinations is the change from INH to OFX suggests that fluoroquinolones could be more powerful than INH. Nuermberger et al.,[12] observed that MXF was able to reduce the time to culture conversion in mice by 2 months when replacing INH in standard TB treatment. However, early bactericidal activity studies showed comparable activity of MXF and INH.[13,14] Moreover, *in vitro* studies found that EMB adversely affects the activity of MXF, showing a reduction to 80%. Nonetheless, this interaction has not been clearly observed in patients on the administration of MXF together with EMB.[13]

In conclusion, both three-drug combinations tested may be useful against drug-resistant isolates, although the combination including OFX showed better efficacy, being of potential use in drug-susceptible and INH-resistant isolates. The present study of three-drug combinations against drug-resistant TB is an interesting approach to better design studies using *in vivo* and *ex vivo* models which will be useful for the design of treatment schedules in resistant cases as well, in the evaluation of treatment regimens including new drugs.

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Objective III

Paper III. *In vitro* effect of three-drug combinations of antituberculous agents against multidrug-resistant *M. tuberculosis* isolates

Rey-Jurado, E., Tudó, G., Puig de la Bellacasa, J., Espasa, M., González-Martín, J. *In vitro* effect of three-drug combinations of antituberculous agents against multidrug-resistant *M. tuberculosis* isolates. "In press" on Int J Antimicrob Agents.

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IN VITRO EFFECT OF THREE-DRUG COMBINATIONS OF ANTITUBERCULOUS AGENTS AGAINST MULTIDRUG-RESISTANT *M. tuberculosis* ISOLATES.

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Running title: Activity of three-drug combinations against multidrug-resistant strains of *M. tuberculosis*

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ABSTRACT

Multidrug-resistance (MDR) has become a problem in the management of tuberculosis leading to an urgent need for research related to new regimens including the drugs currently available. The objectives of this study were first, to study the effect of the following second-choice three-drug combinations against MDR and drug-susceptible clinical isolates: levofloxacin, linezolid, and ethambutol; levofloxacin, amikacin and ethambutol; levofloxacin, linezolid and amikacin; and second, to compare the effect of these combinations with an isoniazid, rifampicin and ethambutol combination against drug-susceptible clinical isolates. A total of nine MDR clinical and 12 drug-susceptible isolates (11 clinical isolates and the H37Rv reference strain) were studied using an adaptation of the chequerboard assay. The fractional inhibitory concentration index (FICI) was calculated as follows: $FICI = FIC_A + FIC_B + FIC_C = A/MIC_A + B/MIC_B + C/MIC_C$ where A, B and C were the MICs of each antibiotic in combination and MIC_A, MIC_B and MIC_C were the individual MICs. The FICI was interpreted as synergism when the value was <0.75. The FICI of all the combinations ranged from 1.5 to 3, showing indifference activity. No differences were found between MDR and drug-susceptible isolates, nor between the second choice or the fourth combination against drug-susceptible isolates. In conclusion, the second-choice drugs are equally effective as the combination of isoniazid, rifampicin and ethambutol.

Key words: three-drug combination, levofloxacin, linezolid, ethambutol, amikacin, multidrug-resistant *M. tuberculosis*

INTRODUCTION

Tuberculosis (TB) remains a major global threat, the control of which has become more complicated since the emergence of multidrug-resistant (MDR) and extremely drug-resistant (XDR) strains of *M. tuberculosis*. Among all the incident cases of TB worldwide, it has been estimated that around 3.6% are MDR [1]. The treatment for susceptible isolates is based on a minimum six-month schedule including isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA) [1]. Nevertheless, the treatment of MDR-TB requires an individualized regimen depending on the type of drug resistance, interactions and toxicity. MDR is defined as resistance to at least INH and RIF, with only a maximum of two drugs of the standard regimen remaining available. Therefore, aminoglycosides such as amikacin (AMK) or kanamycin (KAN), have been described as useful in the treatment of MDR-TB [2]. In recent years, fluoroquinolones and other drugs such as linezolid (LNZ) have also appeared as promising new TB therapies [3]. Moreover, clinical trials have suggested that fluoroquinolones such as moxifloxacin or levofloxacin (LEV) may be useful in shortening the treatment of drug-susceptible TB [4]. Nonetheless, despite the need for research into new antituberculous drugs, few are currently in phases of clinical development [4]. Although the different drugs used act in combination, drug-susceptibility testing in *M. tuberculosis* is done individually for each drug and therefore, there is no *in vitro* marker of this combined effect. From this point of view, this effect could be studied using checkerboard dilution and/or the time-kill method [5-7].

The objectives of the present study were: 1. to study the effect of the three-drug combinations against MDR (3DC-MDR): LEV, LNZ, and EMB; LEV, AMK and EMB LEV, LNZ, and AMK and 2. to compare the effect of the 3DC-MDR combinations with the INH, RIF and EMB combination against drug-susceptible *M. tuberculosis* clinical isolates.

MATERIAL AND METHODS

M.tuberculosis isolates

Nine MDR clinical and 12 drug-susceptible isolates (11 clinical isolates and the H37Rv reference strain) were studied. All the experiments were performed in duplicate with the proportions method [8] in Middlebrook 7H11 solid medium (Becton Dickinson, Sparks, MD, USA) supplemented with 10% OADC (Comercial Bellés, Tarragona, Spain), incubating the plate 21 days at 37ºC in 5% CO_2.

Antimicrobial agents

Amikacin, EMB, INH, LEV, LNZ and RIF were obtained from Sigma-Aldrich (St Louis, MO, USA). Stock solutions of AMK, EMB, and INH were prepared in sterile distilled water. Levofloxacin was dissolved in NaOH (0.1M) and LNZ and RIF in DMSO (final concentrations ranging from 0.00005% to 0.007% for LNZ and from 0.0002% to 0.003% for RIF), and then sterile distilled water was added. All the stock solutions were sterilized by filtration and stored at -20ºC.

Inoculum preparation

The isolates studied were grown in Lowenstein-Jensen medium slants (Becton Dickinson, Sparks, MD, USA). Colonies were suspended with sterile distilled water containing 0.05% Tween 80 (Merck, Darmstadt, Germany) and 5mm glass beads (Afora, Barcelona, Spain), and then vortexed during 45 seconds. The supernatant was harvested and adjusted to 0.5 McFarland with a nephelometer (Becton Dickinson, Sparks, MD, USA).

Determination of the MIC

The individual MICs for AMK, EMB, LEV and LNZ were determined for each isolate using the following ranges of drug concentrations: 0.125mg/L to 4mg/L for LEV and LNZ; 0.31mg/L to 7.5mg/L for AMK and EMB. Moreover, the individual MICs for INH, RIF were determined for drug-susceptible isolates using the following ranges of drug concentrations: 0.0125mg/L to 0.1mg/L for INH and 0.125mg/L to 1mg/L for RIF.

Replicates of the inoculum of each isolate diluted 1/1 and 1/100 were seeded on antibiotic-free Middlebrook 7H11 plates as a growth control. An isolate was considered resistant if greater than or equal to 1% of CFU were observed in the drug containing medium compared to drug free medium

Drug interaction analysis

The MICs in combination with three drugs was studied crossing three concentrations for each drug: the individual MIC found and two concentrations below the MIC. The Fractional Inhibitory Concentration Index (FICI) was calculated as follows: $FICI = FIC_A + FIC_B + FIC_C = A/MIC_A + B/MIC_B + C/MIC_C$ where A, B and C were the MICs of each antibiotic in combination and MIC_A, MIC_B and MIC_C were the MICs of each antibiotic alone. The results of the FICI were analyzed according to the checkerboard method [9] adapted to three drugs in combination. As described previously [5,7], a decrease of two dilutions under the individual MIC was interpreted as synergistic with a FICI <0.75; indifference was determined from 0.75 to 4 and a FICI>4 was considered as antagonistic activity.

RESULTS

The 3DC-MDR combinations showed indifference against drug-resistant isolates. Moreover the four combinations studied showed indifference against drug-susceptible isolates.

The MICs in combination of the INH, RIF and EMB combination against the 11 drug-susceptible isolates decreased the individual MIC from one dilution showing indifference with a FICI value of 1.5 (table 1).

The decreases observed in the MICs in combination in the LNZ, LEV and AMK combination (table 2) were up to one dilution, leading to indifference (FICI=1.5-3). The MICs in combination of the LNZ, LEV and EMB combination (table 2) decreased the individual MIC up to one dilution, being the FICI values from 1.5 to 3. The MICs in combination of most of the isolates studied (90%) of the LEV, EMB and AMK combination (table 2) did not differ from the individual MIC, leading to indifference (FICI=1.5-3).

No differences were observed in the MICs between MDR and drug-susceptible isolates with the use of any of the 3DC-MDR combinations. Likewise, no difference was observed between the INH, RIF and EMB combination and the other combinations studied against drug-susceptible isolates.

DISCUSSION

The most relevant result of this study is that it demonstrates that the drug combinations against MDR-TB studied (3DC-MDR) are equally effective against MDR. Furthermore, these 3DC-MDR combinations are also as effective as the broadly tested three-drug combination of INH, RIF and EMB against drug-susceptible clinical isolates.

Multi-drug resistance has become a problem in the management of TB, with an urgent need for research into new drugs as well as the development of efficacious combinations against MDR-TB. Due to cross resistance, ethionamide and rifabutin, are rarely useful against MDR isolates. On the other hand, levofloxacin, a new generation fluoroquinolone, is widely used to treat MDR-TB and other fluoroquinolones such as moxifloxacin have appeared as promising new drugs to shorten TB treatment [10]. Aminoglycosides such as capreomycin, KAN and AMK are also used to treat MDR-TB, with AMK frequently being the first choice due to the toxicity of the others and its more common use among clinicians. Around 30 promising compounds such as LNZ, PA-824 or TCM207 are currently in clinical development phases to treat TB [11]. As Mitchison described in the 1980s [12], different *M. tuberculosis* populations coexist in a tuberculous lesion, requiring multi-drug therapy aimed to avoid the appearance of resistant mutations, acting over different targets as well as achieving a synergistic effect. However,

although the different drugs used act in combination, drug-susceptibility testing in *M. tuberculosis* is done individually for each drug, therefore there is no *in vitro* marker of the combined effect. To date, most *in vitro* studies on drug combinations in the treatment of TB have involved two-drug combinations [6] since the number of concentrations to study rises exponentially. Thus, to our knowledge, this is the first manuscript studying combinations of three drugs including second line drugs such as LNZ and LEV.

In the present study, three-drug combinations against MDR TB were studied. All the combinations presented indifference against all the isolates, showing all these combinations to be equally effective.

Treatment of TB is based on a first phase of INH, RIF, EMB and PZA during two months and a second phase of INH and RIF during four months [1]. In the present study, the combination of INH, RIF and EMB was tested as a representative of a standard schedule in order to approximate the effect of the first line TB treatment and taking into account that its efficacy had already been demonstrated in the past prior to the inclusion of PZA in the standard schedule. PZA was not included due to its acidic pH activity [13] to which other drugs must be standardized, being logistically complicated.

In conclusion, this study demonstrates that the 3DC-MDR combinations studied are equally effective against MDR isolates. Furthermore, the 3DC-MDR are also as effective as the broadly tested three-drug combination of INH, RIF and EMB against drug-susceptible clinical isolates. The study of three-drug combinations against TB could be useful to predict the efficacy of drugs in clinical development phases as well as to test the regimens of antituberculous drugs currently available.

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TABLE 1. Effect of the isoniazid (INH), rifampicin (RIF) and ethambutol (EMB) combination against drug-susceptible *Mycobacterium tuberculosis* isolates.

Isolate	Susceptibility pattern	MIC alone (mg/L)			INH/RIF/EMB	
		INH	RIF	EMB	MIC (mg/L)	FICI
1S	*	0.1	1	2.5	0.05/0.5/1.25	1.5
2S	*	0.1	1	2.5	0.05/0.5/1.25	1.5
3S	*	0.1	1	2.5	0.05/0.5/1.25	1.5
4S	*	0.1	1	2.5	0.05/0.5/1.25	1.5
5S	*	0.1	1	2.5	0.05/0.5/1.25	1.5
6S	*	0.1	1	2.5	0.05/0.5/1.25	1.5
7S	*	0.1	1	2.5	0.05/0.5/1.25	1.5
8S	*	0.1	1	2.5	0.05/0.5/1.25	1.5
9S	*	0.1	1	2.5	0.05/0.5/1.25	1.5
10S	*	0.1	1	2.5	0.05/0.5/1.25	1.5
H37Rv	*	0.1	1	2.5	0.05/0.5/1.25	1.5

MIC: Minimal Inhibitory Concentration; FICI: Fractional Inhibitory Concentration Index; * Drug-susceptible isolates.

TABLE 2. Effect of three different combinations with levofloxacin (LEV), linezolid (LNZ), amikacin (AMK) and ethambutol (EMB) against drug-susceptible and multidrug-resistant (MDR) *Mycobacterium tuberculosis* isolates.

Isolate	Susceptibility pattern	MIC alone (mg/L)				LEV/LNZ/AMK		LEV/LNZ/EMB		LEV/AMK/EMB	
		LEV	LNZ	AMK	EMB	MIC (mg/L)	FICI	MIC (mg/L)	FICI	MIC (mg/L)	FICI
1R	MDR	0.5	0.5	2.5	2.5	0.25/0.25/1.25	1.5	0.25/0.25/1.25	1.5	0.5/2.5/2.5	3
2R	MDR	0.5	0.5	2.5	2.5	0.25/0.25/1.25	1.5	0.25/0.25/1.25	1.5	0.5/2.5/2.5	3
3R	MDR	0.5	0.5	2.5	2.5	0.25/0.25/1.25	1.5	0.25/0.25/1.25	1.5	0.5/2.5/2.5	3
4R	MDR	0.5	0.5	2.5	2.5	0.25/0.25/1.25	1.5	0.25/0.25/1.25	1.5	0.5/2.5/2.5	3
5R	MDR	0.5	0.5	2.5	2.5	0.25/0.25/1.25	1.5	0.25/0.25/1.25	1.5	0.25/1.25/1.25	1.5
6R	MDR	0.5	0.5	2.5	2.5	0.25/0.25/1.25	1.5	0.25/0.25/1.25	1.5	0.25/1.25/1.25	1.5
7R	MDR	0.5	0.5	2.5	2.5	0.25/0.25/1.25	1.5	0.25/0.25/1.25	1.5	0.5/2.5/2.5	3
8R	MDR	0.5	1	2.5	2.5	0.25/0.5/1.25	1.5	0.25/0.5/1.25	1.5	0.5/2.5/2.5	3
9R	MDR	0.5	1	2.5	2.5	0.25/0.5/1.25	1.5	0.25/0.5/1.25	1.5	0.5/2.5/2.5	3
1S	*	0.5	1	2.5	2.5	0.25/0.5/1.25	1.5	0.25/0.5/1.25	1.5	0.5/2.5/2.5	3
2S	*	0.5	0.5	2.5	2.5	0.25/0.25/1.25	1.5	0.25/0.25/1.25	1.5	0.5/2.5/2.5	3
3S	*	0.5	0.5	2.5	2.5	0.25/0.25/1.25	1.5	0.25/0.25/1.25	1.5	0.5/2.5/2.5	3
4S	*	0.5	0.5	2.5	2.5	0.25/0.25/1.25	1.5	0.5/0.5/2.5	3	0.5/2.5/2.5	3
5S	*	0.5	0.5	2.5	2.5	0.5/0.5/2.5	3	0.5/0.5/2.5	3	0.5/2.5/2.5	3
6S	*	0.5	0.5	2.5	2.5	0.25/0.25/1.25	1.5	0.25/0.25/1.25	1.5	0.5/2.5/2.5	3
7S	*	0.5	0.5	2.5	2.5	0.25/0.25/1.25	1.5	0.25/0.25/1.25	1.5	0.5/2.5/2.5	3
8S	*	0.5	0.5	2.5	2.5	0.5/0.5/2.5	3	0.5/0.5/2.5	3	0.5/2.5/2.5	3
9S	*	0.5	0.5	2.5	2.5	0.25/0.25/1.25	1.5	0.5/0.5/2.5	3	0.5/2.5/2.5	3
10S	*	0.5	0.25	2.5	2.5	0.5/0.25/2.5	3	0.25/0.25/1.25	2	0.5/2.5/2.5	3
11S	*	0.5	0.5	2.5	2.5	0.25/0.25/1.25	1.5	0.5/0.5/2.5	3	0.5/0.5/2.5	3
H37Rv	*	0.5	0.5	2.5	2.5	0.25/0.25/1.25	1.5	0.5/0.5/2.5	3	0.5/2.5/2.5	3

MIC: Minimal Inhibitory Concentration; FICI: Fractional Inhibitory Concentration Index; MDR: multidrug-resistant isolates * Drug-susceptible isolates.

Objective IV

Paper IV. Effect of three-drug combinations of antituberculous agents against multidrug-resistant *M. tuberculosis* isolates in a macrophage model.

Rey-Jurado, E., Tudó, G., Soy, D., González-Martín, J. Effect of three-drug combinations of antituberculous agents against multidrug-resistant *M. tuberculosis* isolates in a macrophage model. Submitted to J Antimicrob Chemother.

EFFECT OF THREE-DRUG COMBINATIONS OF ANTITUBERCULOUS AGENTS AGAINST MULTIDRUG-RESISTANT *M. tuberculosis* ISOLATES IN A MACROPHAGE MODEL.

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Running title: Activity of three-drug combinations against multidrug-resistant strains of *M. tuberculosis* in a macrophage model

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ABSTRACT

Background: Multidrug resistance has become a problem in the management of tuberculosis leading to an urgent need for research on new regimens including the drugs currently available. Macrophage models allow evaluation of the effect of drugs against intracellular bacilli.

Methods: The effect of the following different drug combinations against 6 multidrug-resistant and 6 drug-susceptible clinical isolates of *M. tuberculosis* multiplying inside the human macrophages THP-1 was studied: levofloxacin-linezolid-ethambutol; levofloxacin-amikacin-ethambutol; levofloxacin-linezolid-amikacin. The macrophages were lysed, seeded onto Middlebrook 7H11 plates and the CFU were counted after 21 days of incubation. The interaction of the drugs in combination was interpreted by the effect of the combination compared with the most active single drug alone. The antimicrobial activity of these drugs was evaluated comparing the $\log_{10}$CFU/well with the $\log_{10}$CFU/well of the isolate studied without drug. Drug concentrations within infected-macrophages and in extracellular medium were simultaneously determined by chromatography.

Results: The levofloxacin-linezolid-amikacin and levofloxacin-linezolid-ethambutol combinations showed antagonism against all the isolates after a four-day protocol whereas levofloxacin-amikacin-ethambutol displayed indifference. Levofloxacin alone and levofloxacin-amikacin-ethambutol combined were the most potent antimicrobials, presenting reductions up to 5.49$\log_{10}$ and 5.86$\log_{10}$, respectively. The drug penetration percentages ranged from 7.53% to 11.02%, being 5% for ethambutol. The intracellular concentrations found with the drug alone compared to those found in drug in combination were not significantly different.

Conclusions: All the combinations tested against *M. tuberculosis*-infected macrophages showed antimicrobial activity with combinations including linezolid and levofloxacin showing an antagonism effect which may be explained by efflux transporters or changes in macrophage environment.

INTRODUCTION

Tuberculosis (TB) remains a global threat worldwide with nearly nine million incident cases of TB being estimated in 2010 according to the WHO. Among the 12 million prevalent cases of TB reported worldwide in 2010, the WHO estimated 650,000 cases to be multidrug-resistant TB (MDR-TB).[1] The regimen currently used to treat drug-susceptible TB consists of 2 months of initial intensive phase of isoniazid, rifampicin, ethambutol (EMB) and pirazinamide followed by 4 months of isoniazid and rifampicin. MDR is defined as resistance to at least INH and RIF, with only a maximum of two drugs of the standard regimen remaining available. The inclusion of the following drugs is recommended for the treatment of MDR-TB: pirazinamide or EMB, one injectable agent (kanamycin, capreomycin, amikacin (AMK) or streptomycin), one fluoroquinolone (ofloxacin, levofloxacin (LEV), gatifloxacin or moxifloxacin) and cycloserine or p-aminosalicylic acid if cycloserine cannot be used.[2] Indeed, other drugs such as linezolid (LNZ), imipenem and clarithromycin have been proposed, depending on the drug resistance, to treat some cases of MDR and extremely drug-resistant TB.[3-5] Treatment of drug-resistant TB requires an individualized regimen depending on drug resistance, interactions and toxicity to the patient. Indeed, few drugs are available to treat drug-resistant TB, and especially MDR-TB. The length of the treatment together with drug toxicity makes patient compliance difficult in drug-resistant treatment. Despite drug susceptibility testing being done individually, the different drugs used in TB treatment act in combination. Few studies have been performed to study the efficacy and synergy of drug combinations against *M. tuberculosis*.[6-8]

After inhalation of infected aerosols into the lungs of the host, the first cells to respond to *M. tuberculosis* are the alveolar macrophages and tissue dendritic cells. In contrast to other pathogens, *M. tuberculosis* survives and replicates inside macrophages.[9] Thus, macrophage models have been previously used to test the efficacy of drugs against *M. tuberculosis* inside macrophages. Drug distribution to the tissues and cellular accumulation of the drugs has been shown to be important in the killing of intracellular bacteria, therefore also being important in the final outcome of treatment. Fluoroquinolones have shown good penetration to the tissues, whereas the low solubility of aminoglycosides makes tissue distribution poor.[10-12] Our model allows evaluation of the penetration of the drug inside macrophages and consequently the intracellular efficacy of drugs. Indeed, evaluation of drugs in combination within macrophages may provide useful information about drug penetration into macrophages in the presence of more drugs and on the effect of drug interactions on the intracellular activity against *M. tuberculosis*.

The objectives of the present study were: 1. to study the effect of the three-drug combinations against MDR in a human macrophage model: LEV-LNZ-EMB; LEV-AMK-EMB; and LEV-LNZ-AMK, and 2. to determine drug penetration alone and in combination inside *M. tuberculosis* infected-human macrophages.

MATERIAL AND METHODS

M. tuberculosis isolates

Twelve non-clustered *M .tuberculosis* clinical isolates (6 multidrug-resistant with *kat*G and *rpo*B mutations and 6 drug-susceptible isolates) from the Hospital Clinic of Barcelona were selected for the study.

Preparation of antimicrobial agents

Amikacin, EMB, LEV and LNZ were obtained from Sigma-Aldrich (St Louis, MO, USA). Stock solutions of AMK and EMB were prepared in sterile distilled water. Levofloxacin was dissolved in NaOH (0.1M) and LNZ in DMSO (final concentration ranging from 0.0002% to 0.01%), and sterile distilled water was then added. All the stocks solutions were sterilized by filtration and stored at -20ºC.

Determination of the MIC

The MICs for AMK, EMB, LEV and LNZ were determined for each isolate using the following ranges of drug concentrations: 0.125mg/L to 4mg/L for LEV and LNZ; 0.31mg/L to 7.5mg/L for AMK and EMB. All the experiments were performed in duplicate with the proportions method[13] in Middlebrook 7H11 solid medium supplemented with 10% OADC and incubated for 21 days at 37ºC in 5% CO_2.

Cell culture

The human acute monocytic leukaemia cell line THP-1 (ATCC-TIB-202) was grown in supplemented RPMI 1640 (with L-glutamine, 25nM Hepes buffer) containing 10% heat-inactivated FCS (Lonza, Switzerland) and a 100 U/ml penicillin streptomycin mixture (Sigma), at 37ºC in 5% CO_2. When exponential growth was achieved, a concentration of 200,000 cells/ml were seeded in 24-well plates and differentiated to macrophages by the addition of 100 nM of phorbol-12-myristate-13-acetate (PMA) for 10 hours at 37ºC in 5% CO_2. Thereafter, the supernatant was discarded and PMA was again added and the culture was incubated for 4 days to discard non-differentiated cells.

Experiment design of *M. tuberculosis* infected-macrophages

The experiment design for the isolates studied is explained in Figure 1. Each isolate with its respective control and all the drug conditions were tested in the same experiment to ensure accurate measurement of antimicrobial activity and drug interactions.

***M. tuberculosis* inoculum preparation**

The isolates were grown in 7H9 MGIT medium (Becton Dickinson, MD, USA), supplemented with 0.25% tween 80 (Merck, Germany) to avoid clump formation. When the MGIT was positive, the sample was centrifuged adding 5 mm glass beads and the tube was shaken for 45s and sonicated for 1 min. Lastly, clumps were disaggregated by 14 passages through a syringe (20G 1·0.9X25 mm) and 4 passages through an insulin needle (27G 12" mm). The disaggregation of clumps was checked by Ziehl-Neelsen staining before and after the process of disaggregation. The inoculum density was measured using a nephelometre and making dilutions up to 400,000 bacteria/ml. Fifty µL of this inoculum was plated onto Middlebrook 7H11 plates and incubated at 37ºC in 5% CO_2 for 21 days. Quantitative counts were made thereafter.

Infection protocol

After the four days of cells differentiation, changes in morphology were checked by microscopy. Afterwards, macrophages were washed once with supplemented RPMI 1640, and then 1ml of supplemented RPMI 1640 with 10% heat-inactivated FCS was added. After two hours of incubation, the number of cells was calculated by Trypan blue exclusion. The inoculum of *M. tuberculosis* was added on the macrophages for 3 hours at a multiplicity of infection (MOI) 1:1. The macrophages were washed three times with supplemented RPMI 1640 and then 1 ml supplemented RPMI 1640 with 10% heat-inactivated FCS was added. Afterwards, the macrophages were incubated for 72 hours in order to allow bacteria to grow inside the macrophages.

Drug activity against *M.tuberculosis*-macrophage culture

After the 72 hours of incubation, the wells corresponding to day 0 (Figure 1) were washed with supplemented RPMI 1640 and lysis was performed by adding 0.5% NP40 detergent (Roche, Switzerland). The remaining wells were washed with supplemented RPMI 1640 and thereafter adding 1ml of supplemented RPMI 1640 with 10% heat-inactivated FCS containing the following drug concentrations: 25mg/L for AMK, 5mg/L for EMB, 6mg/L for LEV and 13mg/L for LNZ. The drug concentrations studied were selected according to Cmax in serum in humans as

reported previously[14]. As a control, free-drug macrophages were included. This previous process was repeated daily for the following four days. The macrophages were lysed by adding 0.5% NP40 at day 2 and 4. After two washes with phosphate buffered saline, PBS (Lonza, Switzerland), the cell lysates were seeded onto Middlebrook 7H11 plates and incubated at 37ºC in 5% CO_2 for 21 days. Quantitative counts were made thereafter.

Macrophage viability

To test macrophage viability, the cells were raised with four PBS washes and counted by Trypan blue exclusion at days 2 and 4. The percentage of viability was calculated as number live of cells/ total number of cells * 100.

Determination of drug concentration

The lysates and supernatants from the experiments were filtered and thereafter stored at -80 ºC until use. Five isolates (three drug-susceptible and two MDR isolates) were chosen to determine the drug concentration within infected-macrophages and in extracellular medium of the macrophage culture.

Linezolid and Levofloxacin: The concentrations of LNZ and LEV were determined simultaneously using the ultra performance liquid chromatography (UPLC) Acquity System (Waters Cromatografía, S.A., Spain). The chromatographic separation was performed using an ultrabase 100 ODS2 analytical column (100 x 4.6nm, diameter of 3µm; Akady, Spain). The mobile phases were pump A with acetonitrile and pump B with water and triethylamine at 0.4% adjusting the pH to 4 with H_3PO_4 4M. The gradient of pump B was 0 min-80%, 6 min-80%, 7 min-70%, 13 min-70%, 14 min-20%, 17 min-20%, 19 min-80%, 25 min-80% at a flow rate of 0.4ml/min and injection volume of 15µl. The detection was performed with a diode array detector (210-400nm) with fluorescence detection at the wavelengths of 295nm for LEV and 254nm for LNZ.

Amikacin and Ethambutol: The concentrations of AMK and EMB were determined simultaneously using high performance liquid chromatography (HPLC) Agilent 1100 series (Agilent Technologies, Germany). Chromatographic separation was performed using a zic-hilic analytical column (150 x 2.1 mm, diameter of 3.5 µm). The mobile phases were pump C with ammonium acetate 10mM with formic acid at 0.2% and pump D with acetronitrile with formic acid at 0.2%. The gradient of pump C was 0 min-5%, 1 min-5%, 15 min-99%, 25 min-99%, 26 min-5%, 30 min-5%, at a flow rate of 600 µl/min and injection volume of 25µl. The detection was performed with the triple quadruple API3000 (ABSciex, USA) mass spectrometer (MS). The optimized fragmentation transitions for multiple reaction monitoring (MRM) for EMB were: Q1

m/z 205.20→ Q3 *m/z* 116.20 with a collision energy (CE) of 25 V, a declustering potential (DP) of 40V, a focusing potential (FP) of 200V and an entrance potential (EP) of 10V. The optimized fragmentation transitions for MRM for AMK were: Q1 *m/z* 586.30→ Q3 *m/z* 425.20 with a CE of 28 V, a DP of 35V, a FP of 190V and an EP of 9.5V.

Interpretation of the results

Interaction of the drugs in combination

The results was interpreted by the effect of the combination of drugs in comparison with the most active single drug alone.[15] The activity of the combinations was calculated as follows: $\log_{10}$ CFU/well A – $\log_{10}$ CFU/well B, where A is the combination and B the most active single drug alone of the combination studied. Synergism was defined as a 2- $\log_{10}$ increase in killing at the time point with the combination in comparison with the most active single drug alone. Antagonism was defined as a 2-$\log_{10}$ decrease in killing at the time point with the combination compared with the most active drug. Indifference was defined as less than 2-$\log_{10}$ increase or decrease in killing at the time point with the combination compared with the most active single drug.

Antimicrobial activities

To evaluate the antimicrobial activities of the drugs studied against *M. tuberculosis* isolates residing in macrophages, the $\log_{10}$ CFU/well were compared with the $\log_{10}$ CFU/well of the isolate studied without drug at the same time point, after four days of incubation.

Determination of the penetration of the drugs inside the macrophages

The concentration found in the lysates was considered to be inside the macrophages while the extracellular medium concentration was deemed to be outside. The penetration of the drugs studied at days two and four was calculated as the percentage of drug inside the macrophages compared with the extracellular concentration. The concentration inside the macrophages is represented as µg per 2 x 10^5 cells considering the number of cells per well.

Statistical analysis

Statistical analysis and graphs were performed using GraphPad Prism v5.

RESULTS

The growth curves of two drug-susceptible and two MDR representative isolates of *M. tuberculosis* residing in human macrophages during 4 days of drug incubation are shown in Figure 2. The activity of the drugs studied did not show significant differences between drug-susceptible and MDR isolates of *M. tuberculosis* residing in macrophages (Figure 3).

The activity of all the drugs and drug combinations tested against the 12 isolates of *M. tuberculosis* residing in macrophages at days two and four are represented in Figure 2. The different isolates studied showed a similar pattern in terms of controls and the antimicrobial activity of the drugs studied.

Macrophage viability

The macrophage viability was similar in all the drugs tested. At day 2, the mean viability in drug-free macrophages was 89.2%, being 84.1% in macrophages containing drug. At day 4, the mean viability was 91% and 87.5% in drug-free and drug-containing macrophages, respectively. The differences between macrophages with and without drug were not significant.

MICs of the isolates studied

All the isolates showed the same MIC value for AMK, EMB, LEV and LNZ, being 2.5mg/L, 2.5mg/L, 0.5mg/L and 0.5mg/L respectively.

Interactions of the drugs

The most active drug alone was LEV (Table 1). At day 2, 8 out of 12 isolates showed antagonism of the LEV-LNZ-AMK combination with an increase 2.06 $\log_{10}$ compared with LEV alone. Similarly, 7 out of 12 isolates showed antagonism of the LEV-LNZ-EMB combination with an increase 4.29 $\log_{10}$ compared with LEV alone. Nine out of 12 isolates (75%) in the LEV-AMK-EMB combination showed indifference and the remaining three displayed synergism with a decrease ranging from 1.16 to 2.12 $\log_{10}$ compared with LEV alone.

At day 4 (Table 2), all the isolates studied showed antagonism in the combinations of LEV-LNZ-AMK and LEV-LNZ-EMB with an increase from 2.25 to 4.84 $\log_{10}$ compared with LEV alone. All the isolates showed indifference with the LEV-AMK-EMB combination compared with LEV alone.

Antimicrobial activity

The decrease of $\log_{10}$ of *M. tuberculosis* residing in macrophages on the addition of the drug or drug combinations studied compared with drug-free macrophages in the 4-day protocol are shown in table 3. The activity of the drugs individually was as follows: LEV>EMB>LNZ>AMK. The combination of LEV, AMK and EMB was the most active of the combinations tested, decreasing a mean of 5.84 $\log_{10}$ with respect to drug-free macrophages.

Concentration inside macrophages

The concentrations of the drugs inside macrophages and the percentage of penetration of the drugs studied are represented in table 4. No significant difference was found between the penetration of the drug alone or in combination. No significant difference was observed between days 2 and 4.

DISCUSSION

The most important result of the present study is that all the combinations tested against *M. tuberculosis*-infected macrophages showed antimicrobial activity, with the combinations including LNZ and LEV displaying an antagonism effect.

Three-drug combinations including second-line drugs have been tested against six MDR and six drug-susceptible isolates of *M. tuberculosis* residing in the human macrophage cell line THP-1. To our knowledge, no previous study has evaluated the effect of different drug combinations against *M. tuberculosis* residing in macrophages. The present study mimics conditions *in vivo,* since in our model drugs acting on intracellular bacteria need to penetrate the macrophage membrane, the phagosome membrane and finally the bacteria, the same as in an *in vivo* setting. The differentiated THP-1 macrophages have previously been shown to be a good model in terms of receptor expression, bacterial uptake, survival and replication of *M. tuberculosis*.[16]

All the combinations tested showed antimicrobial activity, having a reduction of at least 1.4 $\log_{10}$ compared with their control at the same timepoint after a four-day protocol. Nevertheless, LEV alone and the LEV-AMK-EMB combination were found to be the most potent antimicrobial activity, presenting reductions of up to 5.49 $\log_{10}$ and 5.86 $\log_{10}$. Additionally, those combinations including LNZ and LEV showed much less activity than LEV alone, thereby demonstrating an antagonism effect. This antagonism effect may be explained

by different reasons. The effect may be caused by the bacteria itself. However, in a previous study by our group assessing the same combinations with the *in vitro* checkerboard assay, these drugs showed an indifferent effect.[17] Another explanation may be that these drugs have difficulties in penetrating macrophages due to active expulsion by efflux pumps. Nonetheless, the penetration of LEV, LNZ and AMK into macrophages is similar, with percentages ranging from 7.53% to 11.02%, being of around 5% for EMB. Moreover, the intracellular concentrations found with the drug alone with respect to those found in drugs in combination did not significantly differ thereby suggesting that these drugs do not interact with each other to efflux LEV outside the macrophages. On the other hand, subcellular localization of drugs and bacilli inside macrophages has previously been described. After phagocytosis, *M. tuberculosis* resides in phagosomes at a pH of around 6.2. Macrophage activation results in phagosome maturation and phagosome-lysosome fusion reaching a pH of 4.5-5.0.[9] This exposes *M. tuberculosis* to host-derived stress including protons from the vacuolar ATPasa, reactive nitrogen intermediates and reactive oxygen intermediates, free ubiquitin-derived peptides and lysosomal hydrolases. Nevertheless, evasion of phagosomal maturation allows *M. tuberculosis* to persist within the macrophages by increasing the pH, inhibiting antigen presentation, or activating apoptotic pathways, among other mechanisms.[18-20] Regarding the subcellular localisation of drugs inside macrophages, in the case of moxifloxacin, most of this drug is localised in the cytoplasm, with only around 6% thereby remaining within the lysosomes.[21,22] This situation may explain why some of the drugs studied in the present work show low antimicrobial activity since the drug is not in the same place as the bacilli. In this way, efflux transporters which are able to efflux drugs have been described in eukaryotic membranes.[23] These efflux transporters may be present in the phagolysosome membrane. Drugs such as the rifampicin and EMB have shown to be substrates for P-glycoprotein, being a well-known transporter protein.[24] In our study, the antagonism found may be explained by LNZ interfering with the penetration of the quinolone into the lysosome or by LNZ possibly acting as a substrate of efflux proteins located in the lysosome membrane. On the other hand, the adverse effect of lactic acidosis has been reported after administration of LNZ.[25,26] Linezolid inhibits bacterial protein synthesis by binding to the ribosome, which is similar to the eukaryote ribosome. In this way, an inhibitory action of LNZ on mitochondrial protein synthesis has been previously reported, detecting reductions in COX activity (a mitochondrial respiratory chain complex) and COX-II protein synthesis (one of the COX subunits translated by mitochondrial ribosomes) in peripheral blood mononuclear cells from patients treated with 600 mg/12h of LNZ.[27] In the present study, we hypothesize that LNZ may cause physicochemical alterations inside the macrophage, thereby reducing the activity of LEV.

In conclusion, all the combinations tested against *M. tuberculosis*-infected macrophages showed antimicrobial activity while the combinations including LNZ and LEV displayed an antagonism effect. This effect may be explained by an efflux transporter within macrophages which does not allow drug access inside lysosomes or by alterations within the macrophage environment prompted by LNZ, thereby leading to inactivation of LEV.

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Competing interests: None declared

Ethical approval: Not required.

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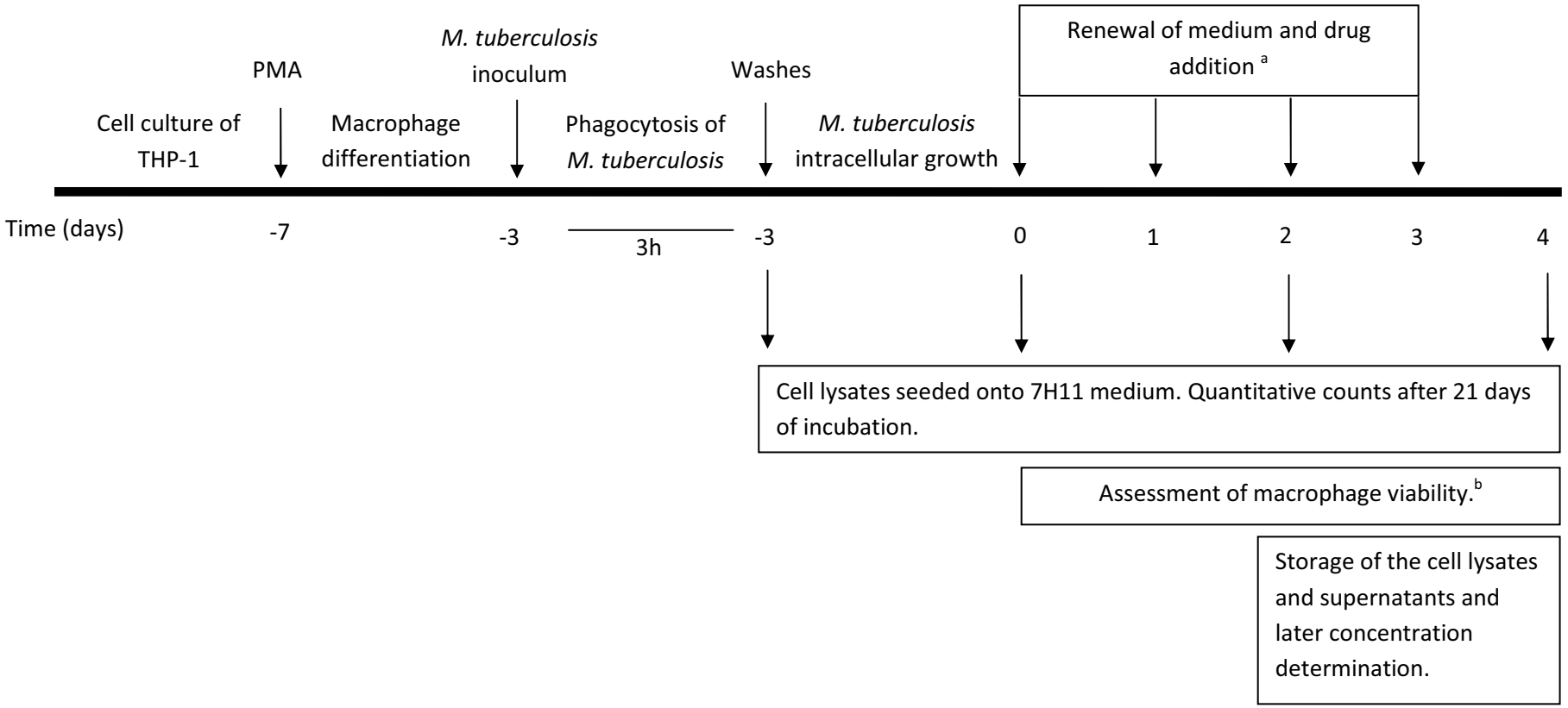
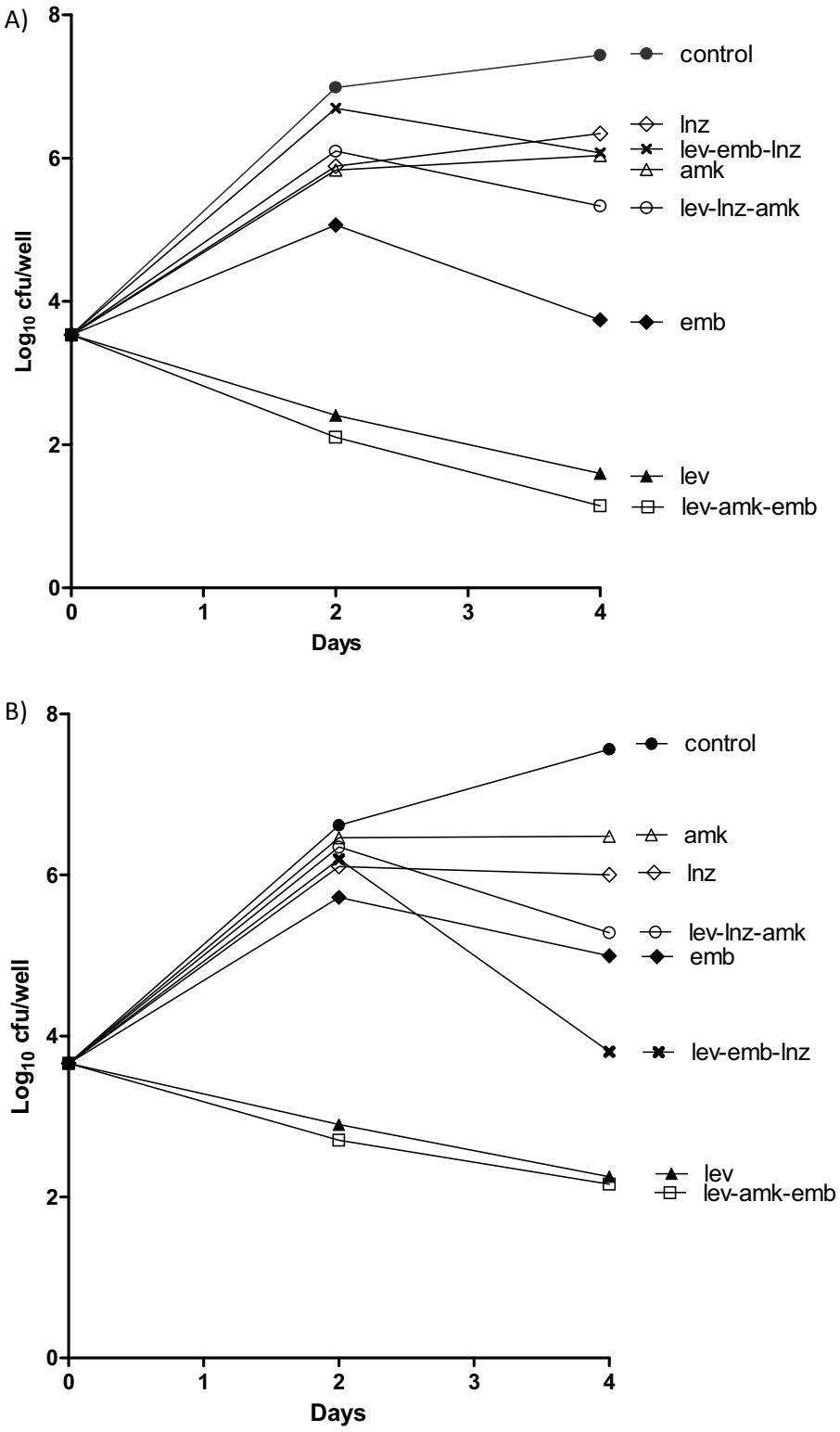
Figure 1. Experiment design of antimicrobial drug activity in a *M. tuberculosis*-infected macrophage model.



[a] Supplemented RPMI 1640 with 10% heat-inactivated FCS medium containing the following drug/s: AMK, EMB, LEV, LNZ, LEV-LNZ-AMK, LEV-LNZ-EMB or LEV-AMK-EMB. Each drug condition was tested in duplicate. Drug-free infected macrophages were added as a control of bacterial growth per triplicate for each timepoint.

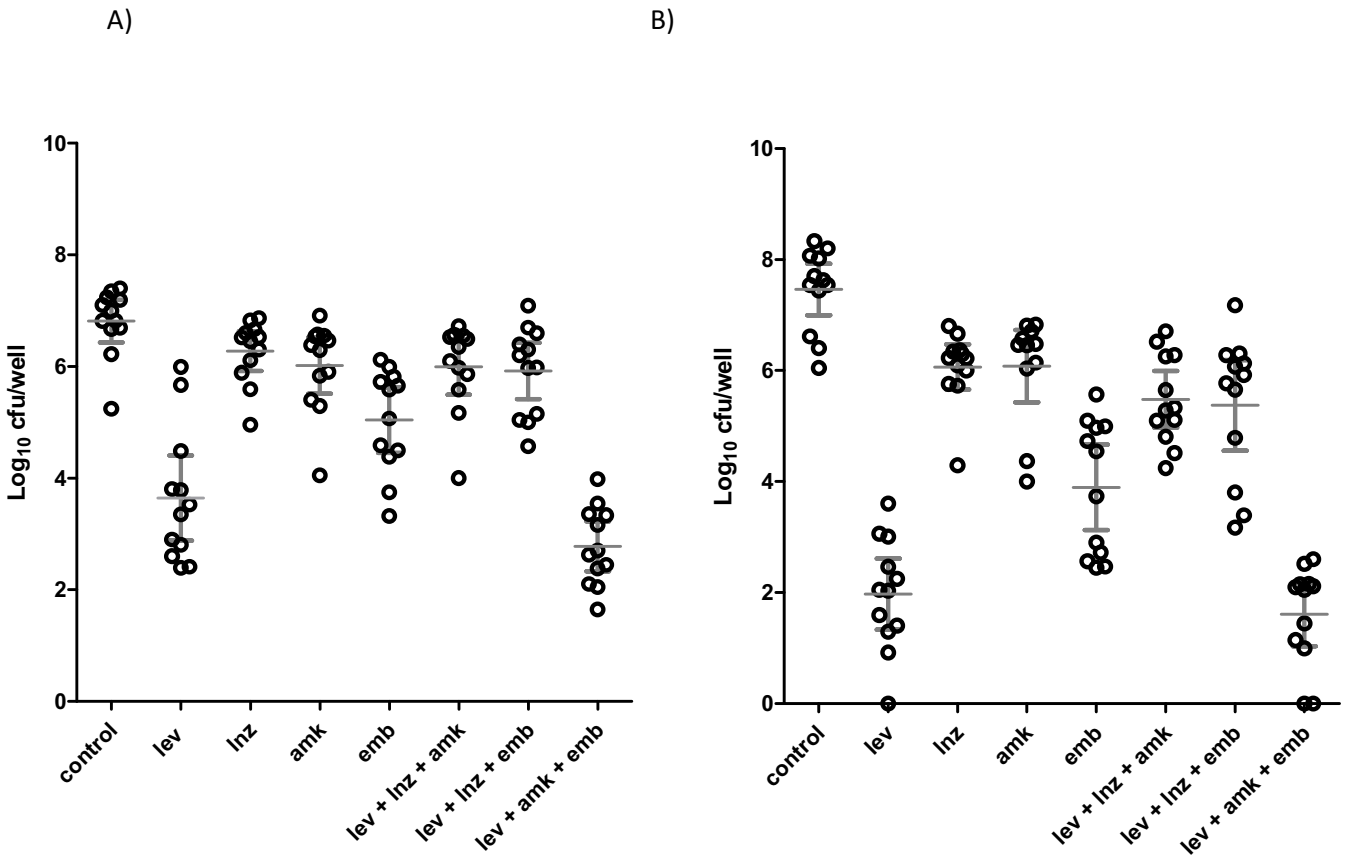
[b] Cellular viability was studied in the following conditions: bacteria-free macrophages under drug conditions, bacteria and drug-free macrophages and infected macrophages under drug conditions was assessed for each timepoint and condition.

Figure 2. Growth curves of *M. tuberculosis* isolates inside THP-1 macrophages during 4-day incubation with different drugs and drug combinations.



A) drug-susceptible isolates and B) MDR isolates. Control: without any drug; lnz, linezolid 13mg/L; lev, levofloxacin 6mg/L; amk, amikacin 25mg/L; emb, ethambutol 5mg/L; lev-lnz-amk, levofloxacin, linezolid and amikacin combination; lev-lnz-emb, levofloxacin, linezolid and ethambutol combination; lev-amk-emb, levofloxacin, amikacin and ethambutol combination. Each plot indicates the mean two experimental replicates.

Figure 3. Activity of drugs and drug combinations tested against the 12 drug-susceptible and MDR *M. tuberculosis* residing in macrophages.



Control: without any drug, lev: levofloxacin, lnz: linezolid, amk: amikacin, emb: ethambutol. Data are represented as the value of each isolate with the median values ±95% confidence interval of the different isolates of each group (n=12). A) Log_{10} of all the isolates studied inside macrophages after two days of drug exposure. B) Log_{10} of all the isolates studied inside macrophages after four days of drug exposure.

Table 1. Interactions of levofloxacin, linezolid, amikacin and ethambutol against drug-susceptible and MDR *M. tuberculosis* residing in macrophages at day two.

Isolate	LEV	LNZ	AMK	EMB	LEV+LNZ+AMK			LEV+LNZ+EMB			LEV+AMK+EMB		
	Log_{10} CFU/well	Log_{10} CFU/well	Log_{10} CFU/well	Log_{10} CFU/well	Log_{10} CFU/well	Δ Log_{10}*	Activity	Log_{10} CFU/well	Δ Log_{10}*	Activity	Log_{10} CFU/well	Δ Log_{10}*	Activity
1S	3.52	6.51	6.58	5.99	6.57	3.05	A	7.09	3.57	A	3.35	-0.17	I
2S	3.35	6.53	5.29	3.32	6.53	3.18	A	5.15	1.80	I	3.33	-0.02	I
3S	5.99	6.60	6.38	6.12	5.98	-0.01	I	6.30	0.31	I	3.16	-2.83	S
4S	5.67	6.87	6.55	5.65	6.49	0.82	I	6.59	0.93	I	3.54	-2.12	S
5S	3.80	6.30	6.54	5.82	6.72	2.92	A	6.40	2.60	A	2.63	-1.17	I
6S	2.41	5.89	5.83	5.06	6.10	3.69	A	6.70	4.29	A	2.10	-0.31	I
1R	3.78	5.59	5.40	4.50	5.16	1.38	I	4.57	0.79	I	3.98	0.20	I
2R	2.90	6.11	6.46	5.72	6.35	3.45	A	6.20	3.30	A	2.70	-0.19	I
3R	4.49	6.82	6.91	4.38	6.55	2.06	A	5.98	1.49	I	2.44	-2.05	S
4R	2.60	6.45	5.91	3.74	5.86	3.26	A	5.04	2.44	A	2.38	-0.22	I
5R	2.39	4.95	4.05	4.59	4.00	1.61	I	5.00	2.60	A	2.05	-0.34	I
6R	2.81	6.66	6.29	5.58	5.58	2.77	A	5.97	3.17	A	1.65	-1.16	I

Each number represents the mean of two experimental replicates. LEV: levofloxacin, LNZ: linezolid, AMK: amikacin, EMB: ethambutol. S: drug-susceptible isolate, R: MDR isolate. A: Antagonism, I: Indifference, S: Synergism. *Log_{10} CFU/well combination – Log_{10} CFU/well with the most active drug of the combination

Table 2. Interactions of levofloxacin, linezolid, amikacin and ethambutol against drug-susceptible and MDR *M. tuberculosis* residing in macrophages at day four.

	LEV	LNZ	AMK	EMB	LEV+LNZ+AMK			LEV+LNZ+EMB			LEV+AMK+EMB		
Isolate	Log_{10} CFU/well	Log_{10} CFU/well	Log_{10} CFU/well	Log_{10} CFU/well	Log_{10} CFU/well	Δ Log_{10}*	Activity	Log_{10} CFU/well	Δ Log_{10}*	Activity	Log_{10} CFU/well	Δ Log_{10}*	Activity
1S	3.01	5.76	6.58	4.73	6.28	3.27	A	5.92	2.92	A	2.52	-0.49	I
2S	2.05	5.73	ND	2.90	5.11	3.06	A	4.79	2.74	A	2.15	0.10	I
3S	3.06	6.80	6.83	5.57	6.52	3.46	A	6.31	3.25	A	2.60	-0.46	I
4S	3.61	6.22	6.71	5.10	6.25	2.65	A	6.28	2.67	A	2.12	-1.49	I
5S	2.04	6.37	6.14	4.97	5.10	3.06	A	5.66	3.62	A	2.05	0.02	I
6S	1.60	6.34	6.04	3.74	5.33	3.73	A	6.07	4.47	A	1.15	-0.45	I
1R	0.00	6.09	4.00	2.47	4.51	4.51	A	3.39	3.39	A	0	0	I
2R	2.25	6.00	6.48	5.00	5.28	3.03	A	3.81	1.55	A	2.16	-0.09	I
3R	2.47	6.30	6.81	2.72	6.71	4.24	A	7.18	4.71	A	2.10	-0.37	I
4R	1.40	6.23	6.46	2.57	5.65	4.24	A	5.77	4.37	A	1	-0.41	I
5R	0.92	4.29	4.37	2.45	4.81	3.89	A	3.17	2.25	A	1.45	0.52	I
6R	1.30	6.66	6.46	4.55	4.25	2.95	A	6.13	4.84	A	0	-1.30	I

Each number represents the mean of two experimental replicates. LEV: levofloxacin, LNZ: linezolid, AMK: amikacin, EMB: ethambutol. S: drug-susceptible isolate, R: MDR isolate. A: Antagonism, I: Indifference, S: Synergism *Log_{10} CFU/well combination – Log_{10} CFU/well with the most active drug of the combination

Table 3. Antimicrobial activity of the drugs studied against the 12 drug-susceptible and MDR *M. tuberculosis* residing in human macrophages at day four.

Drug or Combination	Reduction in growth*
LEV 6mg/L	5.49 ± 0.51
LNZ 13mg/L	1.39 ± 0.81
AMK 25mg/L	1.39 ± 0.63
EMB 5mg/L	3.57 ± 0.99
LEV 6mg/L + LNZ 13mg/L + AMK 25mg/L	1.98 ± 0.47
LEV 6mg/L + LNZ 13mg/L + EMB 5mg/L	2.09 ± 1.02
LEV 6mg/L + AMK 25mg/L + EMB 5mg/L	5.86 ± 0.47

LEV: levofloxacin, LNZ: linezolid, AMK: amikacin, EMB: ethambutol. *Mean ± SD of $\log_{10}$cfu decrease of the 12 isolates studied compared with control

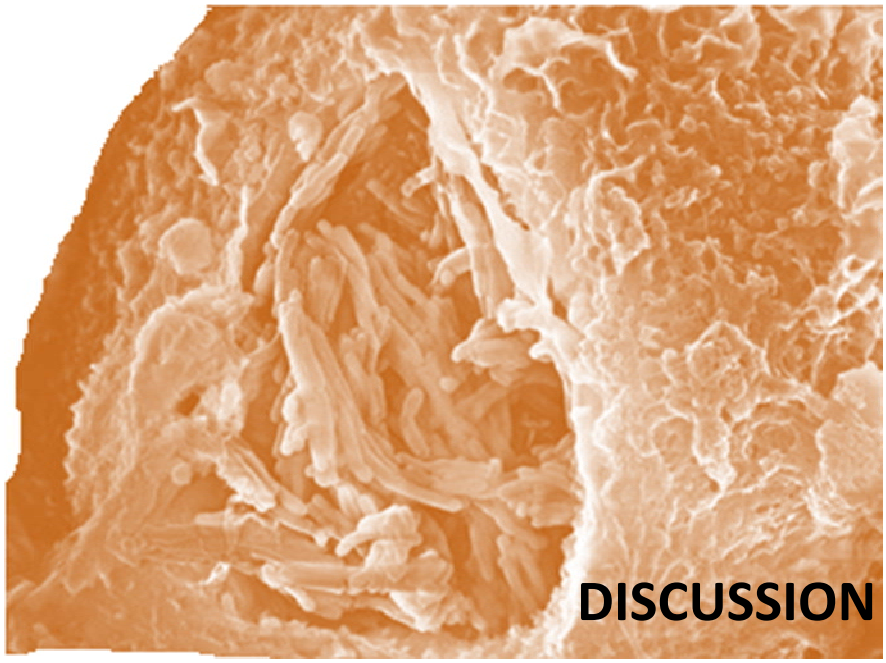
Table 4. Concentrations of the drugs determined inside macrophages.

Drug determined	Drug concentration (µg/200,000 cells)		Drug penetration (%)	
	Day 2	Day 4	Day 2	Day 4
LEV				
Alone	0.40 ± 0.15	0.19 ± 0.06	9.07 ± 1.26	11.02 ± 1.18
With LNZ and AMK	0.40 ± 0.28	0.27 ± 0.15	8.57 ± 1.22	9.39 ± 1.7
With LNZ and EMB	0.43 ± 0.24	0.29 ± 0.07	9.77 ± 1.86	10.22 ± 1.24
With EMB and AMK	0.44 ± 0.09	0.37 ± 0.09	9.42 ± 2.46	11.10 ± 2.16
LNZ				
Alone	0.39 ± 0.22	0.44 ± 0.21	6.93 ± 1.91	8.50 ± 1.65
With LEV and AMK	0.65 ± 0.41	0.41 ± 0.26	8.18 ± 2.06	9.16 ± 2.6
With LEV and EMB	0.87 ± 0.33	0.57 ± 0.27	10.63 ± 3.09	10.30 ± 2.4
AMK				
Alone	0.52 ± 0.34	0.53 ± 0.25	6.72 ± 0.37	7.28 ± 0.48
With LEV and EMB	0.64 ± 0.37	0.48 ± 0.32	6.29 ± 1.72	7.53 ± 1.62
With LEV and LNZ	0.74 ± 0.14	0.53 ± 0.07	7.34 ± 0.64	8.07 ± 1.20
EMB				
Alone	0.12 ± 0.08	0.05 ± 0.04	5.32 ± 4.7	5.13 ± 2.72
With LEV and LNZ	0.10 ± 0.01	0.08 ± 0.04	5.49 ± 3.8	4.22 ± 1.50
With LEV and AMK	0.10 ± 0.05	0.07 ± 0.03	5.13 ± 2.6	5.44 ± 4.03

Each number represents the mean ± SD of five experimental replicates. LEV: levofloxacin, LNZ: linezolid, AMK: amikacin, EMB: ethambutol

Science.... never solves a problem without creating ten more.

George Bernard Shaw



The following discussion tackles the general topics worked in the present thesis since the discussion of each work has been previously developed in each article.

1. MACROPHAGE MODELS

Macrophages are an important part of human defence against initial exposure of *M. tuberculosis* once it is inhaled in the alveoli of the lung. Additionally, the effector phase with macrophages and other immune cells of adaptive response also plays a significant role in the survival and transmission of the bacilli through the body of the host. Only a minority of people develop the disease in spite of being in contact with the bacteria, thereby demonstrating the tremendous efficacy of the human immune system in fighting TB. In the present work, murine J774.2 and human THP-1 macrophage cell lines were used to study the fitness of drug-resistant *M. tuberculosis* and the efficacy of drug-combinations against *M. tuberculosis* within macrophages, respectively. Although no comparative analysis of invasion and intracellular multiplication of *M. tuberculosis* has been undertaken in the two cell lines, the two independent studies in the present thesis provide information about the uses of both cell line macrophages.

Many models have been proposed to mimic their conditions of the above mentioned early stage of the infection. However, do we really know what these conditions are? For instance, the number of bacilli inside macrophages or the time of exposure of the mycobacteria before being phagocytised remains unclear. Macrophage models currently available are from murine to human cells, from cell lines to primary cultures and from monocytic to differentiated macrophages, being used for a range of objectives (See 1.5 section). Such models may therefore vary in the type of growth medium required and exposure time to Mycobacteria or macrophage differentiation. The multiplicity of infection, the MOI, used in the different types of cells range widely in the studies due to the need to adapt the ratio of bacteria/macrophage to the macrophage ability of being infected without undergoing death cell. Nevertheless, the number of bacteria exposed to macrophages may interfere with the number of bacteria capable of penetrating inside macrophages, and consequently the growth inside these

macrophages during the following days may vary. Therefore, comparisons between the different studies published should be made with caution.

J774.2 murine and THP-1 human macrophages have shown to be good models in terms of receptor expression, bacterial uptake, survival and replication (See 1.5 Section). As previously described (Mehta *et al.*, 1996), the concentration of FBS in the tissue culture medium is important in J774.2 cells since the concentration of 10% often used results in detachment and cell lysis. Due to the high the rate of multiplication of J774.2 macrophages the protocol should be of no longer than 6 days in order to maintain the monolayer of macrophages in the culture. To test antituberculous drugs against *M. tuberculosis* in a macrophage model, a large protocol was needed since multiplication inside macrophages, and then drug exposure during a determined time was required. Thus, THP-1 macrophages were chosen as the monolayer cells are maintained in the culture with a protocol of more than six days. Our results in a human macrophage model provide valuable information on the most realistic effects of drugs against the bacilli due to penetration of the drug into the macrophage and then act against *M. tuberculosis*. Therefore, the technique also allows knowledge of the extracellular and intracellular concentrations of the drugs studied, thereby facilitating accumulation or pharmacokinetic characteristics of the drugs analysed.

In conclusion, although macrophage models have limitations, they may be useful to study the survival of *M. tuberculosis* inside macrophages as well as to test the efficacy of any drug combination or new antituberculous drug against *M. tuberculosis in vitro.* These models also allow further studies in animal models or clinical trials.

2. FITNESS OF DRUG-RESISTANCE

Previous studies have found drug-resistance fitness cost, no cost or even enhanced fitness in several microorganisms. β-lactam resistance in enteric bacteria such as *Salmonella typhimurium* is frequently associated with mutations in the *amp*D gene. Indeed, the bacterial peptidoglican is the target of β-lactam drugs, which is not only important to the maintenance and growth of bacteria but also plays an important role in innate immune response. Similar to *M. tuberculosis*, *Salmonella typhimurium* is an intracellular pathogen. In this sense, Folkesson *et al*. (2005) showed that *amp*D mutants were less able to penetrate mouse macrophages than wild type strains and had fitness cost in mice. By contrast, *Neisseria gonorrhoeae* with mutations in the *gyr*A gene have displayed a variety of fitness cost depending on the point of the mutation (Kunz *et al.*, 2012). Quinolone resistance in *N. gonorrhoeae* arises from mutations in the *gyr*A or in the *gyr*A and *par*C genes. Kunz *et al*. (2012) showed a fitness

advantage of isolates with a mutation in the *gyr*A gene, but this advantage was reduced in isolates with a mutation also in the *par*C gene. Moreover, *N. gonorrhoeae* have a multidrug efflux pump which not only recognizes classical antibiotics but also compounds from the host defence such as antimicrobial peptides (Johnson *et al.*, 2011). Jense *et al*. (2003), showed that the lack of this efflux pump caused an inability to sustain vaginal infection in mice. In this sense, this efflux pump may be involved in bacterial resistance, basic metabolism, bacterial fitness and/or pathogenicity.

Prior studies on the virulence of drug-resistant *M. tuberculosis* isolates were carried out by Middlebrook and Cohn (Middlrebrook *et al.*, 1953) who reported less pathogenecity in guinea pigs with INH-resistance compared with drug-susceptible isolates. However, with the genome mapping of *M. tuberculosis* and the knowledge of the mutations conferring resistance to antituberculous drugs, further studies have shown that virulence or fitness may depend on the mutations associated with resistance. In the present thesis (Paper I), the INH-resistant and MDR isolates of *M. tuberculosis* with a mutation in the *kat*G gene had a significantly lower growth inside murine macrophages compared with drug-susceptible isolates. By contrast, the drug-susceptible and MDR isolates had similar growth in human macrophages (Paper IV). This result may be explained by the methodological differences between the two types of cells or by the strains studied. These differences and those found with epidemiological studies showing a fitness cost in some and no fitness cost in others (Burgos *et al.*, 2003; Gagneux *et al.*, 2006; van Doorn *et al.*, 2006) suggest that the impact of the resistance mutations on the *M. tuberculosis* fitness is slight and thus, may be misled by some study approaches. Murine or human macrophages may result in different interactions with the pathogen and variety of cytokine profile, thereby resulting in different strain growth. Indeed, in the last years certain strains has shown varied immune system response (Portevin *et al.*, 2011). On the other hand, eicosanoids have been identified as important host lipid mediators that regulate cell death in both human and murine macrophages (Behar *et al.*, 2011). Different regulation of such lipid mediators in human or mouse models may carry different outcomes of mycobacterial growth. Previous studies have found that *M. tuberculosis* exhibits different strain lineages associated with particular geographic regions and human populations (Gagneux *et al.*, 2007). Indeed, such strain lineages have been reported to be relevant to TB transmission and virulence. For instance, the Beijing genotype, often associated with MDR, has shown high virulence in terms of transmission or progression from latent to active TB. However, the mechanism of acquiring this advantage remains unclear. In the fitness paper (Paper I), clinical isolates displayed a wide range of genotypes without any association with a particular mutation (Paper I, data not

shown in the paper). Thus, although differences in growth inside macrophages may be influenced by the intrinsic characteristics of the strains, there is no evidence of an association with certain lineage or families in our study.

Although the aim of our fitness study was to study the early stage of infection, virulence of *M. tuberculosis* may also depend on the adaptive immune system which plays an important role in the survival of *M. tuberculosis*. The fitness cost found in our study may be present in the early stage of infection and may have the same growth ratio as drug-susceptible isolates afterwards with the appearance of adaptive response to drug-resistant *M. tuberculosis*.

The final outcome of the infection will depend on several factors. The situation in which the patient is infected is important, since factors such as overcrowding or inadequate TB control may favour the transmission of the disease. Thus, the initial transmission would be influenced by the conditions of the patient and the virulence of the strain. The pathogen virulence factors together with the host receptors and inflammatory signals play an important role in the first interactions between the host and pathogen (See 1.2 Section). The later adaptive response and the transmission of the bacilli through the body are also significant factors of the survival of mycobacteria. At this point, the response of the immune system of the host and the ability of the bacilli to evade and cause pathogenecity will also tip the balance in favour of bacilli or the host. Such complexity has led to new approaches of research, such as the systems biology framework which combines mathematical modelling and complements traditional empirical and experimental approaches to biomedical research (Young *et al.*, 2008). These models may be very useful to explore the behaviour of different strains in different settings, being a useful tool of control or transmission TB intervention.

In conclusion, similar to some studies, the present work suggests that a mutation in the *kat*G gene may affect the fitness of resistant isolates and the accumulative mutations in the MDR isolates not shown in the isolates resistant only to RIF may increase the impaired fitness of the isolates. Epidemiological studies have shown two sides, one in which *kat*G315 may have impaired fitness (Burgos *et al.*, 2003) and another in which these isolates are as transmittable as susceptible isolates (Gagneux *et al.*, 2006; van Doorn *et al.*, 2006). Together these studies suggest that the loss of fitness may be slight and the detection of this loss or gain may vary according to the tool used. Nevertheless, these slight differences in fitness may be important in LTBI as well as in its progression to active TB.

3. APPROACHES TO STUDY ANTIMICROBIAL COMBINATIONS

Both checkerboard and time-killing assays have been widely used to study antimicrobial combinations against *Staphylococcus aureus* MRSA, *Candida albicans* and *Acinetobacter baumanii*, among other microorganisms. Similar to *M. tuberculosis*, treatment against these microorganisms requires multiple therapy consisting of drugs with different targets as well as avoiding the appearance of resistance to drugs. The three-dimensional checkerboard assay has been shown to be a reliable method to study three-drug combinations in the two papers presented in this thesis (Paper II and III). Studies of the effect of three-drug combinations are a more realistic approach since the treatment of TB consists of the use of at least three antituberculous drugs. The inclusion of Pirazinamide could be a more appropriate approach, although there are technical difficulties related to environmental pH that make this approach extraordinarily cumbersome. Nonetheless, the literature on these approaches is limited due to the high number of crossed combinations to test in three-drug combinations.

Checkerboard assays are often used to study drug interactions since several concentrations can be studied simultaneously, especially when the microdilution method is chosen. However, in comparison to the macrodilution methods microdilution requires perfect homogenization of innoculum, which is difficult to achieve working with *M. tuberculosis*. In the present work, different combinations were tested using a three-dimensional checkerboard assay with macrodilution method based on agar Middlebrook 7H11, showing synergism or indifference.

In the THP-1 macrophage model for studying drug combinations, we employed the time-killing assay interpretation since this evaluates the effect of drugs by follow-up of *M. tuberculosis*-infected macrophages over a period of time. The antagonism found with time-killing in macrophages is noteworthy (Paper IV) whereas the same drug-combinations and strains resulted in indifference with the checkerboard assay (Paper III). Unlike the checkerboard assay, the dynamic method time-killing curves can evaluate the efficacy of drugs during a period of time. Both methodologies provide valuable information as an approach for designing *in vitro* and *ex vivo* models to further study the efficacy of treatment schedules for TB.

4. EFFECT OF DRUG COMBINATIONS AGAINST *M. tuberculosis*

The drug-resistance of *M. tuberculosis* together with non-adherence to treatment due to its length makes the need for studies on new schedules of treatment as well as new antituberculous drugs urgent.

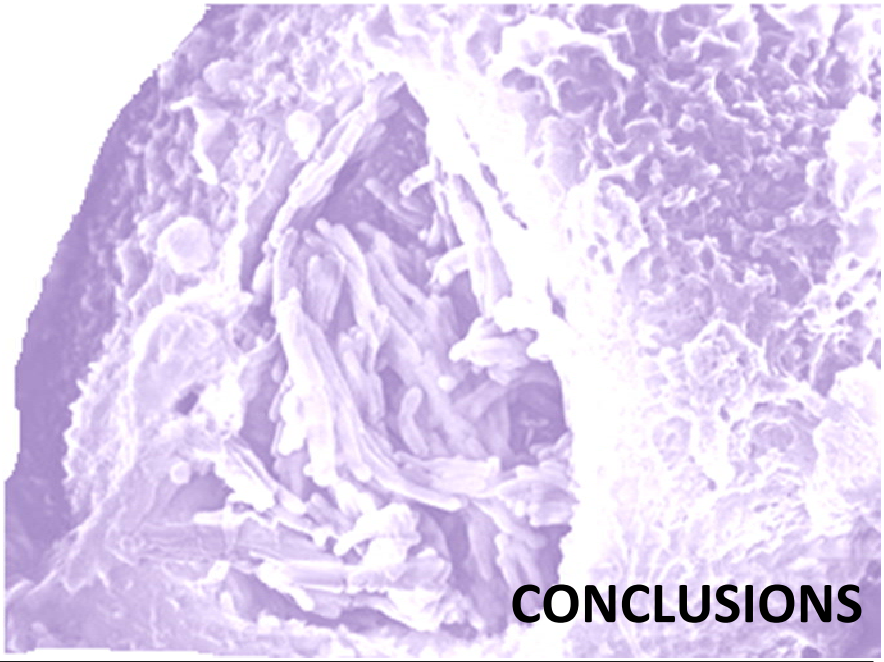
Currently, the standard recommended treatment for drug-susceptible isolates is 6-months of INH, RIF, EMB and PZA. These four drugs have been used for the last 50 years. However, new shorter regimens with new drugs to treat drug-susceptible isolates have recently been proposed. In the present thesis, the OFL, RIF and EMB combination showed significantly more synergism than that of the INH, RIF and EMB combination. Such superiority of fluoroquinolones has been previously shown, supporting the changing one of the drugs of the standard treatment to including one fluoroquinolone. Nevertheless, it is important to know that few new drugs are currently available in case of resistance, overall in low income countries. Thus, the appearance of resistance to fluoroquinolones would further reduce the drugs available. The treatment of drug-resistance requires individualized treatment depending on drug resistance, toxicity to the patient, and drugs available (See figure 15). In the present work, the OFL-RIF-EMB and INH-RIF-EMB combinations showed synergism against INH-resistant isolates. Most of the MIC in combination in the INH-resistant isolates decreased to concentrations approaching drug susceptibility when the INH-RIF-EMB combination was tested. Additional evidence comes from TB cases with a low level of INH-resistance which is not always detected (Tudó *et al.*, 2004), thus indicating the cure of patients with the standard treatment consisting of INH, RIF, EMB and PZA. Nevertheless, these results should be interpreted with caution since, although the synergism against INH-resistant was shown, the bacteria may increase its level of resistance over time, thereby prompting failure and lengthening the need for treatment.

The treatment of MDR-TB further complicates the cure of patients since INH and RIF, considered as potent bactericidal drugs, are withdrawn from the treatment. Second-line drugs to treat the MDR-TB patients are more toxic, expensive and require a longer treatment period than drug-susceptible treatment, which contributes to poor outcomes. Thus, it is important to accurately design efficient treatments to avoid failure/relapse outcomes, transmission of MDR-TB or emergence of resistance. It is important to mention that the availability and price of the drugs depend on the setting, and this should be taken into account when the schedule treatment is designed. Aminoglycosides such as AMK or KAN, fluoroquinolones and other drugs such as LNZ or CLZ have been described as useful for the treatment of MDR-TB. The key is to know if all the possible combinations of these drugs have the same efficacy or if there are some interactions among them which may improve or deteriorate the final outcome. In the present thesis, three-drug combinations including LEV adding LNZ, AMK or EMB were tested against drug-susceptible and MDR *M. tuberculosis* using an *in vitro* checkerboard assay and *ex vivo* macrophage model. Interestingly, the combinations studied showed indifference with the checkerboard assay whereas some combinations displayed antagonism with the macrophage

model. This difference suggests that there is not a clear drug-drug interaction. Drugs have to penetrate into macrophages and then be active to act against the bacteria in the macrophage model. Antagonism was shown in combinations which included LNZ and LEV, decreasing the activity by more than two logarithms compared with LEV alone in all the isolates studied. Since the drugs studied were found to penetrate inside macrophages without differences in intracellular concentration, the following causes could explain the antagonism observed. First, the physiochemical conditions inside macrophages such as pH may alter the activity of some drug combinations. It is well known that a secondary effect of LNZ is metabolic acidosis (Garrabou *et al.*, 2007). An acidic effect inside the cell altering the activity of LEV can not be ruled out. A second explanation could be the efflux transport or a blocking effect within macrophages which avoids drug access inside lysosomes (Hall *et al.*, 2003; Hartkoorn *et al.*, 2007; Vallet *et al.*, 2011). In this sense, no current data are available on the antagonism mentioned between LNZ and LEV. However, it may be underestimated when several antituberculous drugs are evaluated in MDR and XDR-TB treatment, making it difficult to differ possible interactions between drugs. Indeed, few studies have evaluated the efficacy of LNZ since it is relatively new in the treatment of TB. Further studies are required to clarify the mechanism of this antagonism and to determine if it is also present in animal models.

Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world. Science is the highest personification of the nation because that nation will remain the first which carries the furthest the works of thought and intelligence

Louis Pasteur



CONCLUSIONS

1. The two macrophage models designed, J774.2 and THP-1, are good models to study the fitness of *M. tuberculosis* as well the drug activity against intracellular bacilli.

2. The MDR and INH-resistant *M. tuberculosis* isolates with a mutation in the *kat*G gene have a lower growth ratio within murine macrophages compared to drug-susceptible isolates suggesting a lower *fitness* of these isolates.

3. The MDR and INH-resistant *M. tuberculosis* isolates with a mutation in genes other than the *kat*G gene (*inh*A, *rpo*B and/or unknown mutations) did not have significant differences compared with drug-susceptible isolates.

4. A three-dimensional adaptation of the checkerboard assay is feasible for the study of the synergism of three-drug combinations against *M. tuberculosis* isolates.

5. The combination including INH, RIF and EMB could be efficient to treat TB cases with low level INH resistance (MICs ≤ 0.8μg/ml) due to the synergistic effect of the combination.

6. The combination including OFL, RIF and EMB shows better efficacy than that of INH, RIF and EMB, being of potential use in drug-susceptible and in INH-resistant isolates.

7. The combinations including second-choice drugs (LEV-AMK-EMB; LEV-AMK-LNZ; LEV-EMB-LNZ) are equally effective to the combination of INH, RIF and EMB with the checkerboard assay.

8. LEV, AMK, EMB and LNZ penetrate similarly inside human THP-1 macrophages but LEV is the most active drug against *M. tuberculosis* intracellular.

9. The three-drug combinations tested against *M. tuberculosis*-infected macrophages (LEV-AMK-EMB; LEV-AMK-LNZ; LEV-EMB-LNZ) show antimicrobial activity, with the combinations including LNZ and LEV displaying an antagonistic effect.

RESUME IN CATALAN

ABREVIATURES

AMK: Amikacina

BCG: *Bacille Calmette-Guérin*

CIF: Concentració Inhibitòria Fraccionada

CIP: Ciprofloxacina

CMI: Concentració Mínima Inhibitòria

EMB: Etambutol

GAT: Gatifloxacina

IFN-γ: *Interferon- γ*

IGRAs: *Interferon-Gamma Release Assays*

IL: Interleuquina

INH: Isoniazida

LEV: Levofloxacina

Man-LAM: *Mannose-capped Lipoarabinomannan*

MOX: Moxifloxacina

OFL: Ofloxacina

OMS: Organització Mundial de la Salut

PAS: Àcid p-aminosalicílic

PMA: Phorbol Myristate Acetate

PZA: Pyrazinamida

RIF: Rifampicina

STR: Estreptomicina

TB: Tuberculosi

TNF-α: *Tumour Necrosis Factor-α*

VIH: Virus de la Immunodeficiència adquirida

MDR: Multiresistent

XDR: Extremadament resistent

INTRODUCCIÓ

1. Característiques generals de la Tuberculosi

Mycobacterium tuberculosis, l'agent causal de la TB, pertany a la familia *Mycobacteriaceae* i és un dels membres del *M. tuberculosis complex* que té més importància clínica. El gènere *Mycobacterium* presenta un tret que el diferencia de la resta de bacteris, la paret cel·lular amb un alt contingut de lípids, més concretament d'àcids micòlics. El fet de no tenir una membrana externa fa que sigui classificat com a grampositiu ja que la tinció de cristall violeta quedarà retinguda, i a més tindrà la propietat d'àcid-alcohol resistència. *M. tuberculosis* és un bacil de creixement lent i amb una mida de 0,2 -0,4µm d'ample i 1- 4µm de llargada (Murray *et al.*, 2007).

La TB continua sent una de les malalties més importants en salut pública arreu del món. Al voltant de nou milions de casos incidents de TB van ser estimats al 2010. La distribució de la malaltia no és uniforme, el 80% dels casos els trobem en 22 països, entre ells destaquen l'Índia i la Xina amb una alta incidència (WHO, 2011). La OMS ha definit dues categories segons l'historial clínic del tractament del pacient: casos nous, aquells enregistrats com a nou episodi de TB i que no ha rebut tractament previ, i casos tractats com un episodi actual de TB que té una història prèvia de tractament de TB. La TB MDR es defineix com una TB resistent a INH i RIF, i la extremadament resistent, com una TB MDR que a més té resistència a una fluoroquinolona i a un dels injectables de segona línia. L'emergència de la TB MDR, la XDR i la pandèmia de VIH representen obstacles addicionals pel control de la malaltia. Els països d'Europa de l'Est són els que han declarat més casos de TB MDR.

2. Patogènia de la Tuberculosi

La transmissió de la TB es produeix via aerosols i tot i que la forma més freqüent és la TB pulmonar també pot afectar a altres òrgans com la pleura, nòduls limfàtics, tracte genitourinari, la pell, les meninges, les articulacions o els ossos. Una vegada els bacteris arriben a l'alvèol del pulmó, són fagocitats pels macròfags alveolars, on *M. tuberculosis* es multiplica. A continuació, es produeix un flux de limfòcits, cèl·lules dendrítiques i macròfags

activats cap al lloc de la lesió formant la estructura del granuloma. Els bacteris poden esdevenir latents dins d'aquesta estructura durant un llarg període de temps, donant l'anomenada TB Latent, o d'un altre banda la estructura es pot desfer degut a factors immunes de l'host provocant d'aquesta manera la malaltia (Kumar *et al.*, 2011).

El reconeixement de *M. tuberculosis* per part del sistema immune es dona, com amb altres bacteris, per la detecció de patrons d'estructures de microorganismes. En el cas de *M. tuberculosis* aquests patrons els trobem a la paret cel·lular com per exemple el glicolípid Man-LAM. Les cèl·lules del sistema immune tenen uns receptors per tal de reconèixer aquests patrons, els *Toll-like receptors*, on s'han descrit el TLR2, el TLR4 i el TLR9 com importants en el reconeixement de *M. tuberculosis* (Abel *et al.*, 2002; Bafica *et al.*, 2005). Aquest reconeixement juntament amb interaccions amb adaptadors i quinasses acaben donant una cascada de senyals que controlen la regulació de citoquines, quimioquines i altres molècules essencials per la resposta immune adaptativa on s'activen els limfòcits T. La producció de citoquines com la IL-12 i la IL-18 provoquen una resposta Th1 que es caracteritza per la producció de IFN-γ que a la seva vegada activa els macròfags donant la producció de la IL-6 i el TNF-α (Korbel *et al.*, 2008). S'ha descrit la importància del TNF-α per la formació de la estructura del granuloma, una estructura composta per una agregat de cèl·lules del sistema immune: cèl·lules gegants, cèl·lules escumoses, macròfags, cèl·lules dendrítiques, neutròfils, NK i cèl·lules infectades amb *M. tuberculosis* que esdevenen cèl·lules apoptòtiques o necròtiques (Ramakrishnan, 2012).

Després de ser fagocitat, *M. tuberculosis* resideix als fagosomes a un pH al voltant de 6,2. L'activació del macròfag per part del IFN-γ resulta en una maduració del fagosoma i posterior fusió amb el lisosoma arribant a un pH de 4,5-5 (Vandal *et al.*, 2009). Aquesta baixada de pH provoca una alliberació de protons, de reactius intermediaris de nitrogen i reactius intermediaris d'oxigen entre d'altres. Les accions d'aquests reactius com l'òxid nítric alliberats per macròfags i cèl·lules dendrítiques són considerades un potent mecanisme de defensa de l'hoste. La implicació de l'òxid nítric ha estat ben definida en models murins, en canvi, hi ha certa controvèrsia de la seva implicació en humans. S'ha postulat que en humans no s'ha detectat l'òxid nítric degut a la manca de cofactors necessaris per la seva producció en els cultius *in vitro* utilitzats (Bertholet *et al.*, 1999). La supervivència de *M. tuberculosis* dintre dels macròfags depèn de la seva inferència en els processos cel·lulars de l'hoste com són evitar la maduració del fagosoma, inhibir la presentació de l'antigen, activar vies d'apoptosis o generar citoquines pro inflamatòries (Cardona *et al.*, 2011; Lee *et al.*, 2011; Toossi *et al.*, 2012).

3. Diagnòstic de la Tuberculosi

Els símptomes de la TB pulmonar són la febre, tos perllongada, dificultat al respirar, pèrdua de pes, pèrdua de gana i tendència a cansar-se fàcilment. Els infiltrats i/o cavitacions observats en una radiografia de tòrax són signes de TB pulmonar.

3.1. Immunodiagnòstic de la Infecció

La detecció de la infecció de TB es duu a terme a través de marcadors de la resposta immune. Actualment s'utilitzen dos tipus de mètodes, la prova de la tuberculina i aquells basats en la resposta amb IFN-γ. En la prova de la tuberculina s'injecta la proteïna purificada (PPD) en l'avantbraç del pacient, i si hi ha una induració de 6 a 10 mm de diàmetre es considera resultat positiu i per tant, és interpretat com que la persona està infectada amb TB i que té risc de progressar a malaltia (CDC, 2003). Aquesta tècnica pot donar falsos positius en vacunats amb la vacuna BCG, en infectats per altres micobacteris, així com tenir una baixa sensibilitat en pacients immunosuprimits donant falsos negatius.

Els nous mètodes basats en la resposta del IFN-γ han sorgit com alternatives a les limitacions de la prova de la tuberculina (Santín-Cerezalesa *et al.*, 2011). Aquests mètodes es basen en la sensibilització amb cèl·lules T estimulades amb antígens específics de *M. tuberculosis*, no comuns amb els altres micobacteris del complex *M. tuberculosis*. Actualment hi ha dues tècniques comercials, Quantiferon-TB *Gold assays* i el TB-SPOT *test*. El preu més car i la necessitat d'una mostra de sang són desavantatges d'aquests mètodes comparats amb la tradicional prova de la tuberculina.

3.2. Detecció fenotípica de *M. tuberculosis*

Els mètodes més usats per la detecció de la propietat d'àcid-alcohol resistent de *M. tuberculosis* són els basats en la tinció primària amb fucsina, tinció de *Ziehl-Neelsen*, i aquells tenyits amb el fluorocrom Auramina-Rodamina. La tinció és fa directament de la mostra del pacient, essent la més freqüent l'esput tot i que també pot ser pus, líquid cerebroespinal o teixit provinent de biòpsia o qualsevol altra mostra. El diagnòstic definitiu de TB es du a terme amb el creixement en cultiu de *M. tuberculosis* a partir de la mostra del pacient. Aquest continua sent el mètode de referència degut a la seva alta sensibilitat i a que es requereix per fer estudis posteriors com sensibilitat a fàrmacs o tipatge molecular. Hi ha diferents medis de cultius disponibles pel creixement de *M. tuberculosis*, com el medi sòlid *Lowenstein-Jensen* o el líquid 7H9. A més, s'han desenvolupat diferents sistemes comercials tant automàtics com

semiautomàtics per augmentar la qualitat i eficiència del mètode de cultiu com són el BACTEC™ MGIT 960, el Bactec 460TB i el VersaTREK (Alcaide *et al.*, 2011; Parsons *et al.*, 2011).

3.3. Detecció genètica de *M. tuberculosis*

En els darrers anys s'han desenvolupat tot una sèrie de mètodes moleculars per tal d'evitar el retard diagnòstic de la malaltia. Aquets mètodes es basen en l'amplificació de seqüències específiques d'ADN i la observació posterior del fragment amplificat, per hibridació o per seqüenciació. Aquests mètodes inclouen l'*Amplicor M. tuberculosis test*, *Amplified M. tuberculosis direct test*, Genotype *Mycobacterium direct assay* i INNO-LiPA Rif TB kit (Palomino, 2009; Alcaide *et al.*, 2011).

3.4. Diagnòstic de la Tuberculosi resistent a fàrmacs

3.4.1. Mètodes fenotípics

Els mètodes fenotípics per la detecció de resistències de *M. tuberculosis* es basa en l'efecte que fan els fàrmacs davant la multiplicació o metabolisme del bacteri, comparat amb aquells que no han estat exposats als fàrmacs. Els mètodes convencionals per detectar la sensibilitat o resistència als fàrmacs inclouen el mètode de les concentracions absolutes, el mètode de la *ratio* de la resistència i el mètode de les proporcions crítiques. La nova generació de detecció de resistències deriven del mètode de les proporcions crítiques. Els medis utilitzats són en líquid, el 7H9, i en sòlid, el 7H11 i el 7H10 que són derivats del medi anterior. El medi líquid és el més utilitzat degut a que el bacteri creix més ràpid que en cultius sòlids. La soca a estudiar és considerada resistent si més d'un 1% de la població de bacteris és capaç de créixer en presència del fàrmac. El mètode automatitzat reconegut per la OMS és el BACTEC™ MGIT960 que ha aconseguit una alta sensibilitat. En el cas de detectar la resistència a PZA, s'ha adaptat el medi a un pH de 6 per tal d'aconseguir que el metabòlit àcid pirazinoic sigui actiu (Lorian, 2005). S'han descrit altres mètodes, menys utilitzats, per detectar la resistència a fàrmacs com són l'anàlisi de les activitats metabòliques presents en soques resistents o la utilització de fags (Van Deun *et al.*, 2010).

3.4.2. Mètodes genètics

Els mètodes moleculars o genètics per la detecció de resistències es basan en la identificació de les mutacions del genoma bacterià que donen lloc a les resistències. En totes aquestes tècniques es requereix una extracció i una amplificació del gen diana de la mostra. Els mètodes moleculars permeten escurçar de manera molt significativa el temps en tenir el

resultat. La seqüenciació del DNA bacterià és una de les metodologies moleculars més acurades ja que es visualitza la seqüència completa de nucleòtids del fragment amplificat. En els darrers anys s'han desenvolupat diversos *kits* comercials de detecció molecular (Taula 8).

Taula 8. Característiques del principals mètodes genotípics per detectar resistències.

Mètode	**Amplificació**	**Detecció**	**Mutacions detectades**	**Resistències a fàrmacs detectades**
Seqüenciació DNA	PCR	Fluorimètric	*	*
INNO-LiPA Rif-TB	Nested-PCR	Colorimètric	*rpo*B	RIF
Genotype MTBDR plus kit	Amplificació basada en seqüència dels àcids nuclèics	Colorimètric	*Kat*G, *inh*A and *rpo*B	INH, RIF
Genotype MTBRsl	Amplificació basada en seqüència dels àcids nuclèics	Colorimètric	*emb*B, *rrs*, *gyr*A	EMB, aminoglicòsids, fluroquinolones
DNA *microarray*	PCR	Fluorimètric	*	*
GeneXpert MTB/Rif	PCR en temps real	Fluorimètric	*rpo*B	RIF

INH:isoniazida; RIF: rifampicina; EMB: etambutol; *gens escollits en el disseny de la tècnica.

4. Tractament de la Tuberculosi

4.1.Història

La STR va ser el primer fàrmac destinat al tractament de la TB el 1946. Abans d'aquest descobriment, la base del tractament era l'aïllament del pacient en sanatoris i operacions de la zona afectada del pulmó. Al voltant dels anys 50 i 60 nous fàrmacs com el PAS, la INH, la PZA i la RIF van sorgir com efectius i van ser plantejats com una teràpia múltiple per combatre les primeres soques resistents a la STR. El primer tractament proposat va ser de 12 mesos amb la combinació de INH, PAS i STR (Mitchison *et al.*, 2012). Als anys vuitanta diferents tractaments van coexistir, un de 9 mesos que consistia en una primera fase de dos mesos d'INH, RIF i PZA i una segona d'INH i RIF de 7 mesos; un de 8 mesos amb 2 mesos de INH, RIF, PZA i EMB i 2 mesos d'INH i EMB; i un de 6 mesos composat per 2 mesos de INH, RIF, PZA i EMB i 4 mesos d'INH i RIF. Aquest últim va esdevenir el més efectiu i és l'emprat actualment (Jindani *et al.*, 2004).

4.2.Tractament de la Tuberculosi latent

El tractament de la TB latent és clau per evitar la progressió a una TB activa i per tant, una possible transmissió de la malaltia. Aquest tractament preventiu consta de 9 o 6 mesos d'INH que han mostrat eficàcies del 90-93% i el 69%, respectivament (Comstock, 1999). És un tractament recomanat per aquells diagnosticats en TB Latent i que tinguin factors de risc com són infecció per VIH, rebre un teràpia neutralitzant amb TNF-α o edats infantils (Salgado *et al.*, 2011; Kall *et al.*, 2012) o ser contactes molt propers de pacients diagnosticats.

4.3.Tractament de la Tuberculosi activa

La pauta utilitzada actualment per tractar casos de TB sensible a fàrmacs està constituïda d'una fase intensiva inicial de dos mesos amb INH, RIF, EMB i PZA i una de continuació de 4 mesos amb INH i RIF. L'èxit de la teràpia combinada de la TB es basa en els següents aspectes: evitar l'aparició de resistències ja que disminueix la probabilitat de mutacions espontànies dels quatre fàrmacs, actuar simultàniament amb diferents dianes del bacteri i atacar sobre totes les poblacions bacterianes que es troben en diferents estats metabòlics (Mitchison, 2000).

4.4.Tractament de la Tuberculosi resistent a fàrmacs

La resistència a fàrmacs de *M. tuberculosis* és deguda a mutacions espontànies en els cromosomes. La resistència en casos prèviament tractats es defineix per la freqüència de soques aïllades en malalts que prèviament han rebut com a mínim un mes de tractament antituberculós. El tractament recomanat per l'OMS en casos de TB MDR inclou els següents fàrmacs: PZA o EMB, una fluoroquinolona (preferible, si és d'última generació), un dels fàrmacs injectables (kanamicina o AMK, capreomicina) i un o més dels fàrmacs orals de segona línia (cicloserina, o PAS si la cicloserina no es pot usar, etionamida o protionamida). Altres fàrmacs com el LNZ, imipenem i claritromicina s'han proposat darrerament per tractar casos de TB MDR i XDR. La major toxicitat i cost d'aquests fàrmacs de segona línia, així com els llargs tractaments que poden durar fins 20 mesos són obstacles pel compliment del tractament per part del pacient. Per tant, l'optimització de les pautes de tractament en casos de TB resistents a fàrmacs és de vital importància pel control de la malaltia.

4.5.Fàrmacs antituberculosos

4.5.1. Isoniazida

La INH, específica per *M. tuberculosis*, té unes CIMs per soques sensibles que varien entre 0,05 i 2μg/ml. A més, té una bona biodisponibilitat i una unió baixa a proteïna (Global Alliance for TB drug Development., 2008). La INH és un pro-fàrmac que requereix ser activat per l'enzim catalasa-peroxidasa, codificat pel gen *kat*G. A continuació, el seu compost actiu, l'àcid nicotínic, inhibeix la proteïna *enoyl-ACP reductase*, codificada pel gen *inh*A. Aquest procés resulta en una inhibició de la síntesi dels àcids micòlics.

S'han descrit diferents gens implicats en la resistència a INH (Taula 10). Els dos mecanismes més freqüents són les mutacions en el gen *kat*G i en la regió promotora del gen *inh*A, relacionats amb un alt (≥1μg/ml) i un baix nivell de resistència (<1μg/ml) respectivament (Dantes *et al.*, 2012). Una soca es considera resistent si creix a partir de 0,1μg/ml d'INH. La mutació en el codó 315 del gen *kat*G és la més freqüent entre els casos resistents a INH, on descriuen des de el 41,6% en Siria (Madania *et al.*, 2012)al 98,4% en Kazakhstan (Kozhamkulov *et al.*, 2011). L'altre mutació més freqüent és en la regió promotora del gen *inh*A, que s'ha descrit des del 16,9% en Bangladesh (Rahim *et al.*, 2012) al 32% en Siria (Madania *et al.*, 2012). Altres mecanismes implicats en la resistència, tot i que menys freqüents, són la sobreproducció de *inh*A i les mutacions en els gens *kas*A, *ndh, ahpC* i *glf* així com bombes d'expulsió (Lee *et al*., 1999; Cardoso *et al*., 2004).

Taula 10. Mecanismes de resistència de la isoniazida.

Mecanisme genètic	**Diana cel·lular/Efecte**
Mutacions en el *kat*G: D63E, H108Q, T262R, T275P, S315T/R, A350S, G629S	Pèrdua de l'activitat catalasa-peroxidasa Inhibició dels àcids micòlics.
Mutacions en el *inh*A: S94A, I16T, I21V, I47T, I95P Mutacions en la region promotora: C(-15)T Sobreexpressió de *inh*A	Reducció de l'afinitat pel seu cofactor, el NADH, i, finalment, una inhibició dels àcids micòlics.
Mutació *upstream* the *aph*C: C(-81)T, G(-74)A, C(-54)T, G(-51)T, G(-48)T	Compensació de la pèrdua de catalasa-peroxidasa
Mutació en el *ndh*: R268H	Acumulació de NADH, que actua com un competidor per unir-se a *inh*A

4.5.2. Rifampicina

La RIF, de la mateixa manera que la resta de rifamicines, inhibeix les ARN polimerases de les cèl·lules procariòtiques, codificades pel gen *rpo*B (Campbell *et al.*, 2001). Apart de *M. tuberculosis*, la RIF és activa contra altres bacteris Gram-negatius i Gram-positius. La CMI de la RIF per soques sensibles de *M. tuberculosis* és de 0,5μg/ml. Degut a la seva baixa solubilitat, la RIF té una biodisponibilitat variable (Jayaram *et al.*, 2003; Bhise *et al.*, 2010). Més del 95% de les soques resistents a RIF estan associades a mutacions en un *cluster* de 81 parells de bases en el gen *rpo*B. Les mutacions més freqüents es troben als codons 531 i el 516, on s'han descrit el 55-73% i el 13-23% dels casos resistents a RIF, respectivament (Clemente *et al.*, 2008; Bolotin *et al.*, 2009; Wang *et al.*, 2009; Minh *et al.*, 2012). Recentment, s'han descrit mutacions compensatòries en els gens *rpo*A i *rpo*C (Comas *et al.*, 2011; Brandis *et al.*, 2012). La concentració crítica per determinar resistència a la RIF és de 1μg/ml mentre que les CMIs poden variar de 1 a 256μg/ml (Hwang *et al.*, 2003). La majoria de soques resistents a RIF també ho són a la INH, tot i que s'han descrits variacions geogràfiques (Kurbatova *et al.*, 2012; Smith *et al.*, 2012).

4.5.3. Etambutol

L'EMB inhibeix els enzims *arabinosyl transferase* que són necessaris per la síntesi de l'*arabinan*, un polisacàrid essencial per la paret cel·lular del micobacteri (Alderwick *et al.*, 2011). Aquest fàrmac és d'ús específic del gènere *Mycobacterium*. La concentració crítica establerta per determinar resistència a EMB és de 5μg/ml mentre que la CMI per l'EMB de soques sensibles de *M. tuberculosis* pot variar entre 1 i 5μg/ml (Scho *et al.*, 2009). S'ha descrit una absorció pobre i variable de l'EMB (Tappero *et al.*, 2005; Jönsson *et al.*, 2011), tot i que s'ha observat que aquest fàrmac evita l'aparició de resistències a INH i RIF. La mutació en el codó 306 del gen *Emb*B és el mecanisme més freqüent que dóna lloc a resistència a l'EMB i està associada amb alts nivells de resistència (Alcaide *et al.*, 1997; Plinke *et al.*, 2011) mentre la mutació en el codó 406 s'ha vist que predomina a Catalunya (Moure *et al.*, 2008). Tot i així, al voltant del 75% de les soques amb una resistència fenotípica a l'EMB no presenten cap mutació coneguda (Tessema *et al.*, 2012).

4.5.4. Pirazinamida

La PZA és un pro-fàrmac que requereix la amidasa de *M. tuberculosis* així com un pH àcid per convertir-se en la seva forma activa, l'àcid pirazinoic. Degut al requeriment d'un medi àcid es creu que pot ser un fàrmac actiu en les lesions caseoses i en el fagolisosoma (Zhang *et al.*,

2003). La soca de *M. tuberculosis* es considera resistent a PZA quan la CIM és igual o superior a 20µg/ml. La resistència a PZA s'associa amb les següents mutacions en el gen *pnc*A o en la seva regió promotora sense que n'hi hagin unes que predominin sobre les altres: Thr47Ala, Ala146Glu, Gly162Asp, Leu172Pro, Asp8Ala, Phe13Leu, Tyr64Ser, Glu107stop, Ala143Pro and Leu172Arg (Feuerriegel *et al.*, 2012; Stoffels *et al.*, 2012). L'expulsió de l'àcid pirazinoic per part del bacil també ha estat descrit com a mecanisme de resistència a la PZA (Zimic *et al.*, 2012).

4.5.5. Fluoroquinolones

Les fluoroquinolones inhibeixen els enzims topoisomerasa II (ADN girasa) i topoisomerasa IV, responsables de relaxar i enrotllar el ADN durant la seva transcripció, replicació i recombinació. Per tant, són fàrmacs actius contra Gram-positius, Gram-negatius i micobacteris. Les fluoroquinolones descrites com bactericides contra *M. tuberculosis* són la CIP, l'OFL, la LEV, la MOX i la GAT. Les fluoroquinolones de nova generació, la LEV, la MOX i la GAT tenen una CMI de 0,25-1µg/ml mentre que la CIP i la OFL estan entre 0,5 i 2,5µg/ml en soques sensibles. Aquests fàrmacs tenen propietats farmacocinètiques que faciliten el seu transport a teixits (Thwaites *et al.*, 2011) i s'ha vist que tindrien la capacitat de modular la immunitat humoral i cel·lular (Dalhoff, 2005; Shalit *et al.*, 2006). La mutació més freqüent associada a resistència en les fluoroquinolones és a la regió *QRDR* del gen *gyr*A, que codifica per la ADN girasa (Long *et al.*, 2012; Suzuki *et al.*, 2012), i les posicions que predominen són la 89 (Asp89Asn), 90 (Ala90Val) i 94 (Asp94Tyr, Asp94Ala, Asp94Asn, Asp94His) (von Groll *et al.*, 2009; Long *et al.*, 2012). Les mutacions en l'altre gen que codifica per la ADN girasa, el *gyr*B (Pantel *et al.*, 2011), així com bombes d'expulsió també han estat descrits com a mecanismes associats a resistència en les fluoroquinolones (Escribano *et al.*, 2007).

4.5.6. Aminoglicòsids

El mecanisme d'acció dels aminoglicòsids és la inhibició de la síntesi proteica, tenint activitat contra alguns Gram-positius, Gram-negatius i micobacteris. Els aminoglicòsids són fàrmacs amb una baixa solubilitat, per tant tenen dificultats per penetrar en teixits. L'STR, l' AMK, la kanamicina i la capreomicina són els usats pel tractament de la TB resistent i tenen CMIs que varien de 0,25 a 4µg/ml. El 37-85% de les soques resistents a STR tenen mutacions en els gens *rpsl* i/o *rrs*, sent les mutacions en la posició 43 i 88 del gen *rpsl* les més freqüents i associades amb un alt nivell de resistència (Tudó *et al.*, 2010). S'ha descrit la resistència creuada entre els diferents aminoglicòsids (Via *et al.*, 2010). Les mutacions associades a amikacina, kanamicina i capreomicina són encara desconegudes (Georghiou *et al.*, 2012).

4.5.7. Altres fàrmacs de segona línia

La cicloserina i la etionamida, que inhibeixen la paret cel·lular del micobacteri i el PAS la síntesi d'àcid fòlic i el metabolisme del àcid salicílic, són fàrmacs que tot i que són actius contra *M. tuberculosis*, tenen efectes secundaris freqüents i/o severs. A més, hi ha tot una sèrie de fàrmacs destinats a altres bacteris que podrien ser eficaços per tractar casos de TB MDR i XDR com són el LNZ, la clofazimina, l'amoxicilina-clavulànic, la claritromicina o l'imipenem (Chambers *et al.*, 1998; Chambers *et al.*, 2005).

El LNZ té com a diana el ribosoma bacterià, causant una inhibició de la síntesi proteica i té una CMI per *M. tuberculosis* de 0,5µg/ml amb una concentració crítica que determina resistència a partir de 1µg/ml. S'ha descrit una bona biodisponibilitat, absorció i penetració als teixits tot i que també s'han trobat efectes secundaris greus (Dryden, 2011; Wiskirchen *et al.*, 2011). Recentment s'ha avaluat en dos estudis el tractament de TB MDR i XDR incloent el LNZ. En aquests estudis, l'esput es tornava negatiu en al voltant del 70% dels casos (Schecter *et al.*, 2010; Lorenzo *et al.*, 2012). La mutació en la posició 460 del gen *rpl*C s'ha associat amb resistència amb LNZ (Beckert *et al.*, 2012).

4.5.8. Nous fàrmacs

Actualment, al voltant de 30 fàrmacs estan en fase de desenvolupament clínic per tractar la TB. Els derivats del LNZ, el PNU 100480 i AZD 5847, han millorat l'efecte antibacterià (Wallis *et al.*, 2010). El PA-824 que tracta de simular les estratègies del sistema immune provocant la producció d'òxid nítric i reactius intermediaris de nitrogen, ha mostrat efectes primerencs bactericides similars a la pauta estàndard (Diacon *et al.*, 2012). Un altre fàrmac en estudi, el TCM207, que inhibeix la *ATP synthase,* s'ha vist com prometedor per tractar la TB latent (Andries *et al.*, 2005; Dhillon *et al.*, 2010).

4.5.9. Noves pautes de tractament

El tractament actual de la TB és llarg i costós donant lloc a un risc d'incompliment i per tant, a un fracàs terapèutic. Per tal de millorar l'eficàcia del tractament de la TB s'estan avaluant diferents pautes que inclouen fàrmacs com la rifapentina, la clofazimina o les fluoroquinolones. Tot i que la rifapentina té una vida mitja superior a la RIF, alguns estudis han mostrat un potència superior mentre que d'altres no han observat aquesta superioritat (Dorman *et al.*, 2011; Rosenthal *et al.*, 2012). La incorporació en el tractament de GAT, MOX i/o clofazimina així com alguns dels nous fàrmacs comentats anteriorment, han mostrat

propietats esterilitzants i bons percentatges d'esputs que han esdevingut negatius (Van Deun *et al.*, 2010; Williams *et al.*, 2012).

5. Avaluació de combinacions antimicrobianes

La combinació de diferents fàrmacs no és només útil en el tractament de la TB sinó que també juga un paper important en infeccions per *Acinetobacter baumanii*, per *Staphilococcus aureus* en biofilms o per *Candida albicans*. La determinació de sensibilitat als fàrmacs es fa de manera individual per cada fàrmac, sense tenir en compte que en aquests casos els fàrmacs estan actuant en combinació i que per tant, l'èxit del tractament dependrà en part de la interacció entre els diferents fàrmacs.

L'activitat resultant de les interaccions entre els fàrmacs pot ser definida com sinergisme, on la combinació és significativament més activa que cada fàrmac individualment; antagonisme, on els fàrmacs per separat són més efectius que la combinació i indiferència que no hi ha cap mena d'interacció entre els fàrmacs sent la combinació igual d'efectiva que els fàrmacs individuals. El mètode del tauler d'escacs així com les corbes de letalitat són metodologies emprades per estudiar les combinacions de fàrmacs.

5.1.Tècniques *in vitro*

5.1.1. Mètode del tauler d'escacs

El nom de la tècnica ve donat per la forma d'un tauler que donen els tubs o pouets amb múltiples dilucions dels fàrmacs en concentracions superiors i inferiors a la CMI de cadascun dels fàrmacs a estudiar. Aquest mètode permet determinar un gran nombre de concentracions simultàniament. Els resultats són interpretats segons l'isobolograma que resulta de representar el índex de la Concentració Inhibitòria Fraccionada (CIF) en escala aritmètica (Cuenca-Estrella et al., 2004). L'índex de la CIF es calcula amb la següent fórmula: $A/CMI_A + B/CMI_B = CIF_A + CIF_B$, on, A i B són les CMIs en combinació per a cada fàrmac, i CMI_A i CMI_B les CMIs individuals. Segons els resultats obtinguts, la interpretació és la següent: sinergisme si la CFI és menor o igual a 0,5; antagonisme si és més de 4; i indiferència si el valor es troba entre 0,5 i 4. La interpretació de sinergisme es basa en una disminució de la CMI de dos dilucions del fàrmac en combinació respecte la CMI individual (Lorian, 2005). En els estudis de *M. tuberculosis* i de fongs, s'ha avaluat l'activitat de més de dos fàrmacs simultàniament. Per tal d'adaptar la metodologia del tauler d'escacs, s'han proposat variacions en el valor de CIF a

partir del qual s'interpreta com a sinergisme així com gràfics en tres dimensions per avaluar les interaccions de combinacions de tres fàrmacs (Bhusal *et al*., 2005; Dai *et al*., 2010). D'aquesta manera, Bhusal *et al*. (2005) va proposar el valor de 0,75 de la CIF a partir del qual era considerat sinergisme ja que si cada fàrmac en combinació disminueix dos dilucions respecte la seva CMI, el càlcul de la CIF dona el valor de 0,75.

5.1.2. Corbes de letalitat

La determinació de les corbes de letalitat, a diferència de la metodologia del tauler d'escacs, és un tècnica dinàmica que permet detectar l'activitat bactericida enlloc de només la inhibitòria (Mueller *et al*., 2004). Les concentracions que es poden testar en un mateix experiment, en canvi, són limitades. La tècnica es basa en el recompte de bacteris viables durant un temps determinat amb diferents combinacions i concentracions dels fàrmacs a estudiar amb un inòcul establert prèviament (Lorian, 2005). Els resultats s'interpreten com sinergisme si la combinació és 100 vegades més activa i antagonisme si la combinació és 100 vegades menys activa que el fàrmac més actiu individualment. En estudis de *M. tuberculosis*, aquest inòcul així com el temps d'incubació s'han d'adaptar a les seves propietats metabòliques i al seu ritme de creixement.

5.2. Efecte de combinacions de fàrmacs contra bacteris

L'activitat sinèrgica s'ha observat en gran quantitat d'estudis *in vitro*. El sinergisme pot ser degut a una inhibició seqüencial d'una via metabòlica com és el cas de les sulfamides i el trimetoprim (Lorian, 2005). Les bombes d'expulsió presents tant als bacteris com a les cèl·lules eucariotes, poden tenir un rol en la resistència dels fàrmacs per part del bacteri així com afavorir o bloquejar el pas dels altres fàrmacs de la combinació donant d'aquesta manera activitats sinèrgiques o antagòniques (Flores *et al*., 2005; Nguyen *et al*., 2006; Balganesh *et al*., 2012; Adams *et al*., 2011). També s'ha vist com les fluoroquinolones com el GAT i el LEV potencien l'efecte dels fàrmacs de primera línia davant de *M. tuberculosis* (Bhusal *et al*., 2005; Rastogui *et al*., 1996). Mentre que el LNZ combinat amb altres fàrmacs antituberculosos ha donat indiferència davant *M. tuberculosis*, s'ha descrit antagonisme o una tendència a antagonisme quan es combinat amb vancomicina o CIP davant *S. aureus* (Grohs *et al*., 2003; Rodriguez-Diaz *et al*., 2003).

6. *Fitness* de *M. tuberculosis* resistent a fàrmacs

El concepte de *fitness* en un patogen es defineix com la seva habilitat per sobreviure, reproduir-se i ser transmès (Cohen et al., 2003). D'aquesta manera, la *ratio* de multiplicació del bacteri en cultius cel·lulars, la capacitat de disseminar a través dels diferents teixits, l'habilitat per respondre a condicions d'estrès i els casos secundaris en estudis epidemiològics són alguns dels paràmetres que poden ser usats per estudiar la *fitness* de soques resistents de *M. tuberculosis*.

6.1.Metodologies per l'estudi de la *fitness*

6.1.1. Cultius cel·lulars

Els macròfags són àmpliament emprats en l'estudi de la TB degut a que *M. tuberculosis* té la capacitat de multiplicar-se dins d'aquestes cèl·lules i perquè els macròfags alveolars formen part de la primera resposta davant de la infecció. A més, s'ha trobat una associació entre el creixement intracel·lular del bacteri dintre dels macròfags i la seva dinàmica de transmissió entre humans (Zhang *et al*., 1999; Theus *et al*., 2005; Theus *et al*., 2006). Els macròfags utilitzats poden ser cultius primaris que podrien ser més representatius de la situació *in vivo* o línies cel·lulars que presenten una variabilitat menor. Les línies cel·lulars disponibles poden ser d'origen humà, com són les línies cel·lulars THP-1 i la U937, o d'origen murí com són la J774 i la MH-S. Totes aquestes línies han estat descrites com a bons models en termes d'expressió de receptors, multiplicació i supervivència de *M. tuberculosis* (Melo *et al*., 2000; Stokes *et al*., 1999; Passmore *et al*., 2001). Els pneumòcits i les cèl·lules dendrítiques també han estat proposats com a models per l'estudi de *M. tuberculosis* degut a que, al igual que els macròfags, formen part de la resposta immune primerenca a la infecció (Wolf *et al*., 2007; Fine *et al*., 2012). Degut a la gran varietat de tipus cel·lulars i protocols utilitzats en els diferents estudis publicats es fa difícil poder comparar els resultats entre ells així com treure unes conclusions clares.

6.1.2. Models animals

A diferència dels cultius cel·lulars, els models animals ens permeten estudiar no només el primer estadi de la infecció sinó veure efectes més tardans i permetre avaluar la disseminació del patogen pels diferents teixits. Els ratolins, els conills d'índies i els conills són els models més usats degut a la seva infecció per inhalació, la seva resposta innata i adquirida, el seu control bacil·lar inicial de la infecció i la seva darrera sensibilitat a la malaltia (Dharmadhikari *et al*., 2008). Tot i que la TB en ratolins no causa granuloma, el seu baix cost, el bon coneixement

genètic i la disponibilitat de reactius el fan el model escollit per excel·lència. Els conills, d'un altra banda, tenen una histopatologia més semblant al humà però la sensibilitat a *M. tuberculosis* és alta. Els primats presenten moltes similituds amb la malaltia en humans, però el seu ús és reservat per les fases pre-clíniques de desenvolupament de fàrmacs degut al seu alt cos i al requeriment d'una infraestructura específica.

6.1.3. Assaigs competitius

Els assaigs competitius de la *fitness* mesuren el *relative fitness*, que es basa en l'habilitat que té el bacteri en competir per uns recursos limitats donant informació dels avantatges o desavantatges de la soca estudiada a nivell evolutiu. En aquests experiments s'afegeix una soca resistent i una sensible en un medi de cultiu on competeixen pels mateixos nutrients i s'avalua la *fitness* per mitjà del nombre de generacions que té la soca resistent respecte la sensible (Borrell *et al*., 2009; Bhatter *et al*., 2012). Gagneux *et al*. (2006), va utilitzar aquesta metodologia per comparar la *fitness* de soques resistents a RIF amb diferents mutacions associades.

6.1.4. Estudis epidemiològics

L'estudi de contactes així com l'epidemiologia molecular han estat claus per millorar el coneixement de la transmissió de la TB. En termes d'epidemiologia, el cas índex es defineix com el primer pacient que transmet la malaltia, els casos secundaris com els pacients que s'infecten a partir d'aquest cas índex i el *cluster* com el conjunt de pacients d'una determinada població i zona geogràfica durant un temps determinat que estan infectats per la mateixa soca de *M. tuberculosis*. El nombre de casos secundaris generats, la freqüència de casos resistents i la grandària del *cluster* s'han utilitzat per estudiar la *fitness* en soques resistents de TB (Burgos *et al*., 2003; Borrell *et al*., 2009). Tot i que l'epidemiologia és una eina valuosa per avaluar la transmissió, la mesura de *fitness* pot estar influenciada per diferències genètiques de les poblacions o pels programes de control de la malaltia en el lloc de l'estudi.

6.2. Efecte de la resistència a fàrmacs en la *fitness* de *M. tuberculosis*

L'aparició de les primeres soques resistents va obrir un debat sobre la virulència d'aquestes soques. Middlebrook i Cohn van descriure conills d'índies infectats amb soques resistents a la INH amb una menor patogènia que aquells infectats amb soques sensibles (Middlebrook *et al*., 1953). D'un altra banda, més endavant es va veure com les soques clíniques resistents podien tenir un rang molt ampli de virulència (Ordway *et al*., 1995) i que

aquesta variació podria ser deguda a les mutacions associades a la resistència. Les mutacions en el gen *kat*G poden alterar l'activitat catalasa-peroxidasa i per tant, comportar una pèrdua de *fitness* de les soques (Li *et al*., 1998). De totes maneres, aquestes mutacions poden causar des d'un mínim a una pèrdua total de l'activitat i s'ha descrit que el bacteri té la capacitat de compensar aquesta activitat amb una sobreexpressió del gen *aph*C (Sherman *et al*., 1996; Kelley *et al*., 1997). Burgos *et al*. (2003), va observar menys casos secundaris de soques resistent a INH que de soques sensibles i no havia cap de MDR, en canvi, van Doorn *et al*. (2006) va trobar que les soques amb mutació al *kat*G eren en *cluster* amb la mateixa freqüència que les sensibles a INH. Un altre estudi va mostrar que les soques amb la mutació freqüent en *kat*G, la S315T, es transmetien de manera freqüent mentre que aquelles que tenien la mutació en altres posicions o altres canvis del mateix gen *kat*G ho feien en menor grau (Gagneux et al., 2006). Diferents estudis han avaluat l'efecte de la mutació en el gen *rpo*B en la *fitness* del bacteri, descrivint diferències depenen de la posició de la mutació (Billington *et al*., 1999; Gagneux *et al*., 2006). Els llinatges descrits en *M. tuberculosis*, com per exemple les soques Beijing, han estat associats a àrees geogràfiques concretes així com a diferents respostes immunes en l'hoste i a diferents rangs de virulència en models animals (Krishnan *et al*., 2011; Portevin *et al*., 2011; Reed *et al*., 2004).

JUSTIFICACIÓ DEL TREBALL I HIPÒTESI

La preocupació sobre el control de la TB ha augmentat a conseqüència de l'aparició de soques resistents als fàrmacs disponibles actualment. Un millor coneixement de les soques de *M. tuberculosis* resistents a fàrmacs és clau per evitar la transmissió així com per dissenyar pautes de tractament més efectives contra la TB.

1. La resistència a fàrmacs de *M. tuberculosis* és deguda a mutacions espontànies en els cromosomes. Les dianes dels fàrmacs utilitzats afecten al metabolisme del bacteri i per tant, aquestes mutacions poden tenir efectes en processos fisiològics del microorganisme. Aquestes dianes inclouen els gens *kat*G, *inh*A o *rpo*B que són importants per la síntesi de la paret cel·lular del bacteri, per protegir el bacil dels components tòxics i per la síntesi proteica. D'aquesta manera, el bacteri podria tenir dificultats en créixer, causar malaltia o ser transmès en la població humana, tot i que s'ha vist que aquest possible desavantatge podria ser restaurat per mutacions compensatòries.

2. El tractament de la TB requereix una pauta individualitzada per cada pacient depenent de les seves resistències associades, les interaccions entre fàrmacs així com la toxicitat al pacient. A més, en els casos de resistència, especialment en la TB MDR, l'escassa disponibilitat de fàrmacs i la toxicitat juntament amb la llarga durada del tractament provoquen que el pacient abandoni el tractament. Tot i que la susceptibilitat als fàrmacs es du a terme de manera individual, en el tractament els fàrmacs estan actuant en combinació. Pocs estudis han avaluat l'eficàcia i la sinergia de combinacions de fàrmacs davant *M. tuberculosis*. Per tant,

   2.a Els fàrmacs antituberculosos de primera i segona línia podrien tenir una activitat sinèrgica així com mostrar diverses eficàcies.

   2.b L'adaptació del tauler d'escacs a tres fàrmacs podria ser útil per estudiar la sinergia en combinacions de fàrmacs.

   2.c El model de macròfag podria donar un altre enfocament en l'estudi de l'eficàcia de combinacions de fàrmacs degut a que *M. tuberculosis* es multiplica dintre dels macròfags. A més, aquest model podria ser útil per avaluar la penetració dels fàrmacs i de les combinacions als macròfags i per tant poder avaluar l'eficàcia dels fàrmacs individualment i en combinació.

OBJECTIUS

I. Analitzar l'habilitat de soques clíniques de *M. tuberculosis* resistents a fàrmacs de penetrar i créixer dintre de macròfags murins comparat amb soques sensibles.

II.a Avaluar l'habilitat de soques clíniques de *M. tuberculosis* resistents a fàrmacs de penetrar dintre de macròfags murins comparat amb soques sensibles.

II.b Avaluar l'habilitat de soques clíniques de *M. tuberculosis* resistents a fàrmacs de multiplicar-se dintre de macròfags murins comparat amb soques sensibles.

II. Determinar l'activitat sinèrgica *in vitro* de les següents combinacions davant de soques clíniques de *M. tuberculosis* resistents a INH comparat amb les soques sensibles.

a. INH + RIF + EMB
b. OFL + RIF + EMB

III. Determinar l'activitat sinèrgica *in vitro* de les següents combinacions davant de soques clíniques de *M. tuberculosis* multiresistents comparant amb les soques sensibles.

a. LEV + LNZ + AMK
b. LEV + LNZ + EMB
c. LEV + AMK + EMB

IV. Determinar l'activitat antimicrobiana i sinèrgica de les combinacions descrites al objectiu III davant de soques clíniques de *M. tuberculosis* multiresistents i sensible en un model de macròfags humans de la línia cel·lular THP-1.

V.a Determinar l'activitat individual antimicrobiana de l'EMB, l'OFL, l'AMK i el LNZ davant macròfags infectats amb *M. tuberculosis*.

V.b Determinar l'activitat de les combinacions descrites en l'objectiu IV davant macròfags infectats amb *M. tuberculosis.*

V.c Determinar la concentració de fàrmacs sols i en combinació dintre de macròfags infectats amb *M. tuberculosis.*

RESULTATS

Article I. Impaired fitness of *Mycobacterium tuberculosis* resistant isolates in a cell culture model of murine macrophages. *J Antimicrob Chemother* 2011; 66: 2277-2280.

Objectiu: Mesurar l'habilitat de soques clíniques de *M. tuberculosis* resistents a fàrmacs de penetrar i créixer dintre de macròfags murins comparat amb soques sensibles com un indicador de la *fitness*.

Material i mètodes: Es van estudiar 35 soques resistents a diferents fàrmacs i 10 soques sensibles de *M. tuberculosis* en un model de macròfags murins de la línia cel·lular J774.2 en un protocol de 6 dies. Els paràmetres per avaluar la *fitness* van ser l'índex de penetració del micobacteri (MPI) i la *ratio* de creixement (MGR) en els macròfags.

Resultats: Les soques amb la mutació S315T del gen *kat*G de i les soques multiresistents de *M. tuberculosis* van tenir un MGR significativament menor que les soques sensibles. El MPI de les soques amb la mutació S315T del gen *kat*G va ser significativament menor que el de les soques que no tenien aquesta mutació. Es van trobar valors menors, amb una tendència de significació, en els MPI de les soques multiresistents comparat amb les resistents a la isoniazida i amb les sensibles.

Conclusions: Les soques resistent a la INH i les multiresistents amb mutació en el gen *kat*G van mostrar una multiplicació en l'interior dels macròfags disminuïda, suggerint una *fitness* disminuïda de les soques amb aquests patrons de resistència de *M. tuberculosis.*

Article II. Synergistic effect of two combinations of antituberculous drugs against *Mycobacterium tuberculosis*. *Tuberculosis* 2012; 92: 260-263.

Objectiu: Determinar l'activitat sinèrgica *in vitro* de les següents combinacions de tres fàrmacs davant de soques clíniques de *M. tuberculosis* resistents a INH comparat amb les soques sensibles: A) INH, RIF i EMB, i B) OFL, RIF i EMB.

Material i mètodes: Les combinacions de tres fàrmacs es van avaluar en un total de 12 soques resistents a INH i a INH-STR emprant una adaptació del mètode de tauler d'escacs. La Concentració Inhibitòria Fraccionada (CIF) es va calcular amb la següent fórmula: CIF: $A/CMI_A + B/CMI_B + C/CMI_c = CIF_A + CIF_B + CIFc$ on, A, B i C són les CMIs en combinació per a cada fàrmac, i CMI_A, CMI_B i CMI_C les CMIs individuals. LA CIF va ser interpretada com sinergisme quan el valor era igual o menor a 0,75.

Resultats: En la combinació de INH, RIF i EMB, les 11 soques sensibles van reduir la seva CMI individual d'una fins a tres dilucions, mostrant indiferència en el 81,8% (CIF=0,88-1,6) i activitat sinèrgica en el 18,1% (CIF=0,6). La CMI en combinació de totes les soques resistents a isoniazida estudiades van baixar tres dilucions la seva CMI individual, mostrant una activitat sinèrgica (CIF: 0,31-0,38). La combinació d'OFL, RIF i EMB, va mostrar una activitat sinèrgica (CIF: 0,31-0,62) davant de 21 de les 23 soques estudiades, el 91,3%. La combinació d'OFL, RIF i EMB va presentar significativament més sinergisme que la combinació d'INH, RIF i EMB (p=0,007) en soques sensibles.

Conclusions: L'adaptació del mètode de tauler d'escacs, és una tècnica fiable per l'estudi *in vitro* de combinacions de tres fàrmacs. Les dues combinacions testades poden ser útils pel tractament de soques resistents, tot i que la combinació que conté l'OFL presenta una eficàcia major, sent d'ús potencial per tractar casos de soques resistents com de sensibles.

Article III. *In vitro* effect of three-drug combinations of antituberculous agents against multidrug-resistant *M. tuberculosis* isolates. Acceptat al *Int J Antimicrob Agents*

Objectius: Determinar l'efecte de les següents combinacions de fàrmacs de segona línea enfront de soques multiresistents i sensibles: LEV, LNZ i EMB; LEV, AMK i EMB; LEV, LNZ i AMK. Comparar l'efecte d'aquestes combinacions amb la combinació de INH, RIF i EMB davant de soques sensibles.

Material i mètodes: Les combinacions anteriors es van avaluar en 9 soques MDR i 12 soques sensibles (11 soques clíniques i la soca de referència H37Rv) emprant una adaptació del mètode del tauler d'escacs. La Concentració Inhibitòria Fraccionada (CIF) es va calcular amb la següent fórmula: CIF: $A/CMI_A + B/CMI_B + C/CMI_c = CIF_A + CIF_B + CIFc$ on, A, B i C són les CMIs en combinació per a cada fàrmac, i CMI_A, CMI_B i CMI_C les CMIs individuals. LA CIF va ser interpretada com sinergisme quan el valor era igual o menor a 0,75.

Resultats: els índexs CIFs de totes les combinacions es van trobar entre 1,5 i 3, mostrant una activitat d'indiferència. No es van trobar diferències entres les soques multiresistents i les soques sensibles, així com tampoc entres les combinacions de segona línia i la combinació d'INH, RIF i EMB.

Conclusió: *In vitro*, les combinacions de segona línia són igual d'eficaces que la combinació d'INH, RIF i EMB.

Article IV. Effect of three-drug combinations of antituberculous agents against multidrug-resistant *M. tuberculosis* isolates in a macrophage model. Enviat al *J Antimicrob Chemother*

Objectius: Determinar l'efecte de les següents combinacions de fàrmacs de segona línea enfront soques multiresistents i sensibles en un model de macròfags humans: 1. LEV, LNZ i EMB; 2. LEV, AMK i EMB; 3. LEV, LNZ i AMK.

Material i mètodes: L'efecte de les combinacions citades anteriorment es van testar enfront 6 soques multiresistents i 6 soques sensibles de *M. tuberculosis* en un cultiu de macròfags humans de la línia cel·lular THP-1. Els macròfags es van llisar i es van sembrar en plaques de Middelbrook 7H11 i es van fer recomptes quantitatius després de 21 dies d'incubació. La interacció dels fàrmacs es va interpretar com l'efecte de la combinació en comparació a l'efecte del fàrmac sol. L'activitat antimicrobiana dels fàrmacs es va avaluar comparant els $\log_{10}$ UFC/pou amb els $\log_{10}$ UFC/pou de la soca sense fàrmac. Les concentracions en l'interior dels macròfags així com les extracel·lulars es van determinar utilitzant tècniques cromatogràfiques.

Resultats: La combinació LEV-LNZ-AMK i la LEV-LNZ-EMB van mostrar un efecte antagònic davant totes les soques estudiades després d'un protocol de 4 dies, mentre que la combinació LEV-AMK-EMB va ser indiferent. La LEV sola així com la combinació LEV-AMK-EMB van ser les que van tenir un major efecte antibacterià reduint fins a 5.49 $\log_{10}$ i 5.86 $\log_{10}$, respectivament. Els percentatges de penetració dels fàrmacs van variar entre 7.53% i 11.02% per la LEV, l'AMK i el LNZ mentre que en l'EMB van ser al voltant del 5%. Les concentracions intracel·lulars dels fàrmacs individuals van ser similars a les trobades en els fàrmacs en combinació.

Conclusions: Tot i que totes les combinacions testades van mostrar un efecte antimicrobià davant de soques de *M. tuberculosis* multiplicant-se a l'interior dels macròfags, les combinacions que inclouen LNZ i LEV han mostrat un efecte antagònic. Aquest efecte podria ser explicat per proteïnes transportadores o canvis en l'interior del macròfag que afectés a l'activitat dels fàrmacs.

DISCUSSIÓ

MODELS DE MACRÒFAGS

Els macròfags són una defensa clau que té l'hoste com a resposta a l'exposició inicial de *M. tuberculosis*. La immunitat adquirida també tindrà un rol significatiu en la supervivència del bacil així com en la seva disseminació als diferents òrgans. Podríem dir que el sistema immune humà és eficaç davant *M. tuberculosis* ja que només una minoria dels casos infectats de TB desenvolupen la malaltia.

En el treball aquí presentat s'han utilitzat dues línies cel·lulars de macròfags, les murines J774.2 i les humanes THP-1. La primera es va emprar com a model per estudiar la *fitness* de les soques resistents de *M. tuberculosis* i la segona per l'estudi de l'efecte de combinacions de fàrmacs davant de *M. tuberculosis* que està replicant en l'interior dels macròfags. Tot i que no s'ha fet un treball comparant la invasió i multiplicació intracel·lular de *M. tuberculosis* en les dues línies cel·lulars, els dos articles independentment (Article I i Article IV) ens donen una informació valuosa de les utilitats d'aquests models de macròfags.

Han estat molts els models que s'han proposat per tal de reproduir les condicions *in vivo* que es donen a la primera fase de la infecció. Els models de macròfags disponibles actualment van de cèl·lules humanes a murines, de cultius primaris a línies cel·lulars i de monòcits a macròfags diferenciats, tots ells utilitzats per un ampli ventall de finalitats (veure secció 6.1). Aquests models poden variar en el medi de cultiu que requereix, en el temps d'exposició al micobacteri o en el procés de diferenciació dels macròfags. La multiplicitat d'infecció, la MOI, emprada en els diferents models pot variar notablement ja que la *ratio* de bacteri/macròfag s'ha d'adaptar a l'habilitat que tinguin aquells macròfags per fagocitar sense esdevenir a mort cel·lular. D'aquesta manera, el nombre de bacteris que es posen en contacte amb els macròfags podria interferir en el nombre de bacteris capaços de penetrar dintre dels macròfags, i com a conseqüència, el creixement intracel·lular posterior també es podria veure afectat. Per tant, s'hauria d'anar amb prudència quan es comparen treballs on s'han utilitzat diferents models cel·lulars.

Les línies cel·lulars J774.2 i THP-1 han estat prèviament descrites com a bons models, deguts a les seves similituds amb el *in vivo* en termes d'expressió de receptors i de fagocitosis dels macròfags i de supervivència i multiplicació del micobacteri intracel·lular (veure secció

6.1). Mehta *et al*. (1996) ja va descriure que la concentració de SFB en el medi de cultiu cel·lular és important pel correcte creixement dels macròfags J774.2. La concentració que s'usa normalment, el 10%, acaba provocant un creixement massiu i finalment un aixecament de les cèl·lules. A més de la concentració del SFB, s'ha de tenir en compte no allargar el protocol de més de 6 dies per tal de mantenir la monocapa de macròfags en aquesta línia cel·lular. Per tal d'estudiar l'activitat de fàrmacs davant de soques de *M. tuberculosis* es va dissenyar un protocol llarg que permetés la multiplicació del micobacteri dintre dels macròfags i posteriorment la exposició continuada a fàrmacs durant uns dies. D'aquesta manera, es van escollir els macròfags humans de la línia cel·lular THP-1 perquè poden mantenir la monocapa cel·lular durant més de 6 dies. Aquest model permet donar una informació sobre l'actuació del fàrmacs valuosa i més pròxima a la realitat que les tècniques de cultius bacterians, ja que els fàrmac per actuar davant del micobacteri ha d'haver penetrat anteriorment en els macròfags. A més, és una tècnica que també permet determinar les concentracions dels fàrmacs estudiats dintre i fora dels macròfags, facilitant així els anàlisis posteriors d'acumulació i de les característiques farmacocinètiques dels fàrmacs.

En conclusió, tot i que els models de macròfags tenen certes limitacions, aquests poden ser útils per estudiar la supervivència de *M. tuberculosis* dintre dels macròfags així com per avaluar la eficàcia de combinacions de fàrmacs o nous fàrmacs davant de *M. tuberculosis in vitro,* sent una primera aproximació per estudis posteriors en models animals o assajos clínics.

FITNESS DELS BACTERIS RESISTENTS A FÀRMACS

La pèrdua o la potenciació de la *fitness* per l'adquisició de resistència a fàrmacs s'ha descrit en molts microorganismes. La resistència als β-lactàmics en bacteris entèrics com *Salmonella typhimurium* és freqüentment associada a mutacions en el gen *amp*D. D'altra banda, els β-lactàmics tenen com a diana el peptidoglicà del bacteri que no només és important per mantenir la integritat del bacteri sinó que també juga un paper en la resposta immune innata. *Salmonella typhimurium* com *M. tuberculosis*, és un patogen intracel·lular. En aquest sentit, Folkesson *et al*. (2005) va descriure com aquells bacteris amb mutació al gen *amp*D penetraven en menor grau i tenien una *fitness* menor que els salvatges. Pel contrari, *Neisseria gonorrhoeae* amb mutacions en els gen *gyr*A han mostrat diferents comportament en la *fitness* depenen del punt de mutació (Kunz *et al.*, 2012). La resistència a quinolones de *N. gonorrhoeae* ve donada per mutacions en els gens *gyr*A i *par*C simultàniament. Kunz *et al*. (2012) va mostrar que les soques que tenien mutació en el gen *gyr*A tenien un avantatge en la

fitness respecte aquelles que no la tenien, en canvi, aquest avantatge es veia reduït quan se li afegia una mutació en el gen *par*C. A més, *N. gonorrhoeae* presenta bombes d'expulsió de fàrmacs que no només reconeixen antibiòtics clàssics sinó que també són capaces de reconèixer pèptids antimicrobians provinent de la defensa immune de l'hoste (Johnson *et al.*, 2011). Jense *et al*. (2003) va mostrar com la carència d'aquestes bombes provocava la pèrdua de l'habilitat de mantenir una infecció vaginal en ratolins. Per tant, les bombes d'expulsió podrien estar implicades a més de en la resistència a fàrmacs, tenir un paper en el metabolisme bàsic del bacteri, en la seva fitness i/o en la seva patogenicitat.

Middlebrook and Cohn van fer els primers estudis sobre virulència de les soques resistents a fàrmacs de *M. tuberculosis* (Middlrebrook *et al.*, 1953), on veien una menor patogenicitat de les soques resistents a INH en els conills d'índies comparant amb les soques sensibles. Posteriorment, el coneixement del genoma de *M. tuberculosis* i de les mutacions associades a resistències, va donar lloc a estudis posteriors on feien menció com la virulència o el *fitness* podria dependre d'aquestes mutacions. En els resultats de la tesi aquí presentada (Article I), les soques resistents a INH i les MDR amb mutació en el gen *kat*G tenien un creixement intracel·lular significativament menor que les soques sensibles en els macròfags murins. Pel contrari, les soques sensibles i les multiresistents van presentar un creixement dintre dels macròfags humans similar (Article IV). Aquesta discrepància es podria explicar per diferències metodològiques entre els dos tipus cel·lulars o per les soques estudiades. Aquestes i les trobades amb els estudis epidemiològics on alguns han trobat una *fitness* menor i altres no (Burgos *et al.*, 2003; Gagneux *et al.*, 2006; van Doorn *et al.*, 2006), suggereix que el impacte de les mutacions associades a resistència en *M. tuberculosis* és lleuger i que per tant, pot passar desapercebut en alguns estudis. Els macròfags murins així com els humans poden presentar diferents interaccions amb el patogen i una varietat de patrons de citoquines, resultant així en un creixement diferent del bacteris. A més, en els darrers anys s'ha evidenciat que algunes soques poden respondre diferent al sistema immune (Portevin *et al.*, 2011). D'altra banda, els eicosanoids han estat descrits com a importants lípids mediadors que regulen la mort cel·lular tant en macròfags humans com en murins (Behar *et al.*, 2011). La regulació en l'expressió gènica d'aquests lípids poden dirigir la supervivència del micobacteri. Estudis previs han mostrat diferents llinatges de les soques de *M. tuberculosis* que estan associats a particulars regions geogràfiques i poblacions humanes (Gagneux *et al.*, 2007). Aquests llinatges, a més, han estat descrits que poden ser rellevants en la transmissió de la TB i virulència del micobacteri, com és el cas del genotip Beijing. En el primer treball (Article I), les soques clíniques van mostrar un ampli ventall de genotips, sense haver cap que predominés (*Appendix*

1, dades no mostrades a l'article). Per tant, tot i que el creixement de les soques estudiades dintre dels macròfags podria estar influenciat per característiques intrínseques d'aquestes, no hi ha evidència d'una associació entre un genotip en concret amb un grup d'estudi. L'objectiu del nostre estudi de *fitness* era estudiar la virulència de *M. tuberculosis* en la fase primerenca de la infecció. Tanmateix, aquesta podria està també afectada per la resposta adaptativa i per tant, la *fitness* afectada en soques resistents que hem vist en el nostre estudi, podria ser restaurada amb l'aparició de la resposta adaptativa i durant el curs de la infecció.

El resultat final de la infecció dependrà de molts factors. La situació en la que es trobi el pacient és un d'aquests factors, ja que ambients amb poca ventilació i tractament inadequats poden afavorir a una transmissió de la malaltia. El contagi inicial podria estar influenciada per condicions tant del pacient com del micobacteri. Factors de virulència del patogen, receptors de l'hoste i senyals inflamatòries són clau per les interaccions entre el bacteri i l'hoste (Veure secció 2). Una resposta adquirida posterior i la disseminació del bacteri pels diferents òrgans de l'hoste també seran factors que afectaran a la supervivència del micobacteri. En aquest moment, la resposta immune de l'hoste i la capacitat del micobacteri d'evadir-la i causar patogenicitat faran moure la balança a favor de l'hoste o del micobacteri. Aquesta complexitat dóna lloc estudis de la malaltia des d'una visió més global, com el *systems biology framework* que combina models matemàtics, complements tradicionals empírics i experiments de recerca biomèdica (Young *et al.*, 2008). Aquests models podrien ser molt útils per explorar les diferents soques de *M. tuberculosis* en diferents situacions, i ser una eina per millorar el control de la malaltia i facilitar una intervenció.

En conclusió, similar a altres estudis, el treball presentat suggereix que la mutació en el gen *kat*G podria afectar a la *fitness* de les soques resistents, i que l'acumulació de mutacions en les soques MDR, podria empitjorar la *fitness* del micobacteri. Els estudis epidemiològics han mostrat dos situacions, una on les soques resistents amb la mutació *kat*G315 pot tenir danyada la seva *fitness* (Burgos *et al.*, 2003), i un altre on aquestes soques són igual de transmissibles que les sensibles (Gagneux *et al.*, 2006; van Doorn *et al.*, 2006). Per tant, aquestes dades suggereixen que la pèrdua de *fitness* és lleugera i que la detecció d'aquesta varia en funció de la metodologia emprada. Tanmateix, aquestes diferències en *fitness* podrien ser importants en la TB Latent així com en la seva progressió a TB activa.

APROXIMACIONS A L'ESTUDI DE COMBINACIONS DE FÀRMACS

Els mètodes del tauler d'escacs i les corbes de letalitat han estat àmpliament usades per estudiar les combinacions antimicrobianes enfront *Stafilococcus aureus* MRSA, *Candida albicans* i *Acinetobacter baumanii*, entre d'altres. En aquests casos, de manera similar al que passa en *M. tuberculosis*, el tractament requereix una teràpia múltiple on els diferents fàrmacs tenen diferents dianes per evitar l'aparició de resistències. El mètode del tauler d'escacs tridimensional ha mostrat ser un mètode factible per l'estudi de combinacions de tres fàrmacs en dos dels articles presentats en aquesta tesi (Article II i III). L'estudi de l'efecte de combinacions de tres fàrmacs s'aproxima a la realitat ja que el tractament de la TB consisteix en un mínim de tres fàrmacs. Afegir la PZA en les combinacions testades seria una aproximació més propera i acurada, tot i que les dificultats metodològiques d'aquest fàrmac relacionades amb el pH de l'ambient fan que no sigui abordable du-la a terme. Degut al gran número de combinacions creuades que resulten en el tauler d'escacs, hi ha escassos estudis *in vitro* de combinacions davant *M. tuberculosis*.

Un dels avantatges del tauler d'escacs és la gran quantitat de concentracions que es poden testar simultàniament, especialment quan el mètode en microdilució és l'escollit. D'un altra banda, els mètodes en microdilució a diferència dels de macrodilució, requereixen una perfecte homogeneïtzació del inòcul, que és difícil d'aconseguir treballant amb *M. tuberculosis*. En els treballs presentats en aquesta tesi es va utilitzar el tauler d'escacs tridimensional en macrodilució basat en l'agar Middlebrook 7H11, mostrant un efecte sinèrgic o indiferència. En l'estudi de combinacions de fàrmacs en macròfags humans es va utilitzar la interpretació de les corbes de letalitat ja que es va fer un seguiment del creixement de *M. tuberculosis* sotmès a diferents fàrmacs durant un temps determinat. Cal mencionar que l'efecte antagònic que es va trobar en les corbes de letalitat en macròfags (Article IV), va resultar en indiferència pel tauler d'escacs testant les mateixes combinacions i soques (Article III). Les corbes de letalitat, a diferència del tauler d'escacs, és un model dinàmic on permet veure com respon el micobacteri a la exposició de fàrmac durant un període de temps. Ambdues metodologies donen una informació valuosa per poder posteriorment dissenyar pautes més eficaces contra la TB.

EFECTE DE LES COMBINACIONS DE FÀRMACS DAVANT *M. tuberculosis*

La resistència a fàrmacs de *M. tuberculosis* i la no adherència del tractament degut a la seva llarga duració, dóna lloc a una necessitat urgent de estudiar noves pautes de tractament així com nous fàrmacs antituberculosos.

Actualment, el tractament recomanat per pacients infectats amb soques sensibles als fàrmacs és de 6 mesos amb INH, RIF, EMB i PZA. Aquests quatre fàrmacs són els mateixos que es porten utilitzant en els darrers 50 anys. En aquest sentit, recentment s'ha proposat tractaments amb nous fàrmacs que permeten escurçar la duració d'aquests. En l'Article II, la combinació d'OFL, RIF i EMB va mostrar significativament més sinergisme que la combinació d'INH, RIF i EMB. Aquesta superioritat de les fluoroquinolones ja ha estat prèviament descrita, donant suport a la idea de canviar un dels fàrmacs de la pauta estàndard per una fluoroquinolona. Tanmateix, en països en vies de desenvolupament l'accessibilitat de nous fàrmacs és escassa. En aquest sentit, l'aparició de resistència a les fluoroquinolones reduiria els fàrmacs a l'abast per tractar. El tractament de pacients infectats amb soques resistents requereix d'una pauta individualitzada depenen de la resistència a fàrmacs, la toxicitat en el pacient o els fàrmacs disponibles (veure figura 15). En el treball presentat (Article II), la combinació d'OFL-RIF-EMB i la d'INH-RIF-EMB van mostrar sinergisme davant de soques resistents a INH. En la combinació d'INH-RIF-EMB, la majoria de les CMIs en combinació de les soques resistents a INH van decréixer fins concentracions que s'aproximaven als valors de sensibilitat. Aquest fet és evidenciat en casos de resistència baixa a INH que no són sempre detectades (Tudó *et al.*, 2004), indicant que els pacients s'estaven curant amb el tractament estàndard d'INH, RIF, EMB i PZA. De totes maneres, aquests resultats s'haurien d'interpretar amb prudència ja que tot i ser present aquest sinergisme, el micobacteri podria incrementar els seus nivells de resistència en el transcurs del tractament i provocar així un fracàs terapèutic i un allargament de la durada del mateix.

El tractament de la TB MDR complica encara més la curació del malalt ja que l'INH i la RIF, considerats fàrmacs altament bactericides, són retirats del tractament. Els fàrmacs de segona línia per tractar aquests pacients són més tòxics, cars i requereixen allargar més encara el tractament afavorint fracassos terapèutics i abandonaments del tractament. Per això, és clau dissenyar tractaments eficients, evitant així un fracàs terapèutic o recaiguda, la transmissió de la TB MDR o l'aparició de resistències adquirides. Els aminoglicòsids, com la kanamicina o la AMK, les fluoroquinolones i altres fàrmacs com el LNZ i la clofazimina estan considerats en el tractament de la TB MDR i la XDR. És clau aprofundir sobre l'efecte de les diferents pautes

possibles per tractar aquests casos, ja que l'existència d'interaccions entre els fàrmacs de la combinació podria millorar o deteriorar el resultat final del tractament.

En la present tesi, es van estudiar les combinacions de tres fàrmacs que incloïen LEV afegint LNZ, AMK o EMB davant de soques sensibles i multiresistents de *M. tuberculosis* pel tauler d'escacs i pel model de macròfags. Curiosament, es va trobar indiferència en el tauler d'escacs mentre que el model de macròfags va mostrar un efecte antagònic. Aquesta diferència entre el model *in vitro* i el *ex-vivo*, suggereix que no existeix un interacció directa entre els fàrmacs. En el model de macròfags, els fàrmacs tenen que penetrar en aquests i una vegada a l'interior ser actiu davant el micobacteri. L'antagonisme es va trobar en les combinacions que incloïen LNZ i LEV, on l'activitat de la combinació decreixia en més de 2 logaritmes comparat amb el fàrmac més actiu. Els fàrmacs estudiats van penetrar en els macròfags mostrant concentracions intracel·lulars similars. Les següents causes podrien explicar aquest antagonisme provat. Una podria ser que les condicions fisicoquímiques en l'interior dels macròfags com ara que el pH pogués alterar l'activitat d'algun dels fàrmacs de la combinació. En aquest sentit, un efecte advers del LNZ és l'acidosi metabòlica (Garrabou *et al.*, 2007). Una primera hipòtesi podria ser que l'acidificació en l'interior del macròfag o canvis en la seva activitat pogués alterar l'activitat del LEV. Una segona explicació podria ser que hi ha un transport actiu (Hall *et al.*, 2003; Hartkoorn *et al.*, 2007; Vallet *et al.*, 2011) o un efecte que bloquegés l'accés del fàrmac l'interior dels lisosomes on resideixen els bacils. Actualment, no hi ha estudis publicats que observin el mencionat antagonisme entre LNZ i LEV. Tanmateix, l'efecte antagònic d'aquests dos fàrmacs podria estar emmascarada quan s'estudien els tractament de MDR i XDR on una gran quantitat de fàrmacs són administrats i que per tant, no es pot diferenciar en la interacció de dos fàrmacs. Un altre aspecte a tenir en compte és que la utilització del LNZ en el tractament de la TB és relativament nova, existint en aquest sentit un baix nombre de publicacions al respecte. Els resultats de l'últim treball (Article IV) dona lloc a plantejar treballs posteriors per clarificar el mecanisme d'aquest antagonisme i veure si és present en models animals.

CONCLUSIONS

1. Els models de macròfags dissenyats són adients per l'estudi de la *fitness* de *M. tuberculosis* així com per estudiar l'activitat de fàrmacs davant bacils intracel·lulars.

2. Les soques multiresistents i les resistents a la INH amb mutació en el gen *kat*G tenen un creixement dintre dels macròfags menor que els soques sensibles, suggerint una *fitness* menor d'aquestes soques.

3. Les soques MDR i les resistents a la INH amb mutació en altres gens que no són el gen *kat*G (com ara *inh*A, *rpo*B i/o mutacions desconegudes) no tenen diferències significatives en el seu creixement dintre de macròfags comparat amb les soques sensibles.

4. L'adaptació tridimensional del mètode del tauler d'escacs és factible per l'estudi de sinergisme de combinacions de tres fàrmacs davant de *M. tuberculosis*.

5. La combinació que inclou INH, RIF i EMB podria ser eficaç per tractar casos de baixa resistència (CMIs ≤ 0.8μg/ml) degut a un efecte sinèrgic de la combinació.

6. La combinació que inclou OFL, RIF i EMB mostra una millor eficàcia que la combinació d'INH, RIF i EMB, sent d'un ús potencial per tractar casos sensibles i resistents a INH.

7. Les combinacions que inclou fàrmacs de segona línia (LEV-AMK-EMB; LEV-AMK-LNZ; LEV-EMB-LNZ) són igual d'eficaços que la combinació d'INH, RIF i EMB utilitzant el mètode del tauler d'escacs.

8. La AMK, el EMB, el LEV i el LNZ penetren de manera similar dintre dels macròfags humans THP-1 tot i que la LEV és el fàrmac més actiu davant *M. tuberculosis* en creixement intracel·lular.

9. Les combinacions de tres fàrmacs testades (LEV-AMK-EMB; LEV-AMK-LNZ; LEV-EMB-LNZ) davant de macròfags infectats amb *M. tuberculosis* mostren una activitat antimicrobiana, i un efecte antagonista de les combinacions que inclouen LEV and LNZ.

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APPENDICES

APPENDIX 1. **Spoligotyping families of the isolates studied according to associated mutations.**

	Classification according to associated mutations						
	Susceptible (%)	Resistant without mutations in genes studied * (%)	Mutation in the *kat*G gene (%)	Mutation in the *inh*A gene (%)	Mutation in the *rpo*B gene (%)	Mutations in both the *kat*G and the *rpo*B genes (%)	Total (%)
Spoligotyping family and lineages**							
H3	2 (20)	1 (25)	2 (15.4)	2 (40)	0 (0)	2 (25)	9 (20)
LAM9	1 (10)	0 (0)	1 (7.7)	0 (0)	0 (0)	2 (25)	4 (8.9)
T1	1 (10)	1 (25)	2 (15.4)	0 (0)	2 (40)	1 (12.5)	7 (15.6)
LAM7	1 (10)	0 (0)	1 (7.7)	0 (0)	0 (0)	0 (0)	2 (4.4)
Unknown	0 (0)	0 (0)	2 (15.4)	0 (0)	0 (0)	0 (0)	2 (4.4)
Non classifiable	0 (0)	0 (0)	0 (0)	1 (20)	0 (0)	0 (0)	1 (2.2)
Other families***	3 (30)	2 (50)	5 (38.5)	2 (40)	3 (60)	2 (25)	17 (37.8)
Not done	2 (20)	0 (0)	0 (0)	0 (0)	0 (0)	1 (12.5)	3 (6.7)
Total	10 (100)	4 (100)	13 (100)	5 (100)	5 (100)	8 (100)	45 (100)

Spoligotyping of *M.tuberculosis* isolates were carried out as described previously. The international Spo1DB4 database was used to determine the spoligotype families.* *kat*G, *inh*A, *rpo*B. ** Classified according to SpolDB4 database. *** Other families, one or two isolates each, were: CAS1-DELHI, EAI4-VNM, H1, H2/LAM7, H3-LAM9, LAM12-MAD1, LAM2, S1, T3, T5-MAD2, X1, X2.

APPENDIX 2.

IMAGES SOURCE

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The images used throughout the text are:

1. Zielh-Nielssen staining of *M. tuberculosis*. CDC. http://en.wikipedia.org
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15. Checkerboard assay on microtritation tray by alarm blue detection. Karlsruher Institut für Technologie. http://www.ibg.kit.edu/nmr/247.php
16. *M. tuberculosis* infected-macrophage. Confocal microscopy. Own image
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