Universidade Nova de Lisboa

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The dynamics of drug resistance in *Mycobacterium tuberculosis*: exploring the biological basis of multi- and extensively drug resistant tuberculosis (MDR/XDRTB) as a route for alternative therapeutic strategies

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Declaration

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Publications

This thesis is based on original work that was published or is in preparation for publication:

Machado D, Couto I, Perdigão J, Rodrigues L, Portugal I, Baptista PV, Veigas B, Amaral L, Viveiros M. Contribution of efflux to the emergence of isoniazid and multidrug resistance in *Mycobacterium tuberculosis*. PLoS One. 2012; 7: e34538.

Machado D, Perdigão J, Ramos J, Couto I, Portugal I, Ritter C, Boettger EC, Viveiros M. High-level resistance to isoniazid and ethionamide in multidrug resistant *Mycobacterium tuberculosis* of the Lisboa family is associated with *inhA* double mutations. J Antimicrob Chemother. 2013; 68: 1728-32.

Machado D, Pires D, Perdigão J, Couto I, Portugal I, Martins M, Amaral L, Anes E, Viveiros M. Ion channel blockers act as antimicrobial agents and enhancers of macrophage killing activity against drug resistant *Mycobacterium tuberculosis*. *Manuscript in preparation.*

Machado D, Perdigão J, Ramos J, Couto I, Portugal I, Cambau E, Boettger EC, Viveiros M. Exploring the molecular genetic basis of drug-resistant *Mycobacterium tuberculosis* strains in Lisbon, Portugal: correlation between phenotypic and genetic resistance to first and second line antituberculosis drugs. *Manuscript in preparation.*

Machado D, Couto I, Viveiros M. *Mycobacterium tuberculosis*, the etiological agent of tuberculosis. *Manuscript in preparation.*

Other manuscripts for which I have made a contribution during my PhD but that are not included as part of this thesis, are as follows:

Cambau E, Viveiros M, **Machado D**, Raskine L, Ritter C, Tortoli E, Fauville-Dufaux M, Hoffner S, Richter E, Perez del Molino ML, Cirillo D, van Soolingen D, Boettger EC. Revisiting susceptibility testing in multidrug resistant tuberculosis by a standardized quantitative phenotypic assessment in a European multicenter study. 2014; *Submitted*.

Leão C, **Machado D**, Sanches I, Couto I, Viveiros M, Inácio J, Botelho A. Relatedness of *Mycobacterium avium* subspecies *hominissuis* clinical isolates of human and porcine origins assessed by MATR-VNTR analysis. 2014; *Submitted.*

Costa P, Ferreira A, Amaro A, **Machado D**, Albuquerque T, Couto I, Botelho A, Viveiros M, Inácio J. Rapid identification of veterinary-relevant *Mycobacterium tuberculosis* complex species using 16S rDNA, IS*6110* and regions of difference-targeted dual-labelled hydrolysis probes. 2014; *Submitted*.

Perdigão J, Silva H, **Machado D**, Macedo R, Maltez F, Silva C, Jordão L, Couto I, Mallard K, Coll F, Hill-Cawthorne GA, McNerney R, Pain A, Clark, TG, Viveiros M, Portugal I. Unraveling *Mycobacterium tuberculosis* genomic diversity and evolution in Lisbon, Portugal, a highly drug resistant setting. 2014; *Submitted*.

Pedrosa P, Veigas B, **Machado D**, Couto I, Viveiros M, Baptista PV. Gold nanoprobes for multi loci assessment of multi-drug resistant tuberculosis. Tuberculosis. 2014; 94: 332-7.

Machado D, Ramos J, Couto I, Cadir N, Narciso I, Coelho E, Viegas S, Viveiros M. Assessment of the BD MGIT TBc identification test for the detection of *Mycobacterium tuberculosis* complex in a network of mycobacteriology laboratories. Biomed Res Int. 2014; org/10.1155/2014/398108.

Perdigão J, Macedo R, **Machado D**, Silva C, Jordão L, Couto I, Viveiros M, Portugal I. GidB mutation as a phylogenetic marker for Q1 cluster *Mycobacterium tuberculosis* isolates and intermediate-level streptomycin resistance determinant in Lisbon, Portugal. Clin Microbiol Infect. 2013; 10.1111/1469-0691.12392.

Armada A, Alexandru T, **Machado D**, Danko B, Hunyadi A, Dinache A, Nastasa V, Boni M, Ramos J, Viveiros M, Molnar J, Pascu ML, Amaral L. The *in vitro* activity of products formed from exposure of chlorpromazine to a 266nm laser beam against species of mycobacteria of human interest. In vivo. 2013; 27: 605-10.

Viveiros M, **Machado D**, Couto I, Amaral L. Improving on the LJ slope – automated liquid culture. *In*: Tuberculosis - Diagnosis and Treatment (Advances in Molecular and Cellular Microbiology Series), T. D. McHugh, Editor. CABI Publishing, Oxfordshire, UK, 304 pp. 2013. (ISBN 781-84593-807-9).

Viveiros M, Martins M, Rodrigues L, **Machado D**, Couto I, Ainsa J, Amaral L. Inhibitors of mycobacterial efflux pumps as potential boosters for TB drugs. Expert Rev. Anti Infect Ther 2012; 10: 983-98.

Perdigão J, Macedo R, Silva C, **Machado D**, Couto I, Viveiros M, Jordão L, Portugal I. From multidrug-resistant to extensively drug-resistant tuberculosis in Lisbon, Portugal: the stepwise mode of resistance acquisition. J Antimicrob Chemother. 2012; 68: 27-33.

Rodrigues L, **Machado D**, Couto I, Amaral L, Viveiros M. Contribution of efflux activity to isoniazid resistance in the *Mycobacterium tuberculosis* complex. Infect Genet Evol. 2011; 12: 695-700.

Costa SS, Falcão C, Viveiros M, **Machado D**, Martins M, Melo-Cristino J, Amaral L, Couto I. Exploring the contribution of efflux on the resistance to fluoroquinolones in clinical isolates of *Staphylococcus aureus*. BMC Microbiol. 2011; 11:241.

Veigas B, **Machado M**, Perdigão J, Portugal I, Couto I, Viveiros M, Baptista PV. Au-nanoprobes for detection of SNPs associated with antibiotic resistance in *Mycobacterium tuberculosis*. Nanotechnology. 2010; 21: 415101.

Viveiros M, Martins M, Couto I, Rodrigues L, **Machado D**, Portugal I, Amaral L. Molecular tools for rapid identification and novel effective therapy against MDRTB/XDRTB infections. Expert Rev Anti Infect Ther. 2010; 8: 465-80.

Machado D, Couto I, Viveiros M. The interplay between mutations and efflux in acquired resistance to clarithromycin in *Mycobacterium avium* complex. *Manuscript in preparation.*

To my mother

(*in memorium*)

But of opium he said, 'It frequently produces so material an improvement in chronic phthisis, that the patients fancy themselves cured, or almost cured, after taking a few doses.'

Dubos and Dubos, 1952

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Abstract

Keywords: Gene expression, efflux pumps; ion channel blockers; proton motive force; intracellular activity.

Mycobacterium tuberculosis strains with multi- or extensive drug resistance are a major threat for human health. The main objective of this thesis was to understand the basis of the development of resistance in *M. tuberculosis*, in order to design strategies to circumvent its development.

First we started with the description of the molecular basis of drug resistance in *M. tuberculosis* clinical strains circulating in Lisbon through the determination of their resistance patterns and its correlation with mutations associated with resistance. The results showed a clear correlation between the presence of conserved mutation and the resistance phenotype. The description of the resistance profiles of drug resistant strains adds new knowledge for the design of more effective tools for the diagnosis, treatment and management of tuberculosis.

Next, was investigated the contribution of efflux to the emergence of drug resistance in *M. tuberculosis*. The study was based on the *in vitro* induction of an isoniazid resistant phenotype by prolonged serial exposure of *M. tuberculosis* strains to the critical concentration of isoniazid. The results showed that the increased activity of efflux pumps allows a sustainable resistant population to be maintained in the patient under therapy, from which genetically resistant mutants will emerge. The addition of an efflux inhibitor is able to reduce this level of resistance.

Finally, the mechanism of action of the ion channel blockers and inhibitors, verapamil, thioridazine, chlorpromazine, flupenthixol and haloperidol, was evaluated both *in vitro* and *ex vivo*. All compounds exhibit synergistic inhibitory activities when combined with the main first line antituberculosis drugs, demonstrating their role as efflux inhibitors and displaying rapid and high killing activity that could be correlated with a decrease in intracellular ATP levels. Overexpression of efflux genes was detected in response to antibiotics *in vitro* and *ex vivo*, indicating that drug resistance within macrophages is also mediated by the expression of efflux pumps that can be inhibited.

The compounds lead to a decrease in the intracellular mycobacterial load as a result of their ability to induce phagosome acidification with consequent expression of lysosomal hydrolases. We hypothesized that these compounds inhibit the mycobacterial respiratory chain which leads to the dissipation of membrane potential, depletion of ATP, reactive oxygen species generation and cell death. Disruption of the proton motive force (PMF), results in the inhibition of PMF-dependent efflux systems, therefore promoting the retention of antibiotics subject to active efflux. Concerning the host cell, the phagosomal acidification stimulated by these compounds synergize with several components of the host immune response, restricting *M. tuberculosis* intracellular growth. Altogether, we demonstrate that the compounds display a dual inhibitory effect as they target both the bacteria and the host cell.

This thesis aims to contribute to the knowledge on *M. tuberculosis* drug resistance mechanisms. The understanding of the activity of the studied compounds will contribute for the design of new drugs and can provide the basis for the development of new therapeutic strategies to fight drug resistant tuberculosis.

Resumo

Palavras-chave: expressão génica; bombas de efluxo; inibidores de canais iónicos; força motriz protónica; actividade intracelular.

Estirpes de *Mycobacterium tuberculosis* multi- e extensivamente resistentes, constituem uma ameaça para a saúde humana. O principal objectivo desta Tese foi estudar os fenómenos subjacentes ao desenvolvimento da resistência em *M. tuberculosis*, de modo a ser possível desenvolver estratégias para reduzir o seu desenvolvimento.

Iniciamos o estudo com a descrição da base molecular da resistência aos antibacilares em estirpes clinicas de *M. tuberculosis* circulantes em Lisboa, através da determinação dos seus padrões de resistência e sua correlação com as mutações associadas à resistência. Os resultados mostraram uma clara correlação entre a presença de uma mutação e o fenótipo de resistência. A descrição dos perfis de resistência de estirpes resistentes aos antibacilares adiciona nova informação para o desenvolvimento de metodologias mais eficazes para o diagnóstico e tratamento da tuberculose.

De seguida, avaliamos a contribuição dos sistemas de efluxo para o desenvolvimento da resistência aos antibacilares em *M. tuberculosis*. O estudo baseou-se na indução *in vitro* de um fenótipo de resistência à isoniazida por exposição prolongada de estirpes de *M. tuberculosis* à concentração crítica de isoniazida. Os resultados mostraram que o aumento da actividade de bombas de efluxo permite a manutenção de uma população resistente num doente em tratamento, da qual irão emergir mutantes geneticamente resistentes. A utilização de compostos inibidores de efluxo demonstrou ser capaz de reduzir este nível de resistência.

Por último estudamos o mecanismo de acção dos compostos inibidores do efluxo, que partilham a característica comum de serem inibidores de canais iónicos, verapamil, tioridazina, clorpromazina, flupentixol e haloperidol, quer *in vitro* quer *ex vivo*. Todos os compostos apresentaram actividade sinérgica inibitória em combinação com os principais antibacilares de primeira linha e foram capazes de inibir o efluxo activo demonstrando o seu papel como inibidores de efluxo. Igualmente demostraram potente actividade bactericida que pôde ser correlacionada com a diminuição nos níveis de ATP

intracelulares. Foi também detectada sobrexpressão de genes de efluxo em resposta à exposição aos antibióticos *in vitro* e *ex vivo*, indicando que a resistência aos antibióticos em macrófagos é também mediada pela sobrexpressão de bombas de efluxo, a qual pode ser inibida por inibidores de efluxo. Os compostos estudados potenciaram a actividade antimicrobiana de macrófagos infectados e induziram a acidificação do fagolisosoma, com consequente expressão de hidrolases lisossomais. Com estes resultados, foi possível propor, como mecanismo de acção para estes compostos, a inibição da cadeia respiratória micobacteriana. Esta inibição leva à dissipação do potencial de membrana, depleção de ATP, a produção de radicais de oxigénio e morte celular. Por outro lado, e em simultâneo, a disrupção da força motriz protónica (PMF) resulta na inibição dos sistemas de efluxo que dela dependem, promovendo assim a retenção dos antibióticos sujeitos a efluxo activo. No que se refere a célula hospedeira, a acidificação phagosomal estimulada por estes compostos actua em sinergismo com vários componentes da resposta imunitária do hospedeiro, restringindo assim o crescimento intracelular de *M. tuberculosis*. No contexto geral, demonstramos que estes compostos possuem dupla acção, uma vez que actuam quer na bactéria quer na célula hospedeira.

Este trabalho contribuiu para o aumento do conhecimento sobre a resistência aos antibióticos em *M. tuberculosis*. O estudo do mecanismo de acção destes compostos poderá contribuir para o desenvolvimento de novos compostos e pode servir de base para o desenvolvimento de novas estratégias terapêuticas para combater a tuberculose e em particular as suas formas multirresistentes.

Thesis outline

The main objective of this work was to study the mechanisms underlying the development of drug resistance in *M. tuberculosis* as a route for the development of new therapeutic strategies. This thesis is organized so that each chapter can stand on its own as follows:

Chapter I provide a general introductory description on the current knowledge about the main aspects of *M. tuberculosis* biology, epidemiology, diagnosis, drug resistance, and the new therapeutic targets. The review presented gathers and summarizes the information about the recent advances that have been made in the mycobacteriology field that may help us to proper evaluate the important aspects of *M. tuberculosis* drug resistance. This chapter ends with the description of the purpose and objectives of the thesis.

In **Chapter II** we describe the prevalence and pattern of gene mutations associated with resistance to the first and second line antituberculosis drugs, as well as its levels of resistance, in a collection of multi-and extensively drug resistant strains isolated from patients in the Lisbon metropolitan area. The application of a drug resistance quantitative testing allowed us to describe a different mutational pattern associated with resistance to isoniazid among the multidrug resistant strains that circulate in Lisbon. The detailed characterization of the genetic mechanism of resistance to the main antituberculosis drugs for these strains was the basis for the work presented in chapter IV.

In **Chapter III** we assess the contribution of efflux to the emergence of isoniazid and multidrug resistance in *M. tuberculosis*. Here we focus on the understanding of the chain of events leading to the development of resistance during therapy, with specially attention to the differential activity of genes that code for the main *M. tuberculosis* efflux pumps prior to, during and after the establishment of the resistant phenotype and their interaction with the genetically based resistance. The role of efflux inhibitors as adjuvants in tuberculosis therapy for preventing the emergence of drug resistance was also assessed. The identification of the efflux pumps involved in the adaptative response towards resistance and the demonstration that efflux inhibitors can prevent the process

allow us to describe the mechanism of action of these compounds in *M. tuberculosis* clinical strains which is presented in chapter IV.

In **Chapter IV** we investigate the antimycobacterial properties and the mechanism of action of the putative efflux inhibitors verapamil, thioridazine, chlorpromazine, flupenthixol and haloperidol against *M. tuberculosis*, *in vitro* and in human monocyte-derived macrophages. This study provides a description of the molecular mechanisms by which these agents act in *M. tuberculosis* and the results obtained lead us to rename these compounds from efflux inhibitors to ion channel blockers. This chapter contributes with scientific guidance for future research on the improvement and reuse or repurpose of known drugs, on the design and development of new ones, and on their inclusion in future therapeutic regimens against infections caused by drug susceptible or resistant *M. tuberculosis*. The different experimental procedures designed for this work can be applied to characterize and evaluate the bactericidal activity and efficacy of any combination of compounds and antibiotics to be used for this purpose.

In **Chapter V**, the collection of data provided along this thesis is summarized and contextualized in the current context of drug resistance in tuberculosis, the development novel antituberculosis drugs and the description of their mechanism of action for their future therapeutic usage. The results and the conclusions obtained in this work are discussed in light of a mechanistic model that intends to congregate all the findings that drove us to the final conclusion that the compounds studied in this thesis can be useful adjuvants for antituberculosis therapy, preventing the emergence of drug resistance and contributing to the fight against drug resistant tuberculosis. Finally, we propose a number of new scientific approaches for further research and future perspectives on the chemotherapeutics of tuberculosis.

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Chapter IV

List of Abbreviations

ABC	ATP-Binding Cassette
Abl	Abelson
ACR	Acriflavine
AD	Anno Dominni
Ags	Aminoglycosides
AID	Autoimmun Diagnostika
AMK	Amikacin
AMP	Ampicillin
AS	Additional Species
AST	Antibiotic Susceptibility Testing
ATCC	American Type Culture Collection (Virginia, USA)
ATP	Adenosine Triphosphate
BC	Before Christ
BCG	Bacillus Calmette-Guérin
BDQ	Bedaquiline
BP	Before Present
bp	base pair
BSA	Bovine Serum Albumin
^{14}C	Carbon-14
CAM	Chloramphenicol
CAP	Capreomycin
CCCP	Carbonyl Cyanide m-Chlorophenylhydrazone
CDC	Centers for Disease Control and Prevention (USA)
CFU	Colony Forming Units
CIP	Ciprofloxacin
CM	Common Mycobacteria
CO_2	Carbon dioxide
CPZ	Chlorpromazine
Cq	quantification Cycle
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
DPA	Decaprenol phosphoarabinase
DPPR	Decaprenylphosphoryl-β-d-5-phosphoribose
DR	Direct Repeat
DST	Drug Susceptibility Testing
DVR	Direct Variable Repeat
EDTA	Ethylenediaminetetraacetic acid
EF-G	Elongation factor G
EI	Efflux Inhibitor
EMTD	Enhanced *M. tuberculosis* Direct Test
ERDR	Ethambutol Resistance Determining Region
ERY	Erythromycin
ETB	Ethambutol
EtBr	Ethidium bromide

ETH	Ethionamide
ETR	Exact Tandem Repeats
eXIST	extended Individual Susceptibility Testing
FAS-I	Fatty Acid Synthase type I
FAS-II	Fatty Acid Synthase type II
FCS	Fetal Calf Serum
FDA	Food and Drug Administration (EUA)
FIC	Fractional Inhibitory Concentration
FPX	Flupenthixol
FQ	Fluoroquinolones
Fw	Forward
GFP	Green Fluorescent protein
GI	Growth index
Glu	Glucose
GTC	Guanidine Thiocyanate
GTP	Guanosine-5'-triphosphate
GU	Growth Units
H_2O_2	Hydrogen peroxide
HAL	Haloperidol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency Virus
I	Intermediate
ICB	Ion Channel Blocker
IFN	Interferon
IGRA	Interferon Gamma Release Assays
IL	Interleukin
INH	Isoniazid
Ini	Isoniazid-inducible protein
IS	Insertion Sequence
KAN	Kanamycin
kb	Kilo base
LAM	Latino-American-Mediterranean
LSPs	Large Sequence Polymorphisms
MATE	Multidrug and Toxic Compound Extrusion
MB	Middlebrook
Mb	Mega base
M-CSF	Macrophage Colony-Stimulating Factor
MDR	Multidrug Resistant
MDRTB	Multidrug Resistant Tuberculosis
MFS	Major Facilitator Superfamily
$MgCl_2$	Magnesium Chloride
MGIT	Mycobacterial Growth Indicator Tube
MIC	Minimum Inhibitory Concentration
MIRU	Mycobacterial Interspersed Repetitive Unit
miscRNA	miscellaneous RNA
MIT	MIRU International Type
MmpL	Mycobacterial Membrane Protein Large
MOI	Multiplicity Of Infection

mRNA	Messenger RNA
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
MTBDR	*Mycobacterium tuberculosis* drug resistant
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide Hidrogenase
NCBI	National Center for Biotechnology Information (EUA)
ncRNA	non-coding RNA
nd	not done
NO	Nitric Oxide
NVB	Novobiocin
OADC	Oleic acid; Albumin; Dextrose; Catalase
OD	Optical Density
OFX	Ofloxacin
OMPs	Outer Membrane Proteins
ORF	Open Reading Frame
PAS	Paraaminosalicylic acid
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PMF	Proton Motive Force
POA	Pyrazinoic Acid
Prom	Promoter
PZA	Pyrazinamide
PZAse	Pyrazinamidase
qDST	quantitative Drug Susceptibility Testing
QRDR	Quinolone Resistance Determining Region
QUB	Queen's University Belfast
R	Resistant
PCR	Polymerase Chain Reaction
RD	Regions of Difference
rDNA	Ribosomal DNA
RF	Relative Fluorescence
RFF	Relative Final Fluorescence
RFLP	Restriction Fragment Length Polymorphism
RFU	Relative Fluorescence Units
RIF	Rifampicin
RLU	Relative Light Unit
RNA	Ribonucleic Acid
RND	Resistance-Nodulation Division
ROS	Reactive Oxygen Species
RRDR	Rifampicin Resistance Determining Region
rRNA	Ribosomal ribonucleic acid
RT-qPCR	Real time quantitative PCR
Rv	Reverse
S	Susceptible
SD	Standard Deviation
Seq	Sequencing
SIRE	Streptomycin; Isoniazid; Rifampicin; Ethambutol

SMR	Small Multidrug Resistance
SNPs	Single Nucleotide Polymorphisms
SP	Spectinomycin
STR	Streptomycin
TB	Tuberculosis
TCH	Thiophene-2-Carboxylic Acid Hidrazide
TET	Tetracycline
tmRNA	transfer-messenger RNA
TNF	Tumour Necrosis Factor
TPP	Tetraphenylphosphonium
tRNA	transfer RNA
TST	Tuberculin Skin Test
TTD	Time To Detection
TZ	Thioridazine
VAN	Vancomycin
VIT	VNTR International Type
VNTR	Variable Number of Tandem Repeats
VP	Verapamil
WHO	World Health Organization
Wt	Wild type
XDR	eXtensivelly Drug Resistant
XDRTB	eXtensivelly Drug Resistant Tuberculosis

List of Units

ºC	Celsius degree
g	Gravitational force
IU	International Units
kHz	Kilohertz
L	Litre
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromole
mM	Millimole
mg	Milligram
ml	Millilitre
ng	Nanogram
nm	Nanometre
nM	Nanomole
pmol	Picomole
rpm	Revolutions per minute
U	Units

CHAPTER I

Preface to Chapter I.

Phthisis; scrofula; consumption; wasting; white plague; Pott's disease; king's evil; among others. Tuberculosis is one of the oldest and most studied diseases in the world. Known by several names, and being the bearer of a notorious reputation, tuberculosis remains one of the most feared diseases. The causative agent of tuberculosis, *Mycobacterium tuberculosis*, has claimed millions of human lives throughout its history. Today, despite the availability of an effective therapy, tuberculosis remains a global health problem. With the introduction of tuberculosis chemotherapy and implementation of the standardized treatment, it was believed that tuberculosis would decline. However, tuberculosis is still a leading cause of morbidity and mortality in developing countries. In 2012 were estimated that 8.6 million people developed tuberculosis and 1.3 million died from the disease. A further concern is the emergence and spread of multidrug resistant strains of *M. tuberculosis*. In 2012, there were an estimated 450 000 new cases of multidrug resistant tuberculosis worldwide, that resulted in 170 000 deaths. Due to the emergence of drug resistance, tuberculosis has been viewed with a renewed and intense interest. Further research is necessary to improve diagnostics, to develop new drugs and vaccines, implement simple and effective treatment programs, and understand the interaction between the pathogen and the host. This chapter reviews the history, microbiology, pathogenicity, ecology, molecular typing, evolution, epidemiology, laboratory diagnostics, treatment, drug resistance and new therapeutic strategies against *M. tuberculosis* and the disease it causes.

Chapter I. *Mycobacterium tuberculosis*, the etiological agent of tuberculosis

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Introduction

Tuberculosis is one of the most ancient infectious diseases. With records dating back from the Neolithic revolution (8000-6000 BC) (187), his etiologic agent, *Mycobacterium tuberculosis*, is responsible for one of the highest mortalities rates due to only one agent (503). Designated by Hippocrates as *phthisis*¸ consumption, name by which was known until the beginning of the 20th century, tuberculosis reaches pandemics proportions in Europe and North America by the end of the 18th century and first half of the 19th century, being responsible for the death of millions of people. During the 19th century and beginning of the 20th century, *M. tuberculosis* continued to cause several epidemics of tuberculosis. The improvement of sanitation and housing conditions and the implementation of antituberculosis therapy have led, in the mid-twentieth century, to a decrease in the incidence of tuberculosis in developing countries (104; 187). However, by the end of the 20th century, the incidence of tuberculosis raises again, being declared by the World Health Organization (WHO), in 1993, as a global emergence, recognizing by this manner its importance as a menace for public health (496). Nowadays, according to the WHO, approximately one third of the world's population is infected with *M. tuberculosis*. In 2012, were reported 8.6 million new tuberculosis cases and 1.3 million deaths (506). The re-emergence of tuberculosis is related not only with poverty, migration, overcrowding, and increasing of HIV incidence in some regions of the world, but also with the emergence of drug resistant strains, particularly multi- and extensively drug resistant *M. tuberculosis* strains (498). Tuberculosis remains a major public health problem and the emergence of multidrug resistant tuberculosis strongly contributes to this reality. Here, we summarize predominantly the history, epidemiology, biology, pathogenesis, clinical features, laboratory diagnosis, treatment, drug resistance and the new therapeutic strategies against *M. tuberculosis* and the disease it causes, tuberculosis.

A brief history

Tuberculosis has plagued humanity since prehistory and evidences came from both the Old and New Worlds. Characteristic skeletal abnormalities such as Pott's disease and changes in the hip and knee joints are the most frequent forms of tuberculosis found in archaeological remains (25). Spinal tuberculosis is frequently associated with bones deformities due to vertebral body collapse causing angular kyphosis (199). The recognition of such deformities in skeletons indicated that, probably, these people have been affected by tuberculosis. Nowadays, conclusive identification of tuberculosis infection can be achieved by using a combination of ancient DNA analysis and lipid biomarkers (287).

The support of the antiquity of tuberculosis comes from skeletal evidences, mainly from Europe and Africa. There are several cases described in the literature, from a variety of sites worldwide (for a more extensive review of tuberculosis in archaeological remains see ref. 421). The oldest suggestion of tuberculosis in human fossil bones goes back for 8000 BC (187). The definitive evidence of tuberculosis has been found in a skeleton, dated from 5800 ± 90 BC, recovered from the Neolithic cave of Arma dell' Aquila in Liguria, Italy (69). Also dated from the first half of the fourth millennium BC is reported a tuberculosis infection affecting the spine of a Neolithic skeleton discovered in the Arene Candide cave, also in Liguria (156). Further evidence of human tuberculosis dates from 5000 BC with the discovery of a skeleton of a young man showing destruction and fusion of the third and fourth thoracic vertebra in a Neolithic grave near Heidelberg, Germany (314). The earliest case of tuberculosis in Britain was identified during excavations at Tarrant Hinton, Dorset, England, and was dated from the Iron Age, 400-230 BC (273). One of the oldest evidences of typical spinal tuberculosis came from the Late Neolithic-Early Copper Age (fifth millennium BC) site of Alsónyék-Bátaszék, Hungary (219) and recently, Masson *et al*. (262) describes a case of hypertrophic pulmonary osteopathy in a 7000-year-old skeletal from a person who died from tuberculosis, at the Neolithic tell settlement of Hódmezővásárhely-Gorzsa, South of Hungary. This is one of the oldest tuberculosis cases identified to date. Plausible evidence of tuberculosis has also been identified in one adolescent male spine and another in the skeleton of a seven-year-old child dated from 3150-2150 BC in the

Early Bronze Age of Bab edh-Dhra', Jordan (313). Evidence for tuberculosis in skeletons, dated from 600 AD, was recovered from a mass grave in the monastery of John the Baptist near the Jordan River in the Judean desert (524). El-Naijar *et al*. (142) has also reported three potential tuberculosis cases dated from 8100-7600 BP from the Neolithic village 'Ain Ghazal, in the city of Amman, Jordan. In 1998, during the excavation of a Byzantine basilica in the Negev desert at Karkur, Israel, was discovered a calcified fragment of pleura, dating from approximately 600 AD. This was the first demonstration of tuberculosis in non-mummified archaeological tissue other than bone (132). There is no evidence of skeletal remains from sub-Saharan Africa and the majority of archaeological evidences of tuberculosis came from Egypt and Sudan (421). The most convincing case of tuberculosis in Egypt occurred with the discovery of the mummy of the Amun´s priest Nespherehan from the 21st dynasty, in Thebes, that not only exhibits Pott's disease but also a psoas abscess. After this, several other Egyptian mummies have been found that possess evidences of skeletal tuberculosis some of them dating from 4500 BC (526).

In Portugal, the earliest cases of tuberculosis date from the medieval period. The first evidence of tuberculosis was found in a skeleton, dated from 12th/14th centuries, exhumed from S. João de Almedina Church, Coimbra (101; 377). Another two cases found in medieval individuals were described, one from Granja de Serrões, Sintra (376; 377) and the second from Corroios (244; 377). Tuberculosis was the main cause of death at the beginning of the 20th century and several studies indicated that new bone formation on the visceral surfaces of the ribs can be used for the diagnosis of pulmonary tuberculosis in human remains (378). Portugal has rich skeletal collections and the analysis of the skeletons dated from the first half of the 20th century of the collection curated in the Anthropological Museum at the University of Coimbra, by Santos and Roberts (377) allowed the discovery of eighteen skeletons from juvenile individuals who died from tuberculosis affecting different parts of the body. Assis *et al*. (23) when analysed the Coimbra Identified Skeletal Collection described a strong association between hypertrophic pulmonary osteopathy and pulmonary tuberculosis in the skeleton remains. Matos and Santos (266) conducted a research on the Human Identified Skeletal Collection from the Museu Bocage (Lisbon) which represents a Portuguese population from the late 19th-early 20th centuries, and described a strong association between

pulmonary tuberculosis and rib lesions in 76 individuals with tuberculosis as probable cause of death. Recently, Matos *et al*. (265) describes the discovery of a skeleton from a 12-year-old individual exhumed at the necropolis ($13^{th}/14^{th}$-19^{th} centuries) adjacent to the church of São Miguel, Castelo Branco. This case can be the earliest skeletal evidence of tuberculosis in Portugal.

Pulmonary tuberculosis was described in the ancient Greek medical literature, by Hippocrates, as phthisis, which is derived from the Greek "wasting away". Galen provides a remarkable description of the disease due to the extreme emaciation and ulcerated state of the lungs (323). Soranus of Ephesus described the symptoms of phthisis as follows: latent fever, coughing blood, sanious sputa, coarse breathing, emaciation, flushed cheeks and loss of appetite (206). Over time, the disease has been known by several different names, namely *schachepheth* (ancient Hebrew), yaksma, consumption, scrofula, Pott's disease, white plague, king's evil, and so on. Scrofula, a rare manifestation of tuberculosis that affects the lymph nodes, was well documented during the middle age in Europe, when it was believed that the cure resulted from the power of the divine touch of the kings (31). Pott´s disease was characterized by typical skeletal abnormalities, as mentioned before (63). All these several clinical manifestations were assumed to be forms of the same disease as consequence of the studies of Franciscus Sylvius where he described the characteristic lung nodules as "tubercula" (small knots), observing their evolution to cavities (lung ulcers) (55). The first credible hypothesis on the infectious nature of tuberculosis was made by Benjamin Marten in 1722, who proposed that tuberculosis could be transmitted through the breath of a sick person (130). In 1694, the English Doctor Richard Morton used the term consumption to designate tuberculosis (55), and finally, in 1819, René Laennec, the discoverer of the stethoscope, described for the first time the pathogenesis of tuberculosis (45). In 1838, J.L. Schönlein named the disease as tuberculosis due to the presence of tubercles in all manifestations of the disease (187). In 1865, Jean-Antoine Villemin demonstrated formally that tuberculosis is a contagious disease when he successfully transferred pus and fluid from human and bovine lesions to rabbits that then developed tuberculosis (465). In 1882, the German Physician Robert Koch demonstrated for the first time the etiological agent of tuberculosis from crushed tubercles (217) and shown that the organism met the criteria that he described, known as

Koch's Postulates: "To prove that tuberculosis . . , is caused by the invasion of bacilli and conditioned by the growth and multiplication of bacilli it was necessary: (i) to isolate the bacilli from the body; (ii) grow them in pure culture; and (iii) by administering the isolated bacilli to animals, reproduce the same morbid condition." (47). Robert Koch was awarded with the Noble Prize in Medicine or Physiology in 1905 for his discovery. In August of 1890, during The First Ordinary Session of the International Medical Congress, in Berlin, he announced the discovery of an antituberculosis drug, which was called as tuberculin (218). Tuberculin turned to be useless and is nowadays used as a diagnosis tool.

During the 18th century, tuberculosis was known as the White Plague opposite to the Black Death that decimate about one third of the population in Europe from 1346 to the 1800s. At this time the disease became the principal cause of death which leads to its romanticization. Tuberculosis arises with more frequency among young adults between the ages of 18 and 35. In the literature it was named as the "Captain of Death," and is presented as a romantic ideal way to die (140). Tuberculosis was a disease not just of the body, but also of mind and spirit. During the Romantic period, consumption was thought to be the disease of poets and talented people and associated with an aesthetic image, a kind of beauty and melancholy. The pale, slender figure and rosy cheeks due to the disease were highly desirable features among women (140). Prominent people who died of tuberculosis included the English poet John Keats, the Polish composer Frédéric Chopin, all the Brontë sisters (Charlotte, Emily, and Anne), the Russian dramaturge Anton Chekhov, the Italian painter Amedeo Modigliani, and the German writer Franz Kafka, among others (140). As the knowledge about the disease increases and with the discovery of its etiological agent, the social perception of tuberculosis changed from a poetic and aesthetic image to the reality of an infectious disease. In addition, during the 18th-19th centuries tuberculosis was often associated with vampires. The New England folk belief in vampires is based on the capacity of a deceased tuberculosis victim to return from the dead as a vampire and cause the "wasting away" of the surviving relatives (406). The treatment required exhuming the bodies of deceased relatives and checking them for "unnatural" signs, such as "fresh" blood in the heart. Afterwards, the bodies were dismantled in several ways (38; 406).

Despite the development in diagnosis and medicine it was not possible to find an effective cure for tuberculosis until the discovery of streptomycin in 1944. The most popular treatment for tuberculosis in the epoch was based on "open-air treatment". People were often advised to move to warm and humid climates as mountains close to the sea, to get fresh air, sunlight, and do some exercise. With the belief that tuberculosis could be cured by a change of climate quality, in 1859, Hermann Brehmer opened the first tuberculosis sanatorium in Gobersdorf, Germany (477). After this, several other sanatoriums were followed. By the time of Brehmer's death in 1899, there were more than 300 sanatoriums in Germany alone (112). In 1860, Albert Robin postulates that the treatment of tuberculosis had to be not only based on hygienic measures but also requires drug therapy and a proper diet (360). Sanatoriums were able to improve the health of tuberculosis patients and render them less contagious to the community. The contagious nature of the disease makes tuberculosis a matter of public health concern. The Decree of Lucca promulgates in Italy in 1699, for the first time, the notification of the names of patients with phthisis and burning of their belongings after death (47). The advent of antibiotic therapy resulted in a gradual sanatorium close down as the belief among the health authorities was that the fight against tuberculosis was over. Tuberculosis is by no means a disease of the past. Currently it represents a global threat, as it stands as the leading cause of death due to an infectious bacterial agent worldwide.

Tuberculosis was amongst the diseases that most afflicted the Portuguese population in the late 19th century and first half of the 20th century. Tuberculosis originated thousands of victims annually, due to the lack of medical knowledge and impotence of the health authorities. These numbers were aggravated due to unhygienic general practices and overcrowding and industrialization (462). At that epoch, tuberculosis was considered a social disease. According to António de Almeida Garrett, tuberculosis was a disease of the poor (159). The great majority of the diseased people lived with poor social-economic conditions, a factor that contributes to the dissemination of the disease. However, tuberculosis was not restricted to the anonymous and poor population that lived in urban areas, but also extended its arms to prominent people as the King D. Pedro IV and his daughter D. Amélia, the writer Julio Dinis, the poets António Nobre and Cesário Verde, among others (258). By the end of the 19th century the fight against the disease was essentially based on sanitary hygiene measures and included the

construction of dispensaries, preventoriums and sanatoriums (379). In 1853, was funded in Madeira (Funchal) the first Portuguese sanatorium for the treatment of tuberculous patients, by the Empress of Brazil Amélia de Beauharnais, widow of D. Pedro IV, in memory his daughter, the Princess D. Maria Amélia, who died victim of tuberculosis (379; 462). The sanatorium received the name Princess D. Maria Amélia and in 1862 received the first's patients. Around 1880, Dr. Sousa Martins conducted two expeditions to Serra da Estrela to study the climatic conditions on the mountains in order to begin the construction of a sanatorium (462). In 1881, was opened the sanatorium Hospital Príncipe da Beira, Guarda (379). The identification of *M. tuberculosis*, as the causal agent of tuberculosis, totally changed the social concept of the disease. Tuberculosis was no longer seen as a hereditary disease, but as a contagious infectious disease that could be preventable. Based on the preventable condition of the disease it was established prophylactic actions against tuberculosis. In 1895 was held in Coimbra the first National Congress of tuberculosis (462). Following this, other initiatives were created. This included the creation of isolation rooms for the afflicted in the Hospital de Santo António and the Hospital da Marinha in 1896 (462). Regarding charity, the Duchess of Palmela and her assistants dedicated themselves to serve meals to the poorer (379; 462). Several physicians rose against the social problem of tuberculosis with the desire to disseminate concepts of hygiene among the population. In 1897, the Society of Medical Sciences of Lisbon in collaboration with Dr. Miguel Bombarda initiates a campaign against tuberculosis, which led to the creation, in 1899, of the National League against tuberculosis (379; 462). The National League against tuberculosis was constituted by several commissions whose actions gradually took the form of campaigns conducted through national congresses on tuberculosis (1901, 1902, 1904 and 1907) (462). In 1899 was born the National Assistance to tuberculosis patients, a private Foundation directed by the Queen D. Amélia (379, 462). The foundation became a public institution in 1946 (19). The fight against tuberculosis enjoyed political, social, and public acceptance and generous funding, with all services, being provided free of charge; the epidemiological surveillance began in 1951; the prevention program was formally integrated into the primary health care system in 1984; and annual reports have been published since 1988 (19). However, tuberculosis remains a public health problem in Portugal despite the many efforts made to its control.

Mycobacteria and the tubercle bacillus

Mycobacteria are acid-fast bacilli belonging to the genus *Mycobacterium*, family *Mycobacteriaceae*, suborder *Corynebacteriaceae*, and order *Actinomycetales* (144). The name *Mycobacterium* results from the junction of *myces* (fungus) and *bakterion* (small rods) and refers to the fungus-like pellicles that are formed when mycobacteria are grown on liquid media (Figure I.1-I). Presently, the genus *Mycobacterium* comprises 169 species and 13 sub-species (144). It contains the obligate pathogens responsible for tuberculosis, leprosy and Buruli ulcer, whose etiologic agents are *M. tuberculosis*, *Mycobacterium leprae* and *Mycobacterium ulcerans*, respectively. However, the great majority of mycobacterial species are environmental, as they inhabit mainly in grass, soil, marshes, rivers and piped water systems (145). Environmental mycobacteria can cause opportunist diseases in those individuals whose immune systems are debilitated. Until 1950, all acid-fast bacilli other than the tuberculous bacilli were named just as atypical tubercle bacilli (141; 166). The classification of mycobacteria was established in 1959 when Ernest Runyon assigned them to four groups according to their capacity to produce pigment and growth rate on solid media (367). The first three groups include the slow-growing species (colonies in more than seven days) that can be distinguished among them by the production of pigment: Group I: photochromogens (pigment production in the presence of light); II: scotochromogens (constitutive pigment production); and III: nonchromogens. Group IV includes the fast-growing species (colonies in less than seven days). Attending to the optimal growth temperatures, they range from 20ºC to 52ºC depending on the species (446). The morphology of the colonies varies among the species, from rough to smooth. Depending on the conditions of growth and age of cultures, cells may vary in size and morphology, from small cocobacilli to slightly curved rods, which may be dispersed or presented in small clumps. Mycobacteria are aerobic, non-motile, non-sporing, and non-flagellate, with 2-5 μm in length and 0.2-0.5 μm in width. The presence of lipid storage granules may give a distinct banded or beaded appearance to the cells (165). Mycobacteria are called acid-fast bacilli. They do not strain or stain poorly by the Gram staining, and for that they are called Gram-ghosts (448). Due to their almost unique acid-fast property, they can be visualized by using an acid-fast staining technique. The original acid-fast staining was described by Paul Ehrlich in 1882 and later modified by Franz Ziehl and Friedrich

Neelsen (44). The Ziehl-Neelsen staining is the most widely used in clinical mycobacteriology.

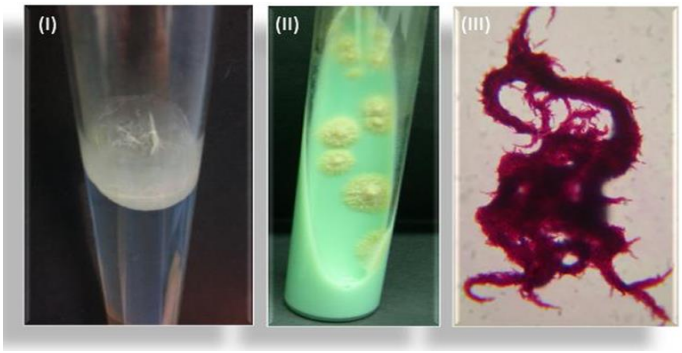

Figure I.1. Macroscopic and microscopic observation of *Mycobacterium tuberculosis*. (I) Formation of a fungus-like pellicle at the surface of MB7H9; (II) development of rough colonies on solid media; and (III) Ziehl-Neelsen staining showing cording formation (DMachado, Grupo de Micobactérias, IHMT).

The tubercle bacillus

'Tuberkelbazillus' (tubercle bacillus) was the name by which *M. tuberculosis* was known at the time of its discovery (263). *M. tuberculosis* was identified in 24 March 1882 by Robert Koch which not only isolated the bacteria but through its postulates established it as the definitive cause of tuberculosis (47; 217). *M. tuberculosis* complex is composed by closely related species that share 99.9% genomic similarity and identical 16S rRNA sequences (50). Nevertheless, species from *M. tuberculosis* complex differ among them with regards to their morphological and biochemical characteristics, host range, pathogenicity and epidemiological behavior. Nowadays, *M. tuberculosis* complex includes nine species: *M. tuberculosis, Mycobacterium bovis, Mycobacterium africanum, Mycobacterium caprae, Mycobacterium microti, Mycobacterium pinnipedii, Mycobacterium orygis, Mycobacterium mungi,* and *Mycobacterium canettii* (10; 20; 94; 457; 458). *M. tuberculosis* strains produce rough colonies on solid media that look like breadcrumbs or cauliflowers (Figure I.1-II). *M. cannettii* is an exception since it forms smooth colonies and is considered an ancestral of *M. tuberculosis* (430; 458). *M. bovis* and *M. caprae* possess a wide host range, from man to domestic animals. *M. africanum* is an important pathogen that is isolated from individuals from equatorial Africa. This species is placed between *M. tuberculosis* and

M. bovis in regard to the geographical area and biochemical characteristics. *M. africanum* type I is found mainly in West Africa, and presents characteristics similar to *M. bovis*; *M. africanum* type II, is found mainly in East Africa, and presents similarities resembling *M. tuberculosis* (107; 305). *M. microti* was first isolated from the vole *Microtus agrestis* (485), and *M. pinnipedii* was first isolated from seals and sea lions in Australia, New Zealand and South America (94), and in an Australian seal trainer (213). *M. orygis*, the oryx bacilli, have been isolated from members of the *Bovidae* family, *i.e*., oryxes, gazelles, deer, antelope, and waterbucks (457). The mongoose bacillus or *M. mungus* was isolated from banded mongooses (*Mungos mungo*) in Botswana (10).

The mycobacterial cell wall

The most distinct characteristic of mycobacteria is their complex and lipid-rich cell wall. The mycobacterial cell wall consists of an inner membrane and an outer membrane that surround the plasma membrane (57). The outer membrane consists of lipids, proteins and polysaccharides. The lipids are often freely associated with the cell wall, with some long- and short-chain fatty acids complementing the short and long chains found in the inner layer. The lipid-linked polysaccharides associated with the outer membrane consist of lipoarabinomannan, lipomannan, phthiocerol-containing lipids such as phthiocerol dimycocerosate, dimycolyl trehalose, sulfolipids, and the phosphatidylinositol mannosides (PIMs) (56). Lipoarabinomannan (LAM) is a branched polysaccharide of the mycobacterial cell wall. In slow-growing, pathogenic mycobacteria, such as *M. tuberculosis* and *M. leprae*, lipoarabinomannan are coated at the terminal β-Ara (arabinose) residue with mannose residues designated as ManLAMs (73; 74). The inner membrane is composed of three covalently attached macromolecules, the peptidoglycan, arabinogalactan, and mycolic acids (57) and known as the peptidoglycan-arabinogalactan-mycolic acids complex. Peptidoglycan, or murein, is composed by peptides and glycan strands. The glycan strand consists of alternating units of N-acetylglucosamines linked to modified N-acetylmuramic acid (96). Next to the peptidoglycan is the arabinogalactan, a branched polysaccharide consisting of arabinan and galactan (275). Arabinogalactan is the major polysaccharide of the mycobacterial cell wall. This polymer is unique as its sugars, arabinan and galactan, are

in the furanose configuration (275). The galactose residues are arranged in alternating 1→5, 1→6 linkages of approximately 30 residues, with the arabinan attached to the position 5 of the galactan in 1→5 linkages (102). Arabinogalactan is covalently attached to the peptidoglycan by a phosphoryl-N-acetylglucosaminosyl-rhamnosyl linkage (276). Next to the arabinogalactan is one of the principal components of the mycobacterial cell wall – the mycolic acids, which are long-chained α-alquil branched -β-hidroxilated fatty acids that contain up to 90 carbon atoms (56). The aliphatic chains of the mycolic acids contain methyl branches, methoxymycolates, unsaturated bonds and cyclopropane rings (33). External to mycolic acid is a layer composed by trehalose dimycolates and sulpholipids (75). Trehalose dimycolates, or cord factor, are thought to be responsible for the characteristic serpentine cording arrangement of *M. tuberculosis* (Figure I.1-III). On the surface of the mycobacterial cell wall can also be found complex molecules as the mycosides. The majority of mycosides are peptidoglycolipids, consisting of peptides, lipids (mycoserosic acids) and carbohydrates (56). The mycobacterial cell wall differs from that of most other bacteria and forms a diffusion barrier which is 100- to 1000-fold less permeable to hydrophilic molecules. The permeability properties are facilitated by channel-forming proteins also designated as porins (Figure I.2).

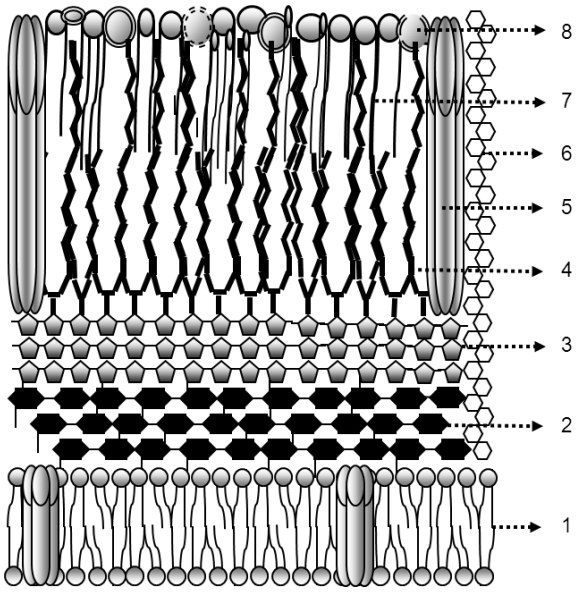

Figure I.2. Simplified schematic representation of the mycobacterial cell wall . 1, Cellular lipidic bilayer with internal porins embebed; 2, peptidoglycan layer; 3, arabinogalactan layer; 4, mycolic acid layer; 5, external porins; 6, lipoarabinomannan (LAM); 7, external lipids and glycolipids embebed in the external face of the mycolic acid layer (trehalose dimycolates and sulpholipids); 8, external layer of mycosides (peptidoglycolipids) (adapted from 57).

Recently, the basic structural model of the mycobacterial cell wall described above was revised and updated according to the ultrastructure findings where a structure more close to the Gram-negative bacteria was described including a periplasmic space with an outer and inner membrane (303; 527). Both the classical and modern models converge in its high complexity and diversity of molecules that contributes to the mycobacterial impermeability to the outer environment and drug resistance (56; 303).

The genome of *M. tuberculosis*

M. tuberculosis H37Rv was isolated in 1905 and classified as type-strain by Kubica *et al*. (226). It has remained pathogenic and because of this is the most widely used strain in tuberculosis research. The complete genome sequence of *M. tuberculosis* H37Rv was obtained in 1998 (89) and re-annotated in 2002 (68). *M. tuberculosis* genome comprises 4 411 532 bp, with a G+C content of 65.9% (68; 89; 242). This includes 4018 genes encoding proteins (more 94 than the original sequence), 13 pseudogenes, 45 tRNA genes, three rRNA genes, 30 ncRNA genes and two miscRNA genes (242). The functional classification of *M. tuberculosis* genes is shown in Table I.1.

Table I.1. Functional classification of *M. tuberculosis* genes (**242***).

Class	Function	Number of genes
0	Virulence, detoxification and adaptation	239
1	Lipid metabolism	272
2	Information pathways	242
3	Cell wall and cell processes	772
4	Stable RNAs	80
5	Insertion sequences and phages	147
6	PE and PPE proteins	168
7	Intermediary metabolism and respiration	936
8	Proteins of unknown function	15
9	Regulatory proteins	198
10	Conserved hypothetical proteins	1042

*Last access May 2014.

The number of sequencing projects and related databases has increased in the last years due to the huge advances in DNA sequencing technology. The access to such data is of

benefit for the field of mycobacteriology since it can increase our knowledge regarding the functions of several gene products. This improvement is reflected on the reduced number of proteins with unknown function on *M. tuberculosis* genome at the present. It is expected that this number continues to decrease in the coming years with the results from the above mentioned projects.

M. tuberculosis 'devolve' to evolve

Progressive genome downsizing is a hallmark of mycobacterial evolution (Figure I.3). When compared with nontuberculous mycobacteria it is observed that *M. tuberculosis* complex has been evolved by reductive evolution. The genome of the non-pathogenic *M. smegmatis* has 7 Mb in size; the genomes of the pathogenic *M. tuberculosis* and *M. leprae* are 4.41 Mb and 3.3 Mb in size, respectively. This suggests that pathogenic mycobacteria have evolving towards pathogenicity by the loss of its genetic material. The adaptation to a pathogenic life style and permanent association with a host has led to the loss of genes indicating that a minimal gene set is needed for a successful obligate intracellular parasitism (297; 384).

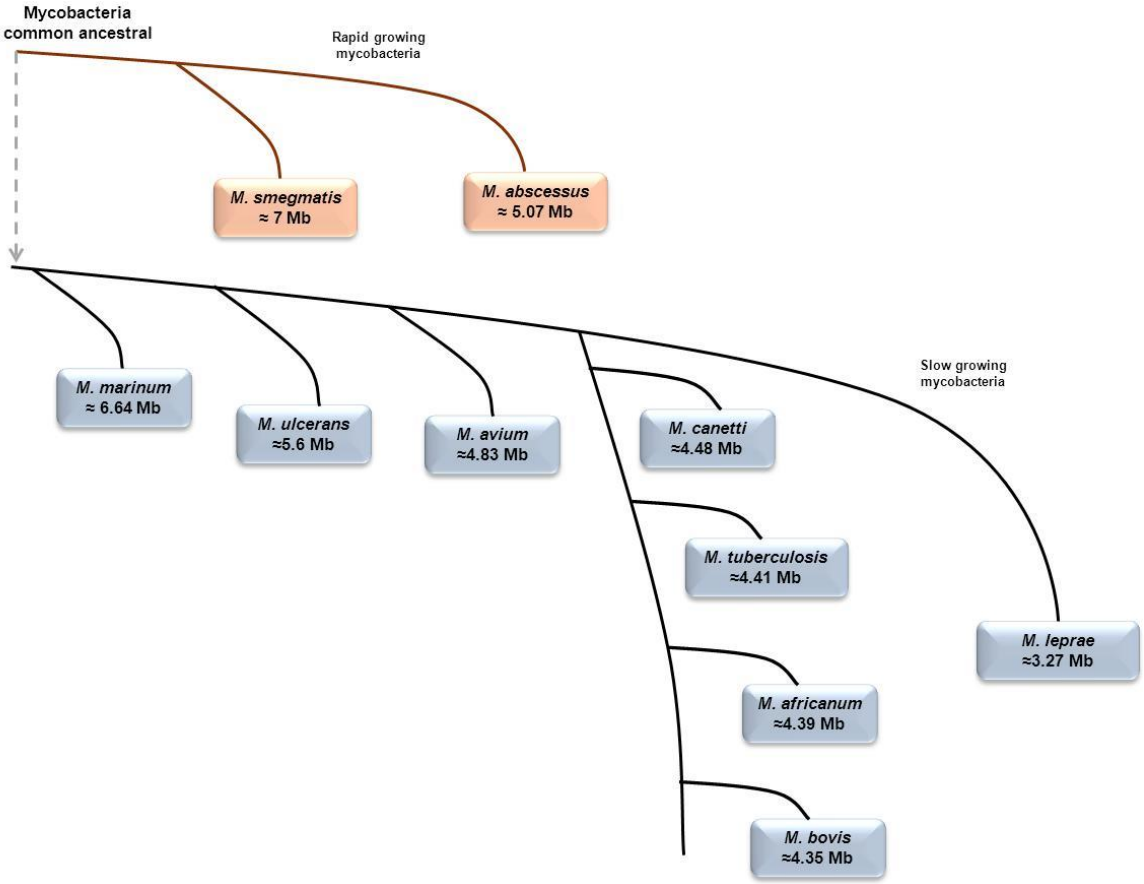

Figure I.3. The devolution of mycobacteria. Diagram shows the reduction of genome size among some environmental non-pathogenic and pathogenic mycobacteria, and obligate pathogens. In the Figure were not considered the time and genetic events preceding speciation. Based on 461a. Genome sizes were obtained from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/, last access May, 2014).

Due to the high degree of conservation among *M. tuberculosis* complex species it has been suggested that they underwent an evolutionary bottleneck at the time of speciation (59). The second suggestion state that the ancestral form of *M. tuberculosis* was *M. bovis* (418) and that the evolution of *M. tuberculosis* from *M. bovis* results in a more restricted host range, from an animal to a human pathogen. However, both hypotheses came before the revolution brought by the characterization of the *M. tuberculosis* genome and also before the comparative genomics uncovered several variable genomic regions in the members of the *M. tuberculosis* complex (59). The differentiation between the various species belonging to the *M. tuberculosis* complex can be made by the analysis of the presence or absence of 20 regions of difference on their genome (Figure I.4). *M. tuberculosis* complex RDs comprise the RD1 to RD14, ranging from two to 12.7 kb (37; 163; 253), six regions related to the H37Rv strain designated as

RvD1 to RvD5 and a *M. tuberculosis* specific deletion, TbD1, that is absent from the strain H37Rv. *M. canettii* possess all RDs, RvDs and TbD1; additionally it have a specific deletion that partially overlaps RD12, RD^{can} (59). *M. africanum* strains possess the six regions absent in H37Rv, but lack RD9 (*M. africanum* type I) and RD9 plus RD7, RD8 and RD10 (*M. africanum* type II); *M. microti* lacks RD7, RD8, RD9 and RD10 and possess a specific deletion, $RD1^{mic}$, that partially overlaps RD1. *M. mungi* do not possess the RD7, RD8, RD9 and RD10 and have a specific deletion, RD^{mun}. *M. orygis*, unlike *M. microti*, the dassie bacillus, and *M. mungi*, has an intact RD1 and a specific deletion of RD12 (457). *M. pinipedii* like *M. orygis*, *M. microti*, the dassie bacillus, and *M. mungi*, do not have the RD7, RD8, RD9 and RD10 but shows a specific deletion, RD^{seal}. *M. caprae* shows the deletion of RD7, RD8, RD9, RD10, RD12 and RD13, and *M. bovis* additionally presents the deletion of RD4. *M. bovis* BCG strains display a large number of deletions, from RD1 to RD14. The dassie bacillus is a rare variant that was not yet officially recognized as a different species.

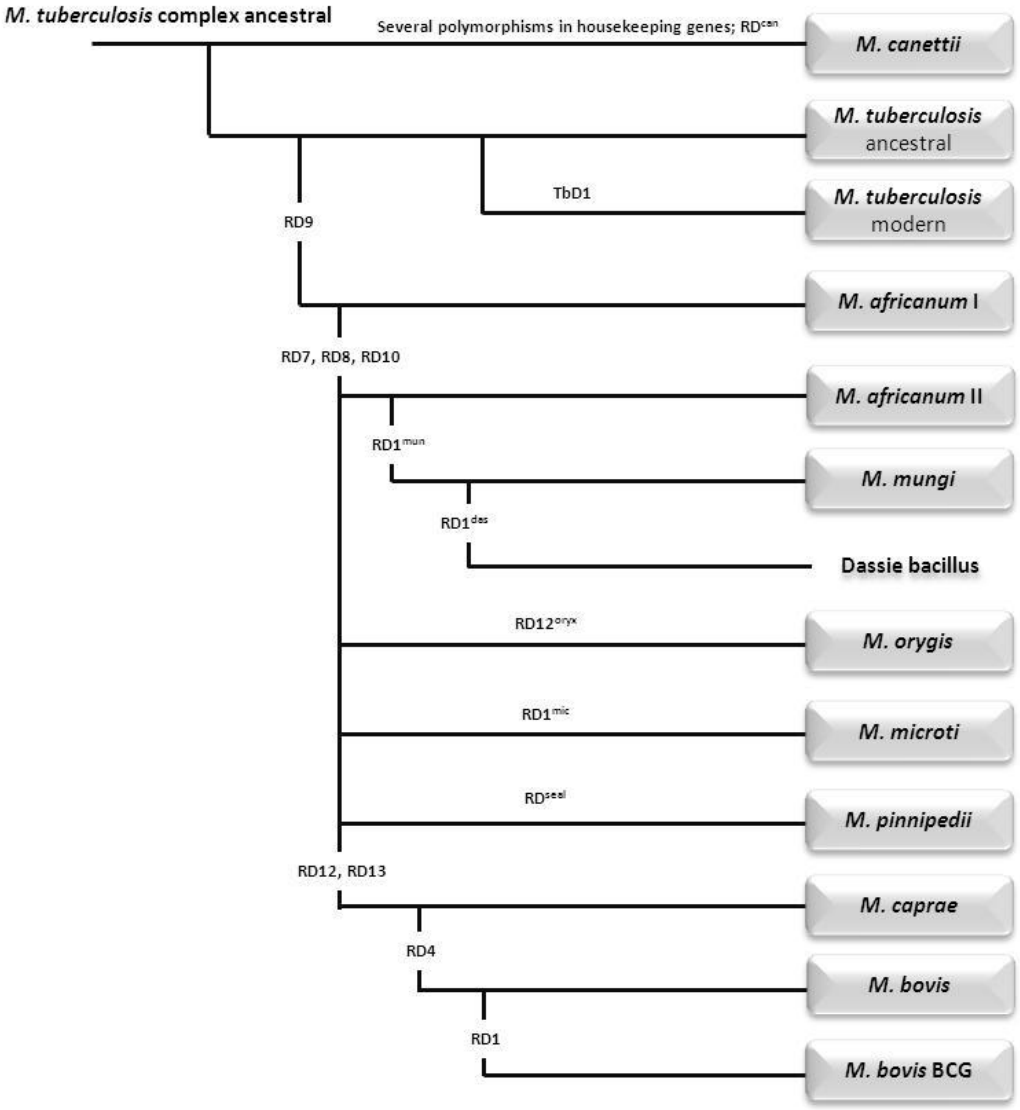

Figure I.4. RD deletion based phylogeny of the *M. tuberculosis* complex. Simplified evolutionary pathway of *M. tuberculosis* complex based on the works of Brosch *et al*. (59) and van Ingen *et al*. (457).

Molecular typing

In the last decades, several genotyping methods have been developed for the differentiation of *M. tuberculosis* strains. The majority of the methods used for molecular typing target repetitive units in the mycobacterial genome, either interspersed repeats (direct repeats or insertion sequence) or tandem repeat (264). The most widely used molecular typing method for *M. tuberculosis* complex isolates is the IS*6110* restriction fragment length polymorphism (IS*6110*-RFLP). The IS*6110*-RFLP typing is a standardized method based on the detection of the IS*6110* insertion sequence, an

insertion element with 1361 bp that is randomly dispersed in the *M. tuberculosis* genome (443; 455). The IS*6110* insertion sequence was first described by Thierry *et al*. (444) and until recently was thought to be exclusive to the *M. tuberculosis* complex. The IS*6110* was found in the genome of *M. smegmatis* and named ISMs*6110* (91). The difference between the ISMt*6110* and ISMs*6110* is that the later lacks one inverted repeat. These transposable elements have been shown to be involved in gene disruption (374). The IS*6110* insertion sequence varies in number (0 to ≈25) and position within the genome generating a high level of DNA polymorphism among strains (459). It is also known that the insertion of IS*6110* can alter bacterial gene expression. Depending on the location, the IS*6110* insertion sequence may upregulate the expression of downstream genes by acting as a promoter (13; 35). This activity has been demonstrated for upregulation of the two-component regulatory system PhoP/PhoR (410). The IS*6110*-RFLP has been used to infer genetic relationships between *M. tuberculosis* strains with high (≥6) IS*6110* copy number. It has low sensitivity for the epidemiological study of strains with low (<6) copy number (97). The IS*6110*-RFLP analysis requires (i) restriction endonucleases (PvuII) to cleave the genomic DNA, originating DNA restriction fragments of different sizes that can be visualized by gel electrophoresis, (ii) hybridization with a labelled DNA probe complementary to the IS*6110* sequence, and (iv) detection of the labelled probes by autoradiography (459). The resulting banding pattern is characteristic of a *M. tuberculosis* strain. Despite its widespread use, this method has several limitations: (i) it can only be applied to cultures since requires large amounts of DNA; (ii) is time consuming and labour intensive; (iii) it cannot accurately differentiate low copy number strains; (iv) is expensive; (v) and does not allow to perform inter-laboratory comparisons with confidence due to the complexity on the interpretation of the results obtained. Nevertheless, this methodology was the basis for the current knowledge about the transmission dynamics of *M. tuberculosis* strains in different regions of the world. Besides, it has the capacity to evaluate (i) exogenous reinfection and mixed infections; (ii) the impact of drug resistance on transmission and pathogenesis; (iii) the geographical distribution and global dissemination of *M. tuberculosis* strains; detect outbreaks and laboratory contamination; and track contacts, among others. To bypass the several limitations associated with IS*6110*-RFLP molecular typing, other techniques were developed,

namely spoligotyping (204) and mycobacterial interspersed repetitive units-variable number tandem-repeats (MIRU-VNTR) typing (427).

Spoligotyping (for spacer oligotyping) is based on the polymorphisms of the chromosomal direct repeat (DR) locus of the *M. tuberculosis* complex genome, that contains a variable number of copies of direct repeats of 36 bp interspersed with unique nonrepetitive sequences (34 to 41 bp) (204), which together are termed direct variable repeat (DVR) sequences. Isolates of *M. tuberculosis* complex generally differ in the presence or absence of one or more spacers and adjacent DRs (456). The DR region of *M. tuberculosis* is also a hotspot region for the integration of the IS*6110* (146; 204; 478). The presence or absence of the DRVs is evaluated through polymerase chain amplification (PCR) of the DVRs with the standard set of primers (DRb and DRa-5′-biotinylated) and subsequent hybridization in a membrane containing 43 different DVR sequences. Following the hybridization, the membrane is incubated with streptavidin-peroxidase and the hybridization signals visualized by chemiluminescence (164). This technique is faster and easier to perform than the IS*6110*-RFLP typing and requires smaller amounts of DNA. Spoligotyping presents high sensitivity to detect and discriminate among isolates with low IS*6110* copy number (164). However, the observation that the DRVs may undergo convergent evolution, leading to epidemiologically unrelated strains sharing identical spoligotype patterns (479) is the main limitation of this molecular typing method. Nevertheless, spoligotyping can be useful to study population structure of *M. tuberculosis* strains in several geographical areas, since the evolution of the DRs created unique spoligotype signatures. The strains that evolved from a common ancestral can be grouped into evolutionary lineages according to unique spoligotype signatures (423). The simplicity of this method has allowed the establishment of an international spoligotype database, originally named as SpolDB (version 1 to 4) (61) and now updated to SITVITWEB, which describes 62 582 clinical isolates from 153 countries (119).

Mycobacterial interspersed repetitive units (MIRU) typing has become the most important method for *M. tuberculosis* complex typing, as it allows high-throughput, discriminatory and reproducible analysis of *M. tuberculosis* clinical isolates. MIRU is a *M. tuberculosis* complex specific name (428; 429), for a multiple locus VNTR (variable number of tandem repeats) analysis (MLVA). Variable number tandem repeats, or

VNTRs, corresponds to a location in a genome where a short nucleotide sequence is organized as a tandem repeat (clustered together and oriented in the same direction) (472). VNTR typing relies on multiplex PCR amplification using specific primers for the flanking regions of the VNTRs. The determination of the sizes of the amplicons is done by electrophoresis. As the length of the repeat units is known, the number of VNTR copies is given by the sizes of the bands obtained (204). The final result is a numerical code, corresponding to the repeat number in each VNTR locus (Figure I.5). VNTRs sequences comprises exact tandem repeats (ETR) (157), MIRUs and QUB (Queen's University Belfast)-VNTRs (364; 405). MIRUs are minisatellite-like structures with 40±100 bp found as tandem repeats and scattered in 41 intergenic regions of the *M. tuberculosis* genome (429). A collection of 186 strains representing the major *M. tuberculosis* lineages was used for implementing a web server, MIRU-VNTR*plus* (http://www.miru-vntrplus.org/).

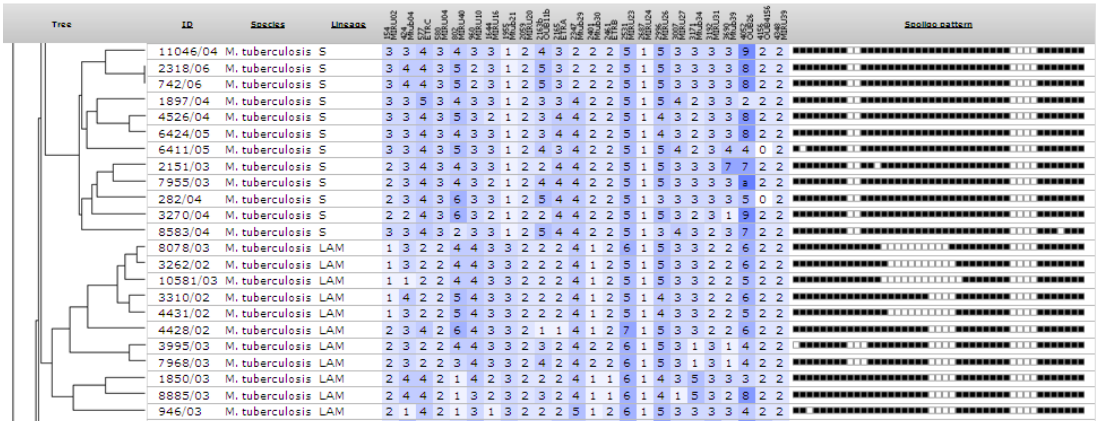

Figure I.5. MIRU-VNTR*plus* data table. For each strain is shown the identification, the species and lineage followed by the MIRU-VNTR pattern, in blue, and the spoligotyping pattern. An UPGMA tree is used for cluster the strains (MIRU-VNTR*plus* web page at http://www.miru-vntrplus.org/, last access May 2014).

The MIRU-VNTR application allows the input of copy numbers for 24 MIRU-VNTR loci, a highly discriminatory subset of 15 loci or the original 12 loci (427; 429), spoligotyping patterns, regions of difference profiles, single nucleotide polymorphisms (SNPs), susceptibility data, and IS*6110* RFLP fingerprint images (12; 427; 487). As a basis for standardized MIRU-VNTR typing of *M. tuberculosis*, the 15 MIRU-VNTR is proposed to be applied to epidemiological studies and the 24 loci for phylogenetic studies (427). Compared with the IS*6110*-RFLP typing, MIRU-VNTR typing possess

several advantages such as rapidness, it's easier to perform, cheap, appropriate for all *M. tuberculosis* isolates, even for IS*6110* low-copy strains and allows easy and rapid inter-laboratory comparison of the results. Several studies show that MIRU-VNTR-based typing possess a discriminatory power similar to that of IS*6110*-RFLP typing which could be further enhanced when combined with spoligotyping (139; 407; 425).

M. tuberculosis complex molecular epidemiology and lineages

The development of molecular techniques for genomic analysis, such as those referred in the previous section, has revealed that species belonging to *M. tuberculosis* complex can be divided into lineages, sub-lineages, clusters or families (92). However, the markers upon which these techniques are based change quite rapidly, making it hard to describe deep phylogenetic relationships (158). The large sequence polymorphisms (LSPs) represent unique event polymorphisms and this analysis can be used to establish *M. tuberculosis* phylogenies (192). Gagneux *et al.* (158) used the LSPs and the regions of difference TbD1, RD7, RD8, RD9, RD10 and RD^{can} plus the 7-bp deletion in the *pks15/1* locus to describe the six main lineages of *M. tuberculosis* complex (Figure I.6) which are associated with particular populations. Lineage 1 corresponds to the Indo Oceanic; lineage 2, East Asia; lineage 3, East African Indian; lineage 4 comprises Euro-American, West Africa, South Africa, and Central Africa; lineage 5, West African-1; and lineage 6, West African-2. With the application of spoligotyping, a total of six lineages and several sub-lineages within the *M. tuberculosis* complex have been defined (119; 151). These lineages correspond to the previously ones identified in the work by Gagneux *et al*. (158) (Figure I.6). Spoligotyping based lineage East African-Indian and Manu corresponds to the previous lineage 1; Beijing lineage, lineage 2; Central Asian (CAS), lineage 3; X, Haarlem, LAM (Latin American Mediterranean) and Uganda, lineage 4; AFRI1 (africanum type 1), lineage 5; AFRI2 (africanum type 2), and BOV, *M. bovis* lineage (151). Demay *et al*. (119) defined the spoligotyping based lineage PINI, which corresponds to *M. pinnipedii* strains. Recently was identified the *M. tuberculosis* lineage 7 which seems to be, so far, associated strictly with the Horn of Africa (154). Other studies have used single nucleotide polymorphisms (SNPs) for the same purpose (27; 172).

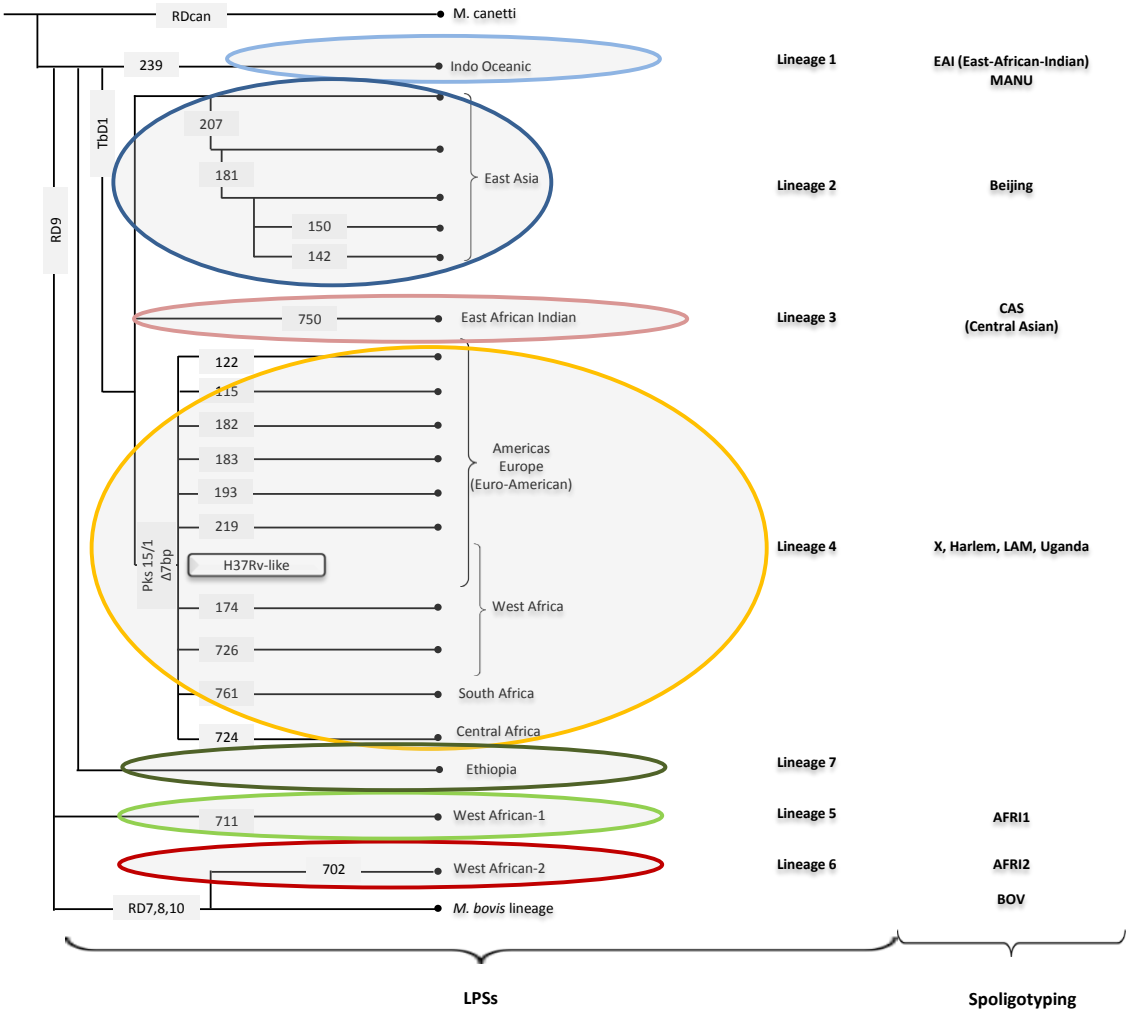

Figure I.6. *M. tuberculosis* phylogenies and the geographic regions associated. At the right it is presented the *M. tuberculosis* phylogenies defined by the analysis of LSPs as defined by Gagneux *et al*. (158) except for lineage 7 that was defined by Fridessa *et al*. (154). Region of difference are shown in rectangles. At the left is shown the spoligotyping based lineages as defined by the work of Filliol *et al*. (151). The spoligotyping based BOV lineage was defined in the work by Demay *et al*. (119).

Spoligotyping, single nucleotide polymorphisms, and large sequence polymorphisms presents with different discriminatory power and not always coincide. Therefore, it becomes challenging to establish *M. tuberculosis* complex lineages. MIRU-VNTR typing has allowed achieving not only a significantly higher discrimination, but also provided with a new insight on *M. tuberculosis* complex evolution (119). This methodology can be used to classify *M. tuberculosis* complex isolates; however, it is needed further analyses to study all the 24 loci individually and search for the association between their allelic diversity and *M. tuberculosis* lineages (119).

Molecular epidemiology of *M. tuberculosis* strains in Portugal

According to the World Health Organization, there were an estimated 8.6 million incident cases of tuberculosis in 2012 and 1.3 million deaths were attributed to the disease. The emergence and spread of drug resistant strains of *M. tuberculosis* is becoming dramatically worse every year. An estimated 450 000 new cases of multidrug resistant tuberculosis with 170 000 deaths associated occurred globally in 2012 (506). Only about 84 000 (19%) of these were laboratory confirmed, in part, because of the lack of laboratory capacity in many resource-limited settings. Conventional drug susceptibility testing involves culturing the bacteria from a clinical specimen and determining if the bacteria can grow in media containing the antituberculosis drugs and that requires an operational and quality assured network of tuberculosis laboratories. The lack of such support associated with tuberculosis control and drug observed therapies programs inadequately implemented are nowadays favoring the selection and spread of drug resistant forms of *M. tuberculosis*. Although theoretically any *M. tuberculosis* strain can develop drug resistance, there are a number of strains into distinct lineages that have demonstrated a particular tendency to become multidrug resistant. It had been shown that strain type plays a role not only in disease outcome, variation in vaccine efficacy but also in the emergence of drug resistance (155). Strain type also influences disease epidemiology as in some settings it is associated with the presence or absence of clustering due to recent transmission (210; 328). Lineage-specific traits in the virulence and predisposition to acquire drug resistance of clinical isolates have been reported within modern lineages, such as Beijing and Euro-American Haarlem strains that are believed to exhibit more virulent phenotypes compared to ancient lineages, such as East African Indian and *M. africanum* strains (155; 355). The molecular mechanisms and genetic factors responsible for the described differences in pathogenesis, virulence and ability to acquire resistance remain largely unknown and it is expected that the recent efforts with the whole-genome sequencing projects will shed some light to this.

In 2002, the Portuguese National Health Authorities recorded a tuberculosis incidence of 41 new cases per 100 000 inhabitants, with 12.7% cases of multidrug resistant tuberculosis (127). By 2013, tuberculosis control efforts appeared to be working as the

number of cases decreased to 20.4 new cases per 100 000 inhabitants with an average of 0.87% cases of multidrug resistant tuberculosis (129). However, despite the significant decline in tuberculosis incidence in the recent years, Portugal hasn't reached, yet, the goal of the < 20 cases/ 100 000 inhabitants to be considered a low incidence country. The main problem is prevalence of multidrug drug resistant tuberculosis and, in particular, extensively drug resistant tuberculosis. From the 0.56% cases of multidrug resistant tuberculosis reported in 2012, 20% corresponded to extensively drug resistant tuberculosis (128). Multi- and extensively drug resistant tuberculosis constitutes a major preoccupation mainly in the three main urban centres, Lisbon, Porto and Setubal (129).

The study of the genetic diversity of the *M. tuberculosis* strains circulating in Lisbon over these years allows us to observe that the majority of the multidrug resistant *M. tuberculosis* strains circulating in Lisbon belong to a genetically related group (324; 326, 327). It was found that the prevalence of this group of strains among multidrug resistant *M. tuberculosis* strains was almost 90% (324; 326). Furthermore, it is now recognized that approximately 20% of all multidrug resistant strains circulating in Lisbon are extensively drug resistant (128). This group of strains was identified in 1998 by IS*6110*-RFLP in a study comprising the characterization of 99 multidrug resistant strains from 14 hospitals of Lisbon (339). It was found that 88.9% of the strains were in cluster. Four clusters were identified, the cluster A, B, C and D, each one harbouring a distinct IS*6110*-RFLP profile. From the four clusters, cluster A was the predominant comprising 75% of the strains. This cluster was at the time associated with an outbreak of multidrug resistant tuberculosis among a majority of HIV-positive patients and drug abusers (340). Strains from cluster A display similar RFLP patterns being characterized by the presence of 11 to 13 IS*6110* bands. Because of this, cluster A was divided in three sub-clusters, A1, A2, and A3. Cluster A is now designated by Lisboa Family (342). So far, were reported 11 different clusters belonging to Lisboa Family (324; 326; 342). Among these, cluster Lisboa3 is the most prevalent. Cluster Lisboa3 can be divided in Lisboa3A and Lisboa3B based on IS*6110*-RFLP patterns; cluster Lisboa3A comprises an extra IS*6110* copy whereas cluster Lisboa3B comprises 3 extra copies of IS*6110* (329). These strains are divided in two related clusters as defined by MIRU-VNTR. The two main genetic clusters correspond to the clusters Lisboa3 and Q1 (329). LAM genotype was previously shown to be the main genotype circulating in Portugal

(110; 324). Lisboa3 can be assigned to LAM1 sublineage and Q1 to LAM4 sublineage (329). Cluster Q1 is genetically similar to the Lisboa family, although it presents less mutational diversity than the Lisboa3 cluster (324; 326). Cluster Q1 corresponds to the original cluster B determined by RFLP. The exact phylogenetic origin of cluster Q1 with respect to the Lisboa family is still under study (Perdigão *et al*., unpublished). Lisboa Family has been associated mostly with multi- and extensively drug resistant tuberculosis; however, it also includes susceptible strains (342). Until now, no correlation has been established between the different clusters and drug resistance, *i.e*., among the same cluster there are strains with different susceptibility patterns (324; 326; 327).

These clusters of strains, mainly detected in the Lisbon region have been disseminated to other regions of the Portugal. The Lisboa Family is a threat to the control of tuberculosis since its prevalence over the years suggests that these strains may possess selective advantages over other strains with different genetic backgrounds (469).

Pathogenesis of tuberculosis

M. tuberculosis infects about one third of the world's population. However, only less than 10% will developed the disease. The majority of the infected individuals successfully contain the infection due to an efficient immune system (451). These individuals are non-infectious and do not present symptoms of the disease. This status is called clinical latency - or latent infection - and can endure through the individual life time. Nonetheless, a perturbation of the host immune system response can lead to the reactivation of the infection (450). The consequences of *M. tuberculosis* infection are dependent on the virulence of the infecting strain as well as the resistance of the infected host.

M. tuberculosis is disseminated by airborne droplet nuclei, which can be transmitted from of a person to another. When an infected individual coughs, the dispersed droplets can be inhaled by a new host (451). The exhaled droplets can remain in the air during several hours and the infectious dose is estimated to one bacterium (368). When the inhaled mycobacteria reach the alveoli of the lungs, it will be phagocytosed by

macrophages and transported across the alveolar epithelium into the lungs (106; 450). The entry of *M. tuberculosis* into the macrophage is mediated by several receptors, including, scavenger receptors, complement receptors, and the mannose receptor (311). In response to the presence of mycobacteria, and at least partly through interactions of mycobacterial components with Toll-like receptors, the macrophages induce a localized pro-inflammatory response that leads to the recruitment of mononuclear cells from neighbouring blood vessels (368; 451). Production of tumour necrosis factor (TNF-α) and inflammatory chemokines from the infected macrophages serves as signals for the recruitment of additional cells (278; 368; 451). These includes neutrophils, natural killer T cells, $CD4^+$ and $CD8^+$ T cells, each of which produce their own set of chemokines and cytokines that increase cellular recruitment and modification of the infection site (368). The migration of macrophages and T and B cells to the site of infection culminates in formation of a localized nodular inflammation, the granuloma, or tubercle, which is the pathological hallmark of tuberculosis (452). The primary parenchymal focus is called as Ghon focus and usually drains via local lymphatics to the regional lymph nodes. The combination of the Ghon focus, local lymphangitis and regional lymph node involvement is known as the Ranke complex (114) and is often morphologically observed as a calcified granulomatous lesion.

Granulomas are organized and dynamic structures composed by infected macrophages surrounded by foamy macrophages, dendritic cells, multinucleated Langhans giant cells, endothelial cells, and a mantle of lymphocytes in association with a fibrous cuff of collagen and other extracellular matrix components that delineates the periphery of the granuloma (106; 368; 452). During infection the granuloma evolves from cellular granulomas to necrotizing granulomas (Figure I.7) (106; 368). The necrotic core, or the *caseum*, due to its cheese-like appearance, results from both host and bacterial cell lysis. The *caseum* initially forms in the center of the granuloma and spreads outwards (Figure I.7-II). As the granuloma matures, it often develops several layers of fibroblasts (fibrous cuff), limiting the spread of the infection. A cavity is formed when the granuloma comes into contact with an airway, which involves transformation of the granuloma into a cavity made of a fibrotic wall filled with liquefied *caseum* that is directly connected to the luminal surface of the respiratory tract (Figure I.7-III) (106). Caseation necrosis

contributes to the accumulation of infectious necrotic material in the lumen that can be coughed up to spread mycobacteria to other individuals (86).

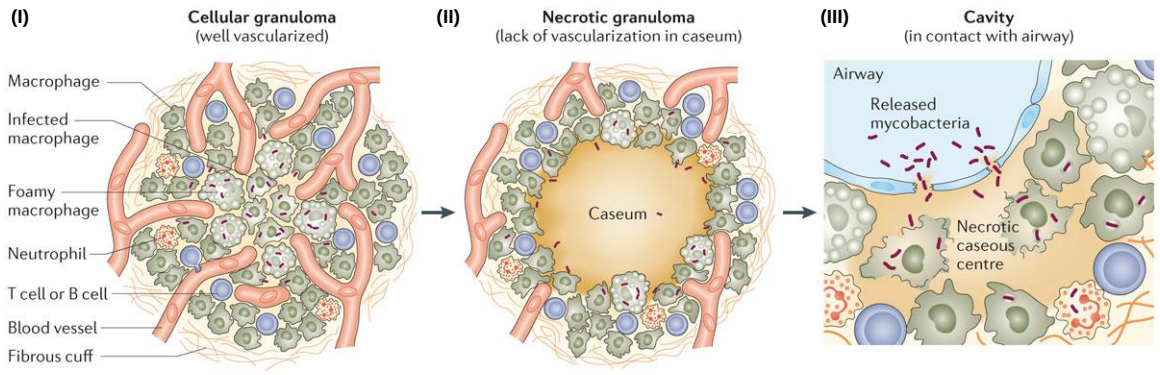

Figure I.7. Maturation of the granuloma. (I) Cellular granuloma where the bacilli reside extracellularly and in activated and foamy macrophages; (II) Necrotic granuloma, bacilli can be found extracellularly in the necrotic *caseum* and in immune cells; (III) Cavity formation, bacilli are found extracellularly in the cavity *caseum* and intracellularly in macrophages, foamy macrophages and neutrophils. Both intracellular and extracellular bacteria are released at the luminal side of the cavity and later appear in sputum. Reproduced with permission from Macmillan Publishers Ltd (106).

Although granuloma formation is regarded as an attempt to contain the spread of the bacilli, several studies have shown that pathogenic mycobacteria use granuloma cells to expand, disseminate and colonize nascent granulomas (113; 288). Infection of macrophages with *M. tuberculosis* can results in necrosis, defined by cell lysis, which allows their escape from macrophages and their spread to the surrounding macrophages (36; 311).

In addition, the granuloma functions as an immune microenvironment to facilitate the interaction between T cells and macrophages and cytokines (368). $CD4^+$ and $CD8^+$ T cells are important producers of Th1-type cytokines, such as IFN-γ (interferon gamma) and TNF-α which act synergistically to activate *M. tuberculosis*-infected macrophages. The activation of infected macrophages will lead to the upregulation of the inducible nitric oxide synthase, resulting in the production of reactive nitrogen intermediates, such as nitric oxide. Reactive nitrogen intermediates, together with reactive oxygen intermediates, exerts antimycobacterial effects, which lead to the reduction of bacterial burden (232). However, this structure can also provide a house for *M. tuberculosis* for a prolonged period. Once *M. tuberculosis* is internalized, it is generated a cascade of

events that results in the formation of a phagosome around the phagocytosed bacteria. Usually the phagocytosed material is degraded in lysosomes, a process called autophagy (122). Phagocytosis of pathogenic microorganisms by macrophages and neutrophils consist in the first step for their eventual degradation, as the phagosome eventually matures into a phagolysosome rich in acid hydrolases with degradative and microbicidal activity (450). However, *M. tuberculosis* inhibits phagolysosomal fusion and persists in the phagosome, preventing acidification of the phagosomal compartment (21; 175). Subsequent studies demonstrate that the vacuoles containing mycobacteria are not acidic (98). The basis for the acidification defect was provided by studies showing that mycobacterium-containing phagosomes exclude the vesicular proton ATPases (424).

The presence of the bacillus within the granulomas hinders drug delivery and represents a further concern to the control of the disease. To reach the center of necrotic granulomas, where quiescent extracellular bacilli are found (39), drugs must diffuse from the periphery to the necrotic center and penetrate in the *caseous* region (106). The presence of intracellular bacteria creates an additional obstacle since the drugs must permeate the cell in which the bacterium resides. To circumvent this problem, some of the innovating approaches rely on liposomes (211; 474) and nano-based drug delivery systems for encapsulation and release of antituberculosis drugs (161; 167; 409). Targeted drug delivery into macrophages can reduce toxicity and increase drug levels inside the lesion. Liposomes and nanoparticles function as particulate carriers that target the macrophages by taking advantage of their phagocytic properties (106). All these drug delivery systems have considerable potential for treatment of tuberculosis.

Macrophages: a central element to control the pathogenesis of tuberculosis

The initial infection of the macrophage results in the production of pro-inflammatory cytokines such as interleukins (IL) IL-6, IL-1β, IL-12 and TNF-α. T-cell responses are shaped by interactions with dendritic cells, which depend on the innate immune response against *M. tuberculosis*. Th1 T cells produce IFN-γ and promote mycobacterial killing by infected macrophages. Th17 T cells secrete IL-17 and are important for protective vaccine-induced responses. The role of Th2 T cells in host defence against *M. tuberculosis* infection is more controversial and less evident but it has been clearly

demonstrated that the key cells and factors for the control of tuberculosis reside in the combination of innate and adaptive responses that influence the macrophage response against *M. tuberculosis* infection as well as the clinical outcome (106; 368; 452). Macrophages are phagocytes with innate immunity functions in non-specific defence but are also the cells that initiate the host specific defence mechanisms (adaptive immunity). Their role is to phagocytose - engulf and then digest - cellular debris and pathogens and also stimulates lymphocytes and other immune cells to respond to pathogens. When a macrophage ingests a pathogen like *M. tuberculosis*, he becomes confined inside the phagosome, which then fuses with a lysosome. Within the phagolysosome, enzymes and toxic peroxides are supposed to promote the acidic digestion of the bacteria. However, *M. tuberculosis*, resist to this programmed elimination in the phagolysosome releasing a number of inhibitors of the antimycobacterial effector functions of the macrophages, such as generation of reactive oxygen intermediates (H_2O_2), the release of reactive nitrogen intermediates (*e.g*. nitric oxide) that prevents the acidification of the phagolysosome, as well promotes the slowdown of the pro-inflammatory response by decreasing the secretion of the above mentioned cytokines (368; 452). The result of this concerted action allows *M. tuberculosis* to survive within the macrophages maintaining a vicious circle of (i) preventing fusion of phagosomes with lysosomes; (ii) avoiding hydrolysis within the acidic phagolysosomes of the activated macrophages; (iii) promoting their replication inside the macrophage until the burst of the infected macrophage and the release of new bacilli to the surrounding. This circle of phagocytosis-apoptosis-necrosis runs uncontrolled until the granuloma emerges and contains the spread or, under immunosuppression, contention is not possible and active disease will outcome (106). At this time, the infection is in the lungs, and with severe cavitary disease the bacteria will, via lymphatic and capillaries, reach and infect other organs (disseminated tuberculosis). In more than 90% of the infected individuals the mycobacterium is contained inside the granuloma with no presentation of clinical symptoms (latent tuberculosis). Up to 10% percent of those with latent *M. tuberculosis* infection will eventually develop clinically active disease that manifests as localized pulmonary infection, or disseminated disease, depending on the innate immune response (452).

The clinical diagnosis of tuberculosis is a paradigmatic example of how the clinician needs the support of complementary exams to confirm the clinical suspicion. From the anamnesis to the physical exam, the gathering of cardinal signs of tuberculosis (cough, weight loss, fever, night sweats, and asthenia) needs to be complemented with strong evidences coming from lung radiology and the immune based tests, the tuberculin skin test (TST) and the interferon gamma release assays (IGRA). Both measure a person's immune reactivity to *M. tuberculosis*. In the TST or Mantoux test (the intradermal injection is termed the Mantoux technique) a standard dose of purified protein derivative (PPD), usually 5 tuberculin units, is injected intradermally and the induration caused read 48 to 72 hours later. A person who has been exposed to *M. tuberculosis* is expected to mount an immune response in the skin, measured by the diameter of the induration raised, proportional to how recent this contact was (334). In the IGRA tests, white blood cells from individuals that have been infected with *M. tuberculosis* will release IFN-γ when mixed with purified antigens derived from *M. tuberculosis* (mainly CPD-10 and ESAT-6) (229). The lymphocytes of a person who has been exposed to *M. tuberculosis* is expected to release IFN-γ at a level proportional to how recent this contact was. Complementary and essential to this diagnostic procedure is the mandatory request of support from the mycobacteriology laboratory for the detection, isolation and characterization of the bacilli causing the disease, the final confirmation that the patient has active tuberculosis. Global efforts to combat tuberculosis continue to be hampered by a lack of readily accessible and rapid laboratory services to establish the diagnosis of tuberculosis and monitor response to therapy. Of particular importance for the clinician or to any health system, is to obtain timely information on the drug susceptibility pattern of the infecting bacilli, with particular importance in countries such as Portugal, where high rates of multi- and extensively drug resistant tuberculosis are noticed, in order to optimize and adapt the multiple drug regimens to the most adequate and effective therapeutic combination.

Laboratory diagnosis of tuberculosis

The main priority of tuberculosis control programs is the rapid and accurate diagnosis and treatment of individuals presenting with active tuberculosis. With this measure, it is

intended to interrupt the chain of transmission of the bacillus and provide appropriate treatment to the patient. Therefore, the mycobacteriology laboratory has a major role in the diagnosis and management of tuberculosis. Diagnosis of active tuberculosis is mainly based on clinical suspicion, chest radiographs, microscopy, culture on solid and liquid media, and molecular identification.

Isolation and identification of *M. tuberculosis* complex – conventional methods

The conventional methods employed for the diagnosis of tuberculosis rely on acid fast staining, culture and phenotypic characterization. *M. tuberculosis* can be isolated from a wide variety of biological samples such as bronchial secretions, sputum, bronchoalveolar lavage fluid, gastric lavage, blood, bone marrow, urine, pleural fluid, cephaloraquidian liquid, biopsies, among others. The detection of acid-fast bacilli is the oldest diagnostic method used for the diagnosis of tuberculosis. Microscopic examination of smears for the detection of acid-fast bacilli by the Ziehl-Neelsen staining is simple, rapid, and inexpensive. In this procedure, the smears are stained with hot carbol fuchsine, decolorized with 25% sulphuric acid and ethanol, and counterstained with methylene blue. Mycobacteria will appear as red cells against a blue background. In spite of the high specificity of the procedure, its sensitivity has been reported to vary from 20 to 80% (419; 420) and frequently negative among HIV-infected patients due to its atypical presentation (320). Other staining methods have been developed to improve the sensitivity and specificity of the detection, which includes the fluorescent auramine-rhodamine staining (419). This technique is more rapid than the Ziehl-Neelsen staining and increases the sensitivity by almost 10% (419). This increased sensitivity allows the reduction in the number of samples analysed, thereby decreasing workload and speeding up diagnosis (449). However, is more expensive due the maintenance of the fluorescence microscope. Besides, these methodologies do not distinguish between *M. tuberculosis* and nontuberculous mycobacteria. The identification of smear-positive patients is of major importance since it is directly related with the degree of the infection and the severity of the disease. As such, culture remains the gold standard for the diagnosis of tuberculosis due to its high sensitivity and specificity.

Mycobacterial culture allows species identification, drug susceptibility testing, monitoring the response to drug therapy and epidemiological studies. Culture is an expensive method, since the majority of these microorganisms do not grow in common culture media, requiring the employment of specific media (for a more detailed description see ref. 471). Besides, *M. tuberculosis* slow growth rate complicates its detection. For isolation of *M. tuberculosis* and other mycobacterial species from biological samples, three main types of media can be employed: egg-based medium (Löwenstein-Jensen), medium with agar (Middlebrook 7H10 and 7H11) and liquid medium (Middlebrook 7H9, 7H12 and 7H13). Among the solid media, the Löwenstein-Jensen medium is the most widely used. Growth detection takes approximately two to six weeks depending on the media used and the species of mycobacteria. Growth of *M. tuberculosis* on Löwenstein-Jensen medium is regarded as rapid and luxurious or eugonic. Contrary, *M. bovis* grows poorly on normal Löwenstein-Jensen medium with small and flat dysgonic colonies (481). The development and implementation of liquid culture media allowed the reduction of the time to detection of positive cultures providing a more rapid diagnosis. The commercial liquid-based culture systems can be manual, semi-automated or automated using radiometric, colorimetric or fluorometric detection methods. The liquid media generally used is the Middlebrook 7H9 or the Middlebrook 7H12/7H13, depending on the system and the source of the specimen. Examples of these systems include the BACTEC®460TB, BACTEC®MB9000, BACTEC®MGIT®960 or 320 and the manual MGIT, the Septi-Chek AFB® systems (Becton Dickinson Instrument systems, Sparks, MD, USA), the ESP® (Extra Sensing Power) Myco-ESPculture System II® (Trek Diagnostic Systems, USA), and the BacT/ALERT MB® (bioMérieux, S.A. Marcy-l'Etoile, France). The most widely used is the BACTEC system, in its two versions: (i) the BACTEC-460TB semi-automated radiometric system based on the works performed by Cummings *et al*. and Middlebrook *et al*. (100; 395). This system uses Middlebrook 7H12 media that contains a radioactive substrate, palmitic acid labelled with ^{14}C, which is metabolized by the mycobacteria. Mycobacterial growth is detected by measuring the release of radioactive CO_2 with the aid of a gas flow radio counter (395, 396). The BACTEC 460-TB system converts the radioactivity released into a growth index (GI), ranging from 0 to 999; (ii) the BACTEC MGIT 960 system is a fully automated fluorometric system and currently is the most

widely used for the isolation of mycobacteria. For mycobacterial growth, is used growth-indicator tubes (MGIT; Mycobacteria Growth Indicator Tube) containing 7 ml of modified Middlebrook 7H9 media and a gel with a fluorochrome (ruthenium) impregnated at the bottom of the tube. The increase in the fluorescence due to oxygen consumption, as a consequence of mycobacterial metabolism, is measured automatically by the system and converted into growth units (GU). Mycobacterial cultures, in combination with smear microscopy, have the potential to increase the sensitivity of the diagnosis of mycobacterial infections and constitute the basis of further testing, which includes drug susceptibility testing and mycobacterial species identification. Classically, the identification of *M. tuberculosis* complex is based on the observation of phenotypic characteristics and a battery of biochemical tests - growth rate, growth at different temperatures, colony morphology, pigment production, niacin production, catalase activity, nitrate reduction, susceptibility to thiophene-2-carboxylic acid hidrazide (TCH), among others (109). In spite of their standardization and application in several reference laboratories, these methods can give false-results, require trained personnel, are time consuming and extremely laborious, resulting in the delay of a result by several weeks. During the last decades, has been developed several molecular methods for the identification of *M. tuberculosis* from cultures or directly from clinical samples thus reducing the time to diagnosis.

Molecular methods for the diagnosis of tuberculosis and drug resistance

Molecular tools for the diagnosis tuberculosis have evolved quickly during the last years. The development of rapid, sensitive and specific molecular techniques for the detection and identification of *M. tuberculosis* and other mycobacterial species constitutes a great advance in the diagnosis of tuberculosis and other mycobacterial diseases. These methods are based on the detection of specific fragments of DNA (or RNA) of the mycobacteria by PCR and includes, among others, RFLP analysis, real-time PCR, DNA sequencing, and nucleic acid hybridization (135; 439). Presently, nucleic acid amplification-based techniques have become widely used for the detection of *M. tuberculosis*. The identification of bacteria by the analysis of gene sequence is routine in many laboratories and can be used for species identification from cultures and

directly from specimens. Sequencing is considered the gold standard for the identification of mycobacteria and the ribosomal 16S rRNA is the most common target (446). The 16S rRNA is highly conserved among different species, however, it contains hypervariable regions that are species-specific and that can be used for a proper identification at species level (174). When the analysis of the 16S rDNA is not enough for species identification, other genes such as the 16S-23S rRNA intragenic region, *rpoB, gyrB, hsp65, recA* and *sodA* can be used (3; 365).

Among the commercially available methods for the direct detention of *M. tuberculosis* complex isolates are the enhanced amplified *M. tuberculosis* direct test (EMTD; Gen-Probe, Inc., San Diego, Ca) and the Amplicor *Mycobacterium tuberculosis* test (Amplicor, Roche Diagnostic Systems Inc., Branchburg, NJ). Both tests are certified for the direct detection of *M. tuberculosis* in clinical specimens (71). The EMTD is an isothermal transcriptase-mediated amplification system and is the first FDA-approved direct test for helping to diagnose smear-positive and negative specimens (72; 76), whereas the Amplicor assay, which uses PCR amplification of the 16S rDNA with biotinilated primers and automatic colorimetric detection with the Cobas Amplicor system, is only validated for the use on respiratory samples with smear-positive acid-fast bacilli (72; 103).

The identification of mycobacterial isolates from cultures was, for many decades, based on biochemical tests and phenotypic characteristics, which are time-consuming, slow, dangerous for the operator due to the high load of bacilli needed to be manipulated, and frequently gives ambiguous results. Several molecular systems were developed to overcome these limitations and are now being used for the identification of mycobacterial isolates from cultures. The AccuProbe (Gen-Probe) uses DNA probes for the identification of *M. tuberculosis* complex, *M. avium* complex, *M. avium*, *M. intracellulare*, *M. kansasii*, and *M. gordonae* from cultures (233). This method is based on the hybridization protection assay methodology by using DNA probes labelled with acridinum ester, a chemiluminescent compound (249). The single-stranded DNA probes are complementary to the mycobacterial rRNA. After a step of sonication, the probes will form a stable DNA-RNA complex. The hybrids can be detected by light emission on a luminometer. The method is safe, easy to perform, highly sensitive and specific (233; 356). The line probe assay technology (hybridization on strips) comprises PCR

amplification with biotinylated primers, reverse hybridization with species-specific DNA probes that are immobilized on a membrane strip, and colorimetric detection of the hybridized probes. Two systems for the identification of *M. tuberculosis* and other mycobacterial species are commercially available: the INNO-LiPA Mycobacteria v2 and the GenoType Mycobacterium CM/AS. The INNO-LiPA Mycobacteria v2 (Fujirebio Europe, Ghent, Belgium) is based on the amplification of the 16S-23S rRNA intragenic region and allows the identification of 17 mycobacterial species: *M. tuberculosis* complex, *M. avium, M. intracellulare, M. scrofulaceum, M. kansasii, M. xenopi, M. chelonae, M. gordonae, M. fortuitum* complex, *M. malmoense, M. genavense, M. simiae, M. smegmatis, M. haemophilum*, *M. marinum*/*M. ulcerans* and *M. celatum*. Besides, it can differentiate subspecies within *M. kansasii* and *M. chelonae* (INNOLiPA V2 package insert, 2005). The GenoType Mycobacterium (Hain Lifescience, Nehren, Germany) includes a multiplex PCR, followed by reverse hybridization on a membrane strip and colorimetric detection of the hybridized probes. There are available three identification systems: (i) the GenoType MTBC for the differentiation within *M. tuberculosis* complex; (ii) the GenoType Mycobacterium CM (common mycobacteria), and (iii) the GenoType Mycobacterium AS (additional species). The GenoType MTBC uses specific fragments of *gyrB* gene, 23S rDNA and the RD1. The analysis of *gyrB* polymorphisms allows the differentiation within *M. tuberculosis* complex (304). *M. bovis* BCG can be detected by the absence of the RD1 (436). The amplification of the 23S rDNA covers the Gram-positive bacteria with a high content of G+C (amplification control) and all members of the *M. tuberculosis* complex (357). The GenoType CM/AS assays have as target the 23S rDNA gene. Combined, the GenoType CM and AS allows the identification of 29 mycobacterial species: *M. tuberculosis* complex, *M. avium, M. intracellulare, M. chelonae, M. abscessus, M. fortuitum, M. gordonae, M. scrofulaceum, M. interjectum, M. kansasii, M. malmoense, M. marinum, M. ulcerans, M. peregrinum, M. xenopi, M. simiae, M. mucogenicum, M. goodii, M. celatum, M. smegmatis, M. genavense, M. lentiflavum, M. heckeshornense, M. szulgai, M. phlei, M. haemophilum, M. gastri, M. asiaticum* and *M. shimoidei* (GenoType Mycobacterium CM/AS, package insert, 2010). The test if rapid, easy-to-perform and interpret, and possess high specificity and sensitivity (95).

Next to the detection of *M. tuberculosis* complex is the detection of mutations associated with resistance to the main first and second line drugs. The methods available are based on the evidence that resistance associated mutations occur in specific regions of the genes whose products are targeted by these antibiotics (discussed in the next section). These include the INNO-LiPA Rif. TB (Fujirebio Europe), GenoType MTBDR*plus*, GenoType MTBDR*sl* (Hain), and the AID TB Resistance assay (GenID, Autoimmun Diagnostika, GmbH, Strassberg, Germany). In 2008, the World Health Organization endorsed two line probe assays for rapid screening of patients at risk of multidrug resistant tuberculosis in smear-positive samples, namely the INNO-LiPA Rif. TB and the Genotype MTBDR*plus* (502). In 2011, the GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA) was endorsed by the World Health Organization for the direct detection of *M. tuberculosis* and resistance to rifampicin in smear-positive and negative samples (504). The INNO-LiPA Rif. TB uses a nested PCR for the detection of *M. tuberculosis* and mutations associated with rifampicin resistance, and can be used directly from clinical specimens and from cultures (467). The nitrocellulose strips contains 10 oligonucleotide probes, from which: one is specific for the detection of *M. tuberculosis* complex strains; five-partially overlapping probes that hybridize with wild type sequences (S1-S5), and four resistance probes (R) carrying the mutations D516V (R2), H526Y (R4a), H526D (R4b), and S531L (R5). The absence of hybridization one or more wild type probes indicates the presence of a mutation that can be detected with the hybridization of one of the mutation probes (INNO-LiPA Rif. TB, package insert, 2011). The GenoType MTBDR*plus* assay enables the simultaneous detection of the *M. tuberculosis* complex, the most common mutations in *rpoB* gene for rifampicin resistance and mutations in *katG* and *inhA* for isoniazid resistance. It uses a multiplex PCR and can be used directly on clinical specimens and cultures (Genotype MTBDR*plus*, package insert, 2009). The GenoType MTBDR*sl* is based on the same principle as the MTBDR*plus* but for the detection of mutations associated with resistance to second line drugs: *gyrA* gene for fluoroquinolone resistance, *rrs* for aminoglycosides and cyclic peptides, *embB* for ethambutol (Genotype MTBDR*sl*, package insert, 2009). This assay can be applied to smear-positive and negative specimens (191). The test was not approved to date for diagnosis due to the scarce evidence on its reliability (528), Recently, a new line probe assay was developed. The

AID TB resistance assays are modular line probe assays for the rapid detection of mutations associated with resistance to isoniazid and rifampicin (module 1), first and second line aminoglycosides and cyclic peptides (module 2), and fluoroquinolones and ethambutol (module 3). This is the only molecular assay that detects mutations associated with streptomycin resistance. These assays can be used directly in clinical specimens and cultures. The evaluation of the three modules for the direct detection of *M. tuberculosis* and mutations associated with resistance on smear positive respiratory and non-respiratory samples showed a high specificity, 100%, 97.1%, and 100%, respectively (359).

The equipment required for line probe assays is the basic equipment available in a molecular biology laboratory, namely, a thermocycler and a water bath. The procedure is divided in four main steps: 1) sample processing; 2) DNA extraction from the decontaminated samples; 3) a nested or multiplex PCR (depends on the system used) with biotinylated primers followed by 4) reverse hybridization. Usually, they can be performed in 48 hours. The GeneXpert MTB/RIF is a fully automated system and is based on a hemi-nested real-time PCR that targets the rifampicin resistance determining region (RRDR) of the *rpoB* gene. It uses plastic cartridges with multiple compartments that are supplied preloaded with buffers and lyophilized reagent beads necessary for sample processing, DNA extraction and for the heminested PCR. Before the analysis, the samples are treated with an inactivation solution (sodium hydroxide plus isopropanol) and incubated at room temperature in order to reduce biohazard risk (231). The results are automatically generated by the system with no operator intervention. The assay can be performed directly from a clinical sample or from decontaminated sputum pellet. Generally it can be done within two hours.

All these molecular methods have been showed to be relatively fast and easy-to-perform while presenting high specificity and sensitivity. However they cannot replace the phenotypic testing due to their inability to detect all mutations associated with drug resistance as well the contribution of the phenotypic traits of the strain of *M. tuberculosis* to the overall resistance level, such as the impermeability of the cell wall and the efflux activity. These methods only detect the most frequent mutations associated with resistance. Therefore, the clinical utility of these assays will depend on

the prevalence of mutations associated with resistance to the drugs covered by these assays, since they vary between different geographical regions (221).

Drug susceptibility testing

Susceptibility testing to first line antituberculosis drugs is essential for the administration of an appropriate therapy. However, if culture is a slow process, drug susceptibility testing is even more time consuming. The traditional methods for *M. tuberculosis* drug susceptibility testing consist on: (i) the absolute concentration method; (ii) the resistance ratio method; and (iii) the proportion method (70). All these methods use agar (MB7H10 or MB7H11) or Löwenstein–Jensen medium. For the absolute concentration method, the antibiotics are incorporated in the media as two-fold dilutions. This method corresponds to a minimum inhibitory concentration determination (MIC) and can be also performed by broth micro- and macrodilution, although standardization is easier with solid media. Resistance is defined as the lowest concentration of the drug that inhibits growth (<20 colonies). The resistance ratio method is very similar to the previous one, however with this method, variations on MICs values are controlled with the simultaneous testing of an isolate with different batches of the same antibiotic. The resistance ratio is defined as the MIC for the strains divided by the MIC for a reference strain. If the ratio is ≤2 the isolate is considered susceptible or resistant if ≥8. The proportion method is based on the premise that every wild type *M. tuberculosis* strain is not a homogenous population but instead contains a mutant subpopulation resistant to the antimycobacterial drugs together with a susceptible subpopulation. The difference between a resistant strain and a susceptible strain is that the proportion of resistant bacteria among the population is much higher in the resistant strain than in the susceptible one (70). The critical concentration of an antituberculosis drug is defined as the concentration that is required to eliminate more than 99% of the population of a control strain that never has been in contact with the drug. A strain is considered susceptible to a given drug tested at their critical concentration if among the population the proportion of resistant cells is less than 1% (critical proportion). The strain is considered resistant when the number of drug-resistant bacteria present in the drug-containing tube is greater than 1%, when compared

with the drug-free growth control. The proportion method correlates well with an effective clinical outcome and is the gold standard for *M. tuberculosis* drug susceptibility testing. However, when performed on solid media these methods provide results only after four to eight weeks of incubation at 37°C. Moreover, the extended incubation time can result in degradation of the drugs. Therefore, the determination of the antibiotic susceptibility testing for *M. tuberculosis* requires more rapid and accurate procedures. The development of broth-based methods and the optimization of the proportion method to be used in these systems reduced significantly the time needed to complete the susceptibility test. Nowadays, the BACTEC MGIT 960 is the system of choice. Within this system, the susceptibility of a strain to a given antibiotic is determined as the ratio of the growth indexes between the tube containing the critical proportion of the test strain (1%) in drug free media and the strain in the presence of an antibiotic at its critical concentration.

Tuberculosis chemotherapy and the emergence of drug resistance

M. tuberculosis is a difficult therapeutic target, mostly due to its long generation time coupled with its ability to establish persistent infections (482). In addition, the localization and the pH of some lesions can limit or inhibit the access of the drugs. The drugs used for the treatment of tuberculosis, streptomycin, isoniazid, rifampicin, ethambutol and pyrazinamide, are designated as first-line, or essential, antibiotics (497) and form the basis of the great majority of drug regimens worldwide. The second-line drugs are reserved for the treatment of drug resistant tuberculosis (498). The purpose of tuberculosis chemotherapy is not only to cure the patients of the infection and prevent the relapse of the disease but also, sterilize the lesions and prevent the emergence of drug resistance. During infection a patient presenting with tuberculosis can harbour three different populations: (i) the first population corresponds to the actively growing extracellular bacteria that are usually present within aerated cavities; ii) the second population consists of intermittently growing bacilli; and (iii) the third population includes dormant bacilli that are present in lesions characterized by an acidic environment and under anaerobic conditions such as in inflammatory lesions or within macrophages (290; 291). Therefore, the antituberculosis drugs to be used must possess

three main properties: bactericidal activity, defined as their ability to kill actively growing bacilli rapidly; sterilizing activity, defined as their ability to kill the semi-dormant bacilli; and capacity to prevent the development of drug resistance (Table I.2) (290; 291). In this context, we can make the distinction between agents that are able to eliminate the bacilli *in vitro* (bactericidal) from those drugs that have the capacity to sterilize the lesions *in vivo*. Among the first-line antituberculosis drugs, the most effective in the three categories mentioned above are isoniazid, rifampicin and pyrazinamide, respectively (290; 291).

Table I.2. *M. tuberculosis* populations and antituberculosis drugs (adapted from 334).

***M. tuberculosis* populations**	**Metabolism**	**Degree of drug activity**	**Type of activity**
Intracavitary (tuberculous caverns)	Neutral pH Aerobic Active growth	STR +++ INH +++ RIF ++ EMB +/ –?	Bactericidal
Intracellular (macrophages)	Acid pH Anaerobic Slow growth	PZA +++ RIF ++ INH + EMB +/ –?	Sterilizing
Solid caseous focus (*caseum*)	Neutral pH Anaerobic Intermittent growth	RIF ++	Sterilizing

Legend: STR, streptomycin; INH, isoniazid; RIF, rifampicin; EMB, ethambutol; PZA, pyrazinamide.
+++ to +/-: level of activity of antituberculosis drugs.

As mentioned above, *M. tuberculosis* populations, irrespective of the location, consists on a mixture of a mutant subpopulation resistant to the antimycobacterial drugs and a drug susceptible subpopulation. Drug therapy can easily lead to the selection of resistant bacilli, since it will act only on the susceptible population. Thus, in order to prevent the selection of drug resistant mutants, the drug regimen should be based on a multiple therapy comprising the three activities simultaneously (bactericidal, sterilizing and prevention of emergence of resistance).

In the past decades, a number of effective drug regimens have been described. Most regimens are given for six months, and this corresponds to the shortest duration of treatment required. The standard regimen recommended by the World Health Organization and International Union against Tuberculosis and Lung Disease

(WHO/IUATLD) comprises an initial intensive phase with duration of two months with four drugs (isoniazid, rifampicin, pyrazinamide, and ethambutol or streptomycin), followed by a continuation phase with rifampicin and isoniazid used together during four months (498). Together, isoniazid and rifampicin eliminate more than 99% of the bacterial population during the first two months (137). During the continuation phase, rifampicin eliminates the dormant bacilli and isoniazid eliminates the rifampicin-resistant mutants when they start growing.

Drug resistant tuberculosis can be classified as acquired or primary drug resistance based on the history of the patient previous treatment. The World Health Organization defines acquired drug resistance as the isolation of drug-resistant *M. tuberculosis* from a patient with record of previous treatment. In patients who have not had prior treatment with antituberculosis drugs, the bacterial resistance is called primary resistance (499). The three main aspects that contribute to the development of drug resistance in *M. tuberculosis* are: the emergence of spontaneous mutations at low frequency, the selection of resistant mutants due to poor management of the disease (including inappropriate drug regimens and non-compliance) and transmission of drug resistant strains.

Unlike other bacteria, *M. tuberculosis* harbours no plasmids and his population structure is essentially clonal, which suggests a limited role for horizontal gene transfer (298). Therefore, *M. tuberculosis* drug resistance arises through the accumulation of particular chromosomal mutations in the genes associated with the resistance to each antituberculosis drug individually (202; 347; 522). This indicates that the chromosomal *loci* responsible for the resistance to various drugs are not linked. Hence, the probability of development of resistance to more than one drug is the product of the probability of emergence of resistance to each one of them. The mutation rate for emergence of resistance to isoniazid and rifampicin in *M. tuberculosis* was estimated to be 10^{-8} and 10^{-9} mutations/bacterium/cell division, respectively (108). Considering that cavitary lung lesions contains an average of 10^8 bacilli and in the solid caseous tuberculous lesions the concentration of bacilli reaches 10^4 to 10^5 (334), it is expected during monotherapy, to occur the selection drug resistant bacteria. For this reason, in order to prevent the emergence of resistance, as stressed before, tuberculosis treatment is and should always be based on a combination therapy including at least four drugs.

Molecular genetic basis of drug resistance

The advances in the molecular biology field and the accessibility to the new information generated by *M. tuberculosis* whole genome sequencing have added significant knowledge concerning the resistance mechanisms to the main antituberculosis drugs. In this subsection, we will address the resistance mechanism for the first-line drugs, isoniazid, rifampicin, ethambutol, pyrazinamide, and streptomycin, and to the mainstay drugs for the treatment of multidrug resistant tuberculosis, fluoroquinolones, aminoglycosides and cyclic peptides.

Isoniazid

In the 1950's, it was noticed that isonicotinic acid hydrazide (later known as isoniazid) had a high bactericidal activity against *M. tuberculosis* (42), that alongside with rifampicin, became the backbone of tuberculosis chemotherapy (498). While other mycobacterial species are less susceptible to this drug, *M. tuberculosis* is highly sensitive with minimum inhibitory concentrations ranging from 0.02 to 0.1 µg/ml.

Isoniazid enters in the cells by passive diffusion and it's primarily target are the mycolic acid biosynthesis (464). Isoniazid is a prodrug that needs to be converted in its active form with antituberculosis activity. This is achieved by the multifunctional catalase-peroxidase enzyme KatG, product of *katG* gene (189; 517). When activated by KatG, isoniazid forms a hypothetical isonicotinic acyl radical that binds to the nicotinamide adenine dinucleotide (NAD) originating a ternary complex, the INH-NAD adduct, essential for the inactivation of the InhA enzyme (345; 366), which is the target of isoniazid (30). The InhA enzyme, encoded by the *inhA* gene, is an enoil-acil (ACP) reductase dependent of the nicotinamide adenine dinucleotide hydrogenase (NADH) belonging to the fatty acid synthase type II system (FAS-II) (123; 259). *M. tuberculosis* has two FAS systems. The FAS-I accounts for the initial synthesis of fatty acids C_{16} to C_{18} and C_{24} to C_{26}, used for the production of phospholipids (346). These C_{16}-C_{18} fatty acids are then extended up to C_{56} by the FAS-II system to make precursors of mycolic acids, which are the essential constituents of the *M. tuberculosis* cell wall (260). KatG-activated isoniazid leads to the ineffectiveness of InhA with consequent accumulation of long chains of fatty acids, inhibition of biosynthesis of mycolic acids and cell death

(432; 464). In addition to its catalase and peroxidase activity, the KatG enzyme serves as both peroxinitritase (486) and NADH oxidase (401). It is also important to mycobacterial pathogenesis, since KatG enzyme counteracts the phagocyte oxidative burst (302).

Isoniazid resistance is thought to be associated with a multiplicity of mutations affecting one or more genes, including the genes *katG*, *inhA*, *ahpC*, *kasA*, *aphC-oxyR* intragenic region and *ndh*. The great majority of the mutations responsible for isoniazid resistance in clinical isolates of *M. tuberculosis* occur in the *katG* gene. The *katG* region of the *M. tuberculosis* genome is highly variable and due to the presence of repeated sequences it becomes unstable. This fact may contribute to the increased likelihood of occurrence of mutations in this gene (518). KatG gene mutations can occur in the N-terminal and C-terminal of the protein. The N-terminal region is associated with the active site of the enzyme (190) and most of the mutations that confer high levels of resistance are located between codons 138 and 350. The most common mutation on the *katG* gene is a point mutation in codon 315, where a serine is substituted by a threonine (S315T). This substitution is responsible for about 50-90% of the isoniazid resistant mutants worldwide and is associated with high-level isoniazid resistance (93; 168; 181). The most common polymorphism associated with the S315T mutation occurs in codon 463, where an arginine residue is replaced by a leucine (168; 190; 202). This substitution is not associated with isoniazid resistance as it occurs in the C-terminal of the protein and does not affect the activity of the protein (190). Several other mutations in *katG* have been reported. These include missense mutations, nonsense mutations, insertions, deletions, truncated regions and, more rarely, the complete deletion of gene (347; 517). *katG* gene mutations may lead to a reduction or the complete loss of the catalase-peroxidase activity (188; 517) which results in the incapacity to activate isoniazid.

The link between the synthesis of mycolic acids and isoniazid was done by Banerjee *et al* in 1994 (30) with the discovery that the *inhA* gene encodes the target of isoniazid, the InhA enzyme. The *inhA* gene is organized in an operon along with *mabA* gene (349). Mutations that occur in *inhA* promote a decreased affinity of isoniazid for NAD (30). Crystallography studies of the complex INH-InhA-NADH shown, that the pyridine ring of isoniazid is covalently bound with the NAD^+ ring inside the active site of the enzyme (366). The activated isoniazid bonds to NADH at the active site of InhA, and it is this

bond (INH-NAD) that is responsible for the inhibition of InhA (354; 366). At least, eight nucleotide substitutions in the local of ligation of INH-NAD to InhA have been associated with isoniazid resistance: I16T, I21T, I21V, I47T, V78A, S94A, I95P and I194T (123; 202; 239). The mutation S94A (the most frequent) seems to affect directly the bond INH-NAD (181; 202), resulting in the incapacity of isoniazid (and ethionamide) to inhibit the mycolic acid synthesis (464). This data is consistent with the structural interactions between isoniazid and InhA and support the importance of these mutations in mechanism of resistance to isoniazid (181). However, mutations in the promoter region of the gene occur more frequently, with a higher incidence for the mutation C-15T (2; 168; 181). This mutation increases the *inhA* mRNA level resulting in InhA overexpression and an eightfold increase in the resistance to isoniazid (463). Mutations in the structural region of the gene can co-exist with mutations in his promoter and/or *katG*, albeit this is a rare event (202; 251). Mutations in *inhA* gene mutations are associated with low and intermediate levels of resistance (202). Overall, clinical isolates with mutations in *inhA* represent approximately 15-30% of the isoniazid resistant cases conferring low-levels of resistance (52). Recently, it was shown that the presence of a mutation in the *inhA* promoter region together with a mutation in the *inhA* structural region can lead to the development of high level isoniazid and ethionamide resistance (251).

A reduced percentage of isoniazid resistant *M. tuberculosis* clinical strains have also been associated with a specific mutation in the type II NADH dehydrogenase (*ndh* gene), the substitution R268H (181; 234), which causes a reduction on the enzyme activity leading to an increased $NADH/NAD^+$ ratio and coresistance to isoniazid and ethionamide (284).

Several other genes have been implicated in isoniazid resistance, *e.g*. (i) the *ahpC* gene that encodes the alkyl hydroperoxide reductase enzyme (AhpC). The AhpC protein is regulated by the *oxyR* gene in most mycobacteria and the loss of *oxyR* in *M. tuberculosis* appears to be linked to the altered expression of the *ahpC* gene (121). Mutations in *oxyR-ahpC* locus increase the expression of AhpC (121) and compensate the loss of catalase activity in *katG* deficient strains (391); (ii) the *kasA* gene, which encodes β-ketoacyl acyl carrier protein synthase, forms part of a five genes (*kasA*, *kasB*, *accd6*, *acpM* and *fabD*) operon that catalyze the initiation of subsequent rounds of acyl

extension by FAS-II (43); and (iii) *furA*, a negative regulator of *katG* (344; 510). However, in each case, the mutations found in these genes were associated with mutations in *katG* and/or in the *inhA* promoter region or were also present in isoniazid susceptible strains.

Rifampicin

Ansamycin derivatives, such as rifampicin, are antibiotics used in the treatment of tuberculosis, leprosy, and diseases caused by Gram-positive bacteria. Ansamycin was isolated in 1957 from *Amycolatopsis mediterranei* (previously known as *Streptomyces mediterranei* and *Nocardia mediterranei*) (387). The ansamycins antibiotics containing an "ansa" chain connecting two nonadjacent carbons of an aromatic nucleus are interesting due to their unusual macrocyclic structures and their remarkable biological activities. Of the ansamycins which contain a naphthalene moiety, the rifamycins and the streptovaricins and their derivatives have received intense study. Ansamycins are structurally characterized by a planar naphthoquinone ring in which positions 3 and 4 have been extensively modified by hemisynthesis to yield commercial antibiotics (318) like rifampicin, rifapentine, and rifabutin.

Rifampicin, a semi-synthetic derivative of rifamycin B, was introduced as antituberculosis drug in 1972 (495). Rifampicin has a broad-spectrum activity and is particularly active against *M. tuberculosis* with minimum inhibitory concentrations ranging from 0.05 to 1 μg/ml. Rifampicin is active against both growing and stationary phase bacilli with low metabolic activity (522). Its rapid bactericidal activity aided to shorten the duration of treatment against drug susceptible *M. tuberculosis* when used in combination with pyrazinamide and isoniazid (125). Currently, rifampicin is a cornerstone of tuberculosis and leprosy therapy and is classified has as essential drug by the World Health Organization (497).

The mechanism of action of rifampicin was first demonstrated by Hartmann *et al*. (177; 178) by using *Escherichia coli* in which was shown that rifampicin inhibits the RNA polymerase. Since then, it was assumed that, in *M. tuberculosis*, rifampicin possesses the same mechanism of action. In 1993, the study performed by Levin and Hatful (240) shown that the mechanism of rifampicin resistance in mycobacteria is due to an altered

RNA polymerase. The RNA polymerase is an oligomer consisting of a core enzyme formed by four chains α2ββ′ in association with the σ subunit to specifically initiate transcription (513). The four different subunits (α, β, β' and σ) are encoded by *rpoA, rpoB, rpoC* and *rpoD* genes, respectively (286). Rifampicin binds in a pocket of the β-subunit of RNA polymerase deep within the DNA/RNA channel, but more than 12Å away from the active site. It directly blocks the path of RNA elongating when the transcript becomes two to three nucleotides in length (66).

More than 95% of *M. tuberculosis* clinical strains resistant to rifampicin harbour a mutation in an 81 base pair core region of the gene known as the rifampicin resistance determining region (RRDR) (440). The RRDR is presumed to be the rifampicin binding site (78; 522). All these mutations are comprised between codons 507 and 533 (*E. coli* numbering) of the *rpoB* gene and are associated with rifampicin resistance as they alter the structural conformation of the RNA polymerase thereby decreasing the binding affinity between rifampicin and the RNA polymerase (66). The RRDR is also known as cluster I (358). Several studies identified a variety of mutations, deletions and insertions in the *rpoB* gene among rifampicin resistant *M. tuberculosis* strains. The level of resistance to rifampicin in *M. tuberculosis* is dependent on the position of the mutation in the *rpoB* gene. Generally mutations in the RRDR convey high-level resistance. Mutations at positions 531 and 526 are among the most frequent mutations found in rifampicin resistant strains and are associated with high-level resistance (>32 μg/ml) and cross-resistance to all rifamycins (186; 202; 347; 467; 522). These mutational hotspots have being used for the prompt identification of rifampicin resistant isolates by molecular methods (52; 202; 347). Conversely, mutations in codons 511, 516, 518 and 522 are associated with low-level resistance to rifampicin (195, 202). Several other mutations along the RRDR have been rarely reported which were shown to be associated with variable levels of resistance (205; 254; 333; 440). Furthermore, it have been described the occurrence of mutations outside RRDR (49; 182; 186; 267; 343; 358; 404).

Recently, Comas *et al*. (90) showed that rifampicin-resistant *M. tuberculosis* strains can acquired, over the time, particular compensatory mutations in *rpoA* and *rpoC* genes that lead to the emergence of multidrug resistant strains presenting a high competitive fitness both *in vitro* and *in vivo*.

Pyrazinamide

In 1945 was demonstrated that nicotinamide (nicotinic acid, a vitamin B3 precursor) had inhibitory activity against *M. tuberculosis* (82). The synthesis of nicotinamide analogues led to the identification of pyrazinamide as the most active compound in a murine model of tuberculosis (521). Pyrazinamide was first synthesized by Dalmer and Walter in 1936 but its tuberculostatic activity was only established in 1952 (228). Pyrazinamide is a crucial component of tuberculosis therapy due to its unique ability to shorten the duration of tuberculosis treatment from nine months to six months when used in combination with isoniazid and rifampicin (521). It has an excellent sterilising effect on semidormant *M. tuberculosis* under acidic conditions (183) but has no activity against *M. tuberculosis* under normal pH environments (438). Besides, pyrazinamide lacks bactericidal activity during the first two days of treatment (521). Pyrazinamide is exclusively active against *M. tuberculosis* complex members, with the exception of *M. bovis* and *M. canettii* that are intrinsically resistant (150; 521). The *in vitro* antimycobacterial activity of pyrazinamide is dependent on the pH of the media. Acidification of the culture media to pH 5.5 enhances the activity of pyrazinamide against *M. tuberculosis*. However, even under such conditions, pyrazinamide exert only bacteriostatic activity with minimum inhibitory concentrations ranging from 6-50 µg/ml. Moreover, its minimal bactericidal concentration is more than 1000 µg/ml (521).

Pyrazinamide is a pro-drug that enters in the cell by passive diffusion (519) where it will be converted into its active form, pyrazinoic acid (POA), by the mycobacterial nicotinamidase/pyrazinamidase enzyme (PZAse) (220). The loss of PZAse activity is observed in *M. tuberculosis* strains that are resistant to pyrazinamide (220). PZAse enzyme is encoded by the *pncA* gene (385) and single mutations in this gene are considered the major cause for pyrazinamide resistance. Approximately 72% to 97% of all clinical isolates resistant to pyrazinamide carry a mutation in the structural gene or in the putative promoter region of the *pncA* gene, which includes missense mutations, insertions and deletions (237; 246; 283; 341; 362; 385). Pyrazinamide resistance has been shown to be strongly associated with multidrug resistance. Mutations in *pncA* are scattered throughout its sequence, however, there is some degree of clustering to the regions that contain the catalytic residues of PZAse (237; 385). The catalytic residues

comprise the active site (D8, K96, A134 and C138), and the metal-binding site (D49, H51 and H71) (138). Mutations affecting the metal-binding residues or the active site are a cause a major reduction of the enzymatic activity and appear to be associated with high-level pyrazinamide resistance (389). However, the involvement of other resistance mechanisms is suggested by the existence of isolates that exhibit a high-level of pyrazinamide resistance without mutations in the *pncA* gene. For instance, *M. bovis* is intrinsically resistant to pyrazinamide due to a single point mutation (C→G) in *pncA* gene at position 169 that produces a non-effective PZAse (385), whereas *M. canettii* is also resistant to pyrazinamide but lacks *pncA* mutations (150).

PZAse is located in the cytoplasm and is constitutively expressed (426; 519). As referred above, pyrazinamide enters the cells by passive diffusion where it will be hydrolysed by PZase to the active bactericidal compound POA. The POA produced intracellularly reaches the cell surface through passive diffusion and a defective POA efflux pump (519). The extracellular acidic pH facilitates the formation of uncharged protonated POA, which then diffuses through the membrane, leading to the accumulation of POA and disruption of the membrane potential. The protonated POA brings protons into the cell and cause cytoplasmic acidification and de-energize the membrane by collapsing the proton motive force (PMF), which affects membrane transport (521). In *M. tuberculosis*, at an acidic external pH, the rate of passive transmembrane equilibrium of POA seems to overwhelm that of active efflux, resulting in the accumulation of POA within the cells (519). Moreover, the decrease of the intracellular pH due to the accumulation of POA was initially thought to cause the inactivation of the fatty acid synthase I (*fasI*) (525). This hypothesis was denied in the study by Boshoff *et al*. (51) where was shown that FAS-I is the target of 5-Cl-PZA and not of pyrazinamide or POA. Therefore, the molecular target of pyrazinamide remains unknown. To clarify this subject, Shi *et al*. (393) identified four proteins as potential targets of POA. Among these, the authors identified the ribosomal protein S1 (RspA, Rv1630) as a target for POA. RpsA is involved in translation and ribosome-sparing process of trans-translation (393). Shi *et al*. elegantly demonstrated that POA binds to RpsA and inhibits trans-translation and not translation. When POA binds to RpsA, it prevents the binding of tmRNA, which therefore cannot rescue stalled ribosomes. Trans-translation is dispensable during active growth but essential for bacteria in

handling stalled ribosomes or damaged mRNA and proteins under stressful conditions (300; 442). This finding helps to explain how different stress conditions, such as starvation, acid pH, hypoxia, and energy inhibitors and other drugs could potentiate pyrazinamide activity (521). Mutations in *rpsA* have been found in some pyrazinamide resistant isolates without mutations in *pncA* gene (150; 400). However, they are present only in a minority of strains indicating that the analysis *rpsA* mutations lacks sensitivity restricting its use for the routine testing of pyrazinamide susceptibility testing exclusively by molecular means (9; 400). Furthermore, it was shown that not all mutations in *rpsA* are associated with pyrazinamide resistance (150). Nevertheless, it can be a useful tool for the study of pyrazinamide resistance in strains presenting positive pyrazinamidase activity without detectable mutations in *pncA*. Curiously, Feuerriegel *et al*. (150) found that *M. canettii* strains possess mutations in the *rpsA* gene. The authors suggest that altered trans-translation might be the mechanistic basis for the intrinsic pyrazinamide resistance of this species. Furthermore, the quest for a new target associated with pyrazinamide resistance leads to the identification of PanD as a possible new mechanism of pyrazinamide resistance (516). PanD encodes an aspartate decarboxylase, which is involved in synthesis of β-alanine, a precursor of pantothenate and co-enzyme A biosynthesis (81). Zhang *et al*. (516) identified different mutations occurring in *panD* gene in 5 pyrazinamide resistant mutants generated *in vitro*. The authors also identified a *panD* mutation in a *M. canettii* strain and in a pyrazinamide resistant clinical isolate. More studies are needed to address the role of *rpsA* and *panD* mutations on pyrazinamide resistance.

Pyrazinamide susceptibility testing is complicated by the fact that pyrazinamide is only active in an acidic media. Among the conditions that can affect the results of susceptibility testing to pyrazinamide are (i) the effect of the inoculum size: large inoculum (10^{7-8} bacilli/ml) can increase the pH of the media and originate false resistance; (ii) the effect of bovine serum albumin (BSA): susceptibility of the bacilli to pyrazinamide is reduced in the presence of BSA, because of its neutralizing effect of the pH of the medium and binding of POA; (iii) the age of the cultures: old cultures are more susceptible to pyrazinamide than a 4-day log phase culture since actively growing bacilli can increase the pH of the media; (iv) efflux of POA: the efflux inhibitor reserpine, an inhibitor of the POA efflux pump, increase the susceptibility to

pyrazinamide (520). Therefore, all phenotypic pyrazinamide resistant isolates should be confirmed by *pncA* gene mutation analysis and, if possible, pyrazinamidase testing.

Ethambutol

Ethambutol was synthesized in 1961 (490) and its *in vitro* and *in vivo* activity against *M. tuberculosis* was revealed in the same year (207; 208; 445). Ethambutol is only active against mycobacteria (215) and, currently, is a key component of the antituberculosis therapy.

Ethambutol inhibits the transfer of mycolic acids to the cell wall but do not inhibit its synthesis (433). Ethambutol induces cellular accumulation and subsequent leakage of trehalose monomycolate, trehalose dimycolate, and free mycolic acids (216). Later, these results were associated with the inhibition of biosynthesis of arabinogalactan (434) and the arabinan of lipoarabinomannan (120). The inhibition of arabinan leads to the accumulation of decaprenol phosphoarabinase (DPA) (492) which is an arabinosyl donor for arabinan biosynthesis, establishing ethambutol as an inhibitor of the arabinosyltransferases (236).

In *M. tuberculosis* and *M. smegmatis* were identified three homologous genes (sharing 65% homology with each other) that encode for arabinosyltransferases: the genes *embC*, *embA* and *embB* who are organized in a 10 kb operon, designed as *embCAB* (441). The EmbCAB are integral membrane proteins with 12 transmembrane domains and a C-terminal globular region of approximately 400 amino acids (441). Structural changes in the enzyme, caused by mutations in the recognition site, may prevent the binding of ethambutol leading to the development of resistance. The mode of action of ethambutol seems to be different on each Emb protein and can be explained by their involvement in different pathways. While the EmbC protein is involved in the formation of arabinan in lipoarabinomannan, the enzymes EmbA and EmbB participate in the synthesis of arabinan in arabinogalactan. According to Telenti *et al*. (441) the development of resistance to ethambutol occurs gradually, involving multiple targets. The mutational hotspot occurs between the amino acids 238-436 of the *embB* gene, known as Ethambutol Resistance Determining Region (ERDR), a region highly conserved in *M. tuberculosis* (8). Resistance to ethambutol is frequent among isolates of *M. tuberculosis*

resistant to one or more antibiotics (180, 198, 325; 390) and monoresistance is rare (252; 319; 390). Several studies suggest that 69% of ethambutol-resistant *M. tuberculosis* strains carry a mutation in the *embB* gene, where the mutation in the codon 306 is the most frequent (180, 198; 325; 336; 348, 370, 412, 413; 414, 417, 441). Five distinct structural mutations in codon 306 of *embB* gene have been described. The most common corresponds to the substitutions of the wild type methionine by isoleucine, leucine, or valine. The role of the *embB306* mutation was established as a cause of low and moderate-level (two to seven times the wild type) (370, 371). For strains with the substitution M306L or M306V the minimal inhibitory concentrations are generally higher than those with the M306I substitutions (348; 412).

Recent studies describe the occurrence of mutations in *embB*306 of *M. tuberculosis* isolates phenotypically susceptible to ethambutol (180; 235; 295; 325; 348; 414; 515). These studies suggest that mutations in this codon are just a common polymorphism that is present in clinical strains of *M. tuberculosis* that are predisposed to the development of resistance to other antituberculosis drugs. Several others mutations have been described in the *embB* gene, as well in *embA* and *embC* (198; 337; 348; 414; 441). Additionally, the codons *embB406* and *embB497* could represent additional hotspots for mutation (319; 348; 371). These two mutations are associated with high-level resistance (≥30 mg/l) to ethambutol comparable to those determined for strains caring the *embB306* mutations (348; 370, 412). Nonetheless, the *embB406* was already found in ethambutol susceptible clinical isolates (235; 350; 413). Safi *et al*. (371) showed that, similar to the *embB306* alteration, mutations other than *embB306* confer only a low to a moderate increase in ethambutol minimal inhibitory concentration.

Recently, it was shown that mutations in *embC* or very high *embC* expression confer the highest resistance levels (372). The UbiA enzyme synthesizes decaprenylphosphoryl-β-d-5-phosphoribose (DPPR), which is a precursor of DPA. Mutations in *ubiA* gene (Rv3806c), which encodes the DPPR synthase, UbiA (193) were associated with high-level resistance and had additive effects with *embB* mutations on the resistance levels. The authors assume that ethambutol resistance is acquired through the acquisition of mutations that interact in complex ways to produce a range of minimum inhibitory concentrations, from those falling below breakpoint values to the ones representing high-level resistance (372). Overall, these considerations indicate multiple molecular

pathways leading to the ethambutol resistant phenotype which make the study on the development of resistance to this antibiotic far from being deciphered.

Aminoglycosides

The great majority of aminoglycosides are bactericidal, possess predictable pharmacokinetics, and frequently act in synergy with other antibiotics, properties that make them valuable as antimicrobial agents (222). The significant clinical toxicities of aminoglycosides are ototoxicity, nephrotoxicity and less frequently neuromuscular toxicity (26). In spite of this, these antibiotics are an important component of our modern antimicrobial arsenal. Most of aminoglycosides are naturally produced by members of the genus *Streptomyces* and *Micromonospora*. By convention, those originating from the genus *Streptomyces* are labelled '-mycins', whereas those originating from the genus *Micromonospora* are labelled '-micins (512).

The antimicrobial activity of these drugs results from an interaction with the bacterial ribosomes where they bind to the A-site and disrupt protein synthesis. They also decrease the fidelity of mRNA, causing incorporation of incorrect amino acids into the growing polypeptide chain (111). The ribosome is composed of two subunits, 30S and 50S. The primary intracellular target of aminoglycosides is the 30S subunit, although some aminoglycosides bind to the 50S subunit of the ribosome.

Among this class of drugs, the aminoglycosides streptomycin, kanamycin and amikacin are those that are used in tuberculosis treatment. Streptomycin is a first-line antibiotic whereas kanamycin and amikacin are second-line drugs reserved to the treatment of multidrug resistant tuberculosis. However, the extensive clinical use of aminoglycosides, mainly as monotherapy, has led to the emergence of resistance.

Streptomycin was isolated from *Streptomyces griseus*, in 1944 by Waksman team (382) and was the first antibiotic demonstrating activity against *M. tuberculosis* (375). Streptomycin binds to the 30S ribosomal subunit (347). The 30S subunit consists of the 16S rRNA (encoded by *rrs gene*) and a set of unique proteins: S1 to S21 (S from small subunit). Resistance to streptomycin in *M. tuberculosis* is usually mediated by mutations in *rrs* and *rpsL* genes. The majority of mutations occur in the ribosomal protein S12, encoded by the *rpsL* gene (347). In this gene, the most common mutations

occur at position 43 (AAG → ACG) and 88 (AAG →AGG) (152; 347). A second type of mutations associated with resistance to streptomycin occurs in the *rrs* gene, especially in region 530 (the most common) and region of codon 915 (347). Generally, bacteria have multiple copies of the *rrs* gene. However, this is not the case of *M. tuberculosis* that only has one copy of this gene. Mutations in *rpsL* and *rrs* have been described in approximately 65-67% of the streptomycin resistant clinical isolates (347). Resistance to streptomycin can be categorized into three types: high-level, intermediate and low-level resistance. High-level resistance is found only in isolates with mutations in the *rpsL* gene; intermediate resistance is mostly associated with mutations in the gene *rrs*; and low-level resistance is found in isolates that do not present mutations in *rpsL* or *rrs* (281). Isolates with low-level resistance corresponds approximately to one-third of the clinical isolates with resistance to streptomycin without detectable mutations (152; 280; 411). These data suggest the existence of other targets or mechanisms associated with the resistance to this drug.

The *gidB* gene, which encodes for a conserved 7-methylguanosine (m^7G) methyltransferase specific for 16S rRNA, was identified as being potentially involved in low-level resistance to streptomycin (312). The product of this gene is involved in the methylation of the 16S rRNA, more specifically at position G527 of *E. coli* (312). The corresponding nucleoside in *M. tuberculosis* is G518, a residue that is found within the 530 loop of the 16S rRNA A-site and which interacts directly with streptomycin (309). Recently, the study by Wong *et al*. (493) provided the first microbiological evidence for the contribution of this gene to streptomycin resistance by showing that *gidB* mutations confer low-level streptomycin-resistance. Moreover, it was also demonstrated that the methylation of G518 affects protein translation and that methylation significantly enhances streptomycin binding. In the absence of the methyl modification, due to mutations in GidB, which is responsible for G518 methylation, streptomycin binds to the ribosome with less affinity, and confers low-level resistance (494). Due to the variety of mutations that can occur in *gidB* and since the impact of specific mutations on the resistance level of the isolate remains unclear, phenotypic drug susceptibility testing remains the most reliable way of predicting therapeutic response to treatment with streptomycin (493).

Kanamycin has been used for the treatment of tuberculosis caused by streptomycin resistant strains; however, the availability of less toxic alternatives such as amikacin and capreomycin (discussed below) has rendered it obsolete. Kanamycin was isolated from *Streptomyces kanamyceticus* and was identified in 1957 (453). Kanamycin comprises three components, kanamycin A (major component) and kanamycin B and C (minor components). Amikacin is a semi-synthetic derivative of kanamycin A with increased potency against M. *tuberculosis* (85). As referred above, streptomycin resistance is mediated by point mutations in *rpsL* or the *rrs* genes, however, no cross resistance between streptomycin and kanamycin or amikacin has been observed (7). This indicates that whereas these drugs bind to the ribosome they bind to a different region. In *E. coli*, ribosomal binding of kanamycin is affected by a mutation in the 1400 region of the *rrs* gene (292). It was also demonstrated that mutations in this region produce resistance to several aminoglycosides (124). Alangaden *et al*. (7) demonstrates that *M. tuberculosis* strains with high-level kanamycin resistance commonly harbour mutations in 1400 region of *rrs* gene, particularly the A1401G substitution. The mutations most commonly reported to cause resistance to kanamycin and amikacin include the A1401G, C1402T, and G1484T substitutions (*E. coli* numbering; *M. tuberculosis* positions 1408, 1409 and 1491, respectively) in the *rrs* gene (7; 60; 67; 148; 191; 194; 203; 214; 271; 326; 403; 431). Both the A1401G and G1484T mutations are associated with high-level amikacin resistance (MIC ≥80 μg/ml) and cross resistance to kanamycin, whereas low-level amikacin resistance is rarely observed (271). Resistance to kanamycin can be classified either as high (MIC ≥80 μg/ml) or low (MIC < 80 μg/ml) level (271). The C1402T mutation is associated low-level kanamycin resistance.

Zaunbrecher *et al*. (511) shown that low-level kanamycin resistance in *M. tuberculosis* is caused by point mutations in the promoter region of the enhanced intracellular survival gene, *eis*. The *eis* gene encodes an aminoglycoside acetyltransferase that was initially thought to be specific for kanamycin. The increase in *eis* transcript levels and the corresponding increase in the levels of the enzyme acetylate and inactivate kanamycin (511). Recently Chen *et al*. (77) demonstrated that Eis has an unprecedented ability to acetylate multiple amines of several aminoglycosides, including amikacin but not streptomycin. The acetylation mechanism promoted by Eis is supported by a complex tripartite fold that includes two general control non-derepressible 5 (GCN5)-

related N-acetyltransferase regions. This represents a unique and efficient strategy of deactivation of aminoglycosides by multiacetylation. Recently, it was shown that mutations in *eis* promoter are also associated with low-level resistance to amikacin (MIC < 4 µg/ml) and high-level cross resistance to kanamycin (328). However, the susceptibility levels are dependent on the methodology used. Gikalo *et al*. (162) reported different results for kanamycin susceptibly testing with the absolute concentration method (susceptible) or with the proportion method on BACTEC MGIT 960 (resistant). Due to the disparity between the different methodologies used for drug susceptibility testing it is difficult to assess the level of resistance due to *eis* mutations, mainly for low-level resistance. Moreover, the sensitivity of the Genotype MTBDR*sl* assay can be considerably affected by mutations in the *eis* gene as it cannot detect low-level amikacin resistance (Machado *et al*., unpublished) and kanamycin resistance (194).

Cyclic peptides

Capreomycin and the structurally similar compound viomycin, belong to the tuberactinomycin class of antibiotics. Viomycin, also known as tuberactinomycin B, was isolated in 1951 from *Streptomyces puniceus* and *S. floridae* (34; 153). Capreomycin was first isolated from *Streptomyces capreolus* in 1960 by Herr *et al*. (184). The tuberactinomycin antibiotics are essential components used for the treatment of multidrug resistant tuberculosis (505). Although viomycin was the first member of this family to be discovered, only capreomycin is commonly used clinically as a second-line antibiotic against multidrug resistant *M. tuberculosis* (505).

The mechanism of action of tuberactinomycins is unique among the ribosome-targeting antibiotics as they affect both ribosomal subunits. Tuberactinomycins are cyclic peptide antibiotics which target bacterial protein synthesis by binding to the well-conserved intersubunit bridge B2a, formed by the interaction between helix 69 (H69) of the 23S rRNA and helix 44 (h44) of the 16S rRNA (416). Viomycin interacts with both ribosomal subunits (507) and prevents the translocation of the ribosome along the mRNA (294). During translocation, a deacylated tRNA moves from the P site to the E site and the peptidyl-tRNA moves from the A site to the P site, releasing the A site for

the next aminoacylated tRNA. Elongation factor G (EF-G) promotes the movement of two tRNAs and the mRNA through the ribosome. Viomycin completely inhibits translocation but does not interfere with EF-G binding to the ribosome or GTP hydrolysis by EF-G (294).

Capreomycin consists in a mixture of four isoforms, IA, IB, IIA, and IIB (394), all of which are active *in vitro* against mycobacteria (185). The differences in composition between isoforms I and II are attributed to the absence of β-lysine in the latter; and B differs from A in the exchange of 1 amino acid: it have the serine of the cyclic pentapeptide core reduced to alanine. The main difference between the binding of viomycin and capreomycin to the 70S ribosome is in the β-lysine side chain of each drug (394).

Mutations in the positions 1401, 1402, and 1484 of 16S rRNA are associated with different degrees of resistance to capreomycin and viomycin in *M. tuberculosis*, while simultaneously conferring cross-resistance to the aminoglycosides amikacin and kanamycin (148; 203; 271; 403). The *rrs* mutation A1401G causes low-level resistance to capreomycin and high-level resistance to amikacin and kanamycin; the C1402T mutation is associated with capreomycin and viomycin resistance and low-level kanamycin resistance. The mutation G1484T is responsible for high-level resistance to amikacin, kanamycin, capreomycin and viomycin (136; 203; 271; 388; 403; 431; 437).

In addition, several single nucleotide polymorphisms in *tlyA* gene have been also associated with capreomycin resistance. The *tlyA* gene encodes a 2'-O-methyltransferase (rRNA methyltransferase) and the loss of 2'-O-methylation of C1920 in H69 and that of C1409 in h44 by TlyA is thought to reduce susceptibility to capreomycin (201; 272). *Thermus thermophiles* TlyA modifies only the C1920 in H69 of 23S rRNA, indicating that sensitivity to capreomycin does not involve the methylation of C1409 (296). Moreover, the inactivation of the *tlyA* gene in *T. thermophilus* does not affect its sensitivity to capreomycin (296), suggesting that modification of C1409 is the relevant determinant of increased drug susceptibility due to 2'-O-methylation (6). Mutations in *tlyA* are associated with low-level capreomycin resistance (Machado *et al*., unpublished).

The genotypes associated with resistance to the cyclic peptides capreomycin and viomycin and the aminoglycosides kanamycin and amikacin are overlapping. For this reason, using a combination of a cyclic peptide (capreomycin or viomycin) and an aminoglicoside (kanamycin and amikacin) is equivalent to using a single drug with respect to the development of resistance. Furthermore, due to indication that the various mutations associated with resistance mediate different degree of susceptibility to capreomycin, it is indispensable to correlate the phenotypic drug susceptibility testing with the genetic basis of capreomycin resistance.

Fluoroquinolones

Quinolones were discovered in 1962 when Lesher and his colleagues identified accidently nalidixic acid as a by-product of the synthesis of the anti-malaria drug chloroquine (238). Since their discovery, this class of antibiotics has generated considerable clinical and scientific interest. However, the importance of quinolones only started in the early 1980s, with the finding that a combination of a fluorine molecule at position 6, a piperazine group at position 7, and a carbon associated group at position 8, resulted in a wide and potent antimicrobial activity. This combination leads to the development of norfloxacin, the first of a new generation of antibiotics (17). Since then, the development of new quinolones evolved with third (*e.g.* ofloxacin and ciprofloxacin) and fourth (*e.g*. levofloxacin, gatifloxacin and moxifloxacin) generations of antibiotics, and is regarded today, as an important group of antimicrobial agents, with a wide gamut activity (17). The newer fluoroquinolones, such as levofloxacin, gatifloxacin and moxifloxacin, have demonstrated high *in vivo* and *in vitro* activities against multidrug resistant strains and ofloxacin and ciprofloxacin resistant strains (282; 338; 363; 523).

Fluoroquinolones are widely used for the treatment of infections caused by non-mycobacterial agents. In addition, they are also active against nontuberculous mycobacteria and, in particular, they display inhibitory activity against *M. tuberculosis*. Presently, fluoroquinolones are essential for the treatment of patients with multidrug resistant tuberculosis. The World Health Organization recommends the inclusion of a fluoroquinolone in the antibiotic cocktail for the treatment of multidrug resistant

tuberculosis, preferentially, a new-generation fluoroquinolone (505). They are also recommended for the treatment of extensively drug resistant tuberculosis even when resistance to ofloxacin is present (501). Furthermore, moxifloxacin is recommended for the treatment of patients who are intolerant to the first-line antibiotics (48). The inclusion of fluoroquinolones in the therapeutic regimen for multi- and extensively drug resistant tuberculosis can improve treatment outcome and shorten the duration of the treatment (310). Nevertheless, the development of fluoroquinolone resistance is one of the causes of treatment failure and death (65).

The significance and effectiveness of the newer fluoroquinolones in tuberculosis treatment is threaten by the extensive use of the older fluoroquinolones, ofloxacin and ciprofloxacin, in general medical practice, for the treatment of other bacterial infections such as the case of empirical treatment of community-acquired pneumonia. This kind of practice contributes for the emergence of resistance since the exposure to fluoroquinolone monotherapy in a patient with undiagnosed tuberculosis can result in the emergence of fluoroquinolone resistance. Besides this, there is the problem of cross-resistance between the older and new generation of fluoroquinolones as they share the same molecular target, DNA gyrase (241).

Fluoroquinolones act by inhibiting the type II topoisomerases DNA gyrase and topoisomerase IV (134). The molecular mechanism of action of quinolones was discovered in 1975 by Crumplin and Smith, which demonstrated that nalidixic acid, inhibits a critical enzyme for bacterial multiplication (99). This enzyme was later purified by Gellert *et al*. (160) and designated as DNA gyrase. Topoisomerase IV was discovered in 1990 by Kato *et al*. (209). DNA gyrase and topoisomerase IV possess almost 40% amino acid sequence identity (209) and are essential enzymes since both are required for cell growth and division and control of DNA topology. Quinolones exert their toxicity on the bacterial cell by stabilizing the double-stranded break in DNA created by DNA gyrase and inhibiting religation (32). The inhibition of DNA gyrase by quinolones will block transcription and DNA replication, which results in cell death (491). For this reason, fluoroquinolones are classified as bactericidal antibiotics. Unlike other bacterial species, *M. tuberculosis* lacks topoisomerase IV (89) making DNA gyrase the unique target of fluoroquinolones. DNA gyrase is an ATP dependent tetrameric protein composed by two GyrA and two GyrB subunits forming an A_2B_2

complex (330). GyrA and GyrB are encoded by *gyrA* and *gyrB*, respectively (241). GyrA, a protein of 97 kDa, is involved in double-strand breakage and reunion reaction, and GyrB, 89.9 kDa, is responsible for the ATPase activity of the enzyme (241). The N-terminal of the subunit A contains the catalytic site of DNA gyrase (64) and also the 120 bp quinolone resistance determining region (QRDR) comprised between the nucleotides 220 and 339 (nt 67 to 106 *E. coli* numbering system). The QRDR is the interaction site between DNA gyrase and quinolones.

Mutations in the QRDR of *gyrA* and, to a lesser extent, *gyrB* genes are responsible for fluoroquinolone resistance (230; 392; 435; 473). The most common mutations in *gyrA* occur in position 94 (D94G or D94A), 90 (A90V), 91 (S91P) and 88 (G88C) (24; 53; 148; 392; 397; 454; 473). Mutations in *gyrB* are less frequent and some nucleotide substitutions have been described in this gene: D472H, R485C, S486F, D495N, N533T, N538T, T539P, D500 (A, H, or N), G509A, N510D and E540 (V or D) (15; 24; 148; 335; 473). However, the correlation between mutations in *gyrB* and fluoroquinolone resistance needs to be further validated. For example, the mutations R485C and S486F were already identified in isolates susceptible to quinolones (316). By using gyrase inhibition and mutagenesis assays, it was demonstrated that the mutations D500A and N538T, located in the *gyrB* QRDR, and T539P and E540V, located outside the QRDR, are implicated in fluoroquinolone resistance (255; 317). Other mutations have been found that are not associated with resistance. The mutation *gyrA* S95T is found in several isolates but is considered to be a natural polymorphism and an evolutionary marker (15; 148; 397).

The level and pattern of resistance to fluoroquinolones varies among the different mutations, the different methods employed to their determination and the nature of fluoroquinolone tested. The difficulty to perform an accurate evaluation of the levels of fluoroquinolone resistance highlights the need for the standardization of fluoroquinolone drug susceptibility testing.

As seen in the main antibiotics used to treat tuberculosis and multidrug resistant tuberculosis, the genetic background of the *M. tuberculosis* strain determines significantly its pattern of resistance mainly due to mutations present in the targets of the antibiotic. Nevertheless, other mechanisms related with the physiological traits of

M. tuberculosis such as active drug efflux, also play an important role in the overall drug resistance levels and contribute significantly to the emergence of drug resistance (466, 470). Understanding the mechanisms underlying *M. tuberculosis* drug resistance not only enables the development of more rapid and accurate tools for the guidance of antituberculosis therapy but also provides new knowledge for development new therapeutic strategies.

Drug efflux, mycobacterial metabolism and new therapeutic targets and strategies

Drug efflux is generally described as an intrinsic or natural characteristic involved in drug resistance. In mycobacteria, intrinsic resistance to the majority of compounds is usually attributed to reduced permeability of the cell wall, in particular to its complex structure and constitution that acts synergistically with the activity of efflux pumps present in the membrane (118; 307; 466). Therefore, the intracellular concentration of an antibiotic is a balance between influx and efflux of the drug (247). Hydrophobic compounds, such as rifampicin and quinolones, can cross the hydrophobic bilayer through passive diffusion, whereas hydrophilic compounds, such isoniazid and ethambutol, use porin-like channels (306). Porins, or outer membrane proteins (OMPs), are water-filled channel-forming proteins that allow the passage of a large variety of hydrophilic compounds into the cell without requiring energy consumption (40). In response to the presence of toxic compounds, the cell can down-regulate their expression or decrease their synthesis (468). Contrary to other bacterial species, porins are less abundant in mycobacteria. OmpA of *M. tuberculosis* and the porin MspA of *M. smegmatis* were shown to be integral OMPs which are accessible at the cell surface (386; 415). More recently, two novel putative Omp-like porins (Rv1698 and Rv1973) have been identified in *M. tuberculosis* (408).

Efflux pumps are now broadly recognized as playing an important role in natural and induced drug resistance since they can expel a wide range of structurally dissimilar compounds, before they reach their target, being associated with multidrug resistance (332). The development of phenotypic drug resistance depends on the constitutive or induced expression of efflux systems (307; 332; 369) where the antibiotics act like

inducers through the regulation of the expression of efflux pumps (468). Efflux pumps are cytoplasmic transmembrane proteins involved in the extrusion of toxic compounds from within cells into the external environment by using cellular energy, ATP or the PMF (483). They are also involved in several physiological processes such as cell-to-cell communication, bacterial virulence, cellular homeostasis, detoxification of intracellular metabolites, and intracellular signal trafficking (261). Efflux pumps are found both in Gram-positive and Gram-negative bacteria, however, in the later, the resistance mediated by efflux is more complex due to the molecular architecture of the cell envelope (227). In this case, drug resistance is attributed to the synergy between reduced drug influx mainly due to the reduced permeability of the outer membrane (308), and active drug efflux.

Bacterial efflux systems can be grouped into five superfamilies or families, according to their structure, energy source, and phylogenetic relationships: (i) the ATP-binding cassette (ABC) superfamily; (ii) the small multidrug resistance (SMR) family; (iii) the multidrug and toxic compound extrusion (MATE) family; (iv) the resistance-nodulation division (RND) family; and (v) the major facilitator superfamily (MFS) (332). These transporters can be classified as primary transporters (ABC), which are energized by the hydrolysis of ATP, or secondary transporters (MFS, SMR, RND, and MATE), which act based on the electrochemical gradient generated by protons that are transported and distributed to the surface of the cell, or PMF (227; 332). These transporters differ in size, ranging from about 1000 amino acids for the RND transporters, through 400-450 amino acids for the MFS and MATE transporters and to 100 amino acids for the SMR transporters (274). The role of efflux mechanisms in drug resistance in mycobacteria have been demonstrated and extensively reviewed over the last years. Is reviewed in detailed in two recent papers (46; 470), thus will be only briefly discussed in this section.

The study of efflux systems in *M. tuberculosis* is mainly based on the characterization of transporter proteins through genetic manipulation and protein expression by using *M. smegmatis*, since is non-pathogenic, fast-growing, and easier to manipulate than *M. tuberculosis*. Using this strategy, several transporters from different classes have been characterized as been involved in drug transport in *M. tuberculosis*, and other mycobacterial species. The pumps energized by protons under the control of the PMF

identified in *M. tuberculosis* belong to the MFS, SMR and RND families and the fourth family (ABC family) uses the hydrolysis of ATP by the ATPase to provide energy for active transport. Overall, *M. tuberculosis* genome encodes for 267 transporters, of which, 129 corresponds to ABC transporters, 30 MFS, 14 RND, and 1 SMR (447). The most well-characterized efflux pumps described to date in mycobacteria are summarized in Table I.3.

Table I.3. Main putative efflux pumps described in *M. tuberculosis*.

Pump	Gene	Description	Substrates	Family	Energy source	Reference
Rv0194	*Rv0194*	Drug transport transmembrane ATP-binding protein	AMP; CAM; STR; NVB; TET; VAN	ABC	ATP	89; 105
DrrABC	*Rv2936-Rv2937-Rv2938*	Daunorubicin transport ATP-binding proteins	TET, STR, ETB, RIF	ABC	ATP	84; 89; 315
Rv1218c	*Rv1218c*	Probable tetronasin transport ATP-binding protein	β-lactams; NVB, biarylpiperazines, pyridines, bisanilinopyrimidines, pyrroles, pyrazolones	ABC	ATP	28; 89; 126
BacA	*bacA* (*Rv1819c*)	Drug transport transmembrane ATP-binding protein	INH	ABC	ATP	89; 200
Rv1747	*Rv1747*	Conserved transmembrane ATP-binding protein	INH	ABC	ATP	54; 89
Rv2686c-Rv2687c-Rv2688c	*Rv2686c-Rv2687c-Rv2688c*	Antibiotic transport ATP-binding protein	FQs	ABC	ATP	89; 321
PstB	*pstB* (*Rv0933*)	Phosphate transport ATP-binding protein	INH; RIF; ETB; CIP	ABC	ATP	54; 89; 380
Mmr	*mmr* (*Rv3065*)	Integral membrane efflux protein	TTP, EtBr, ERY, ACR, Safranin O, Pyronin Y	SMR	PMF	54; 89; 116
EfpA	*efpA* (*Rv2846c*)	Integral membrane efflux protein	INH, FQ, EtBr, Dyes, ACR	MFS	PMF	89; 133; 243
P55	*P55* (*Rv1410c*)	Aminoglycosides/tetracyclines transport integral membrane protein	INH; RIF; TET; AGs	MFS	PMF	89; 200; 399
Rv1258c	*Rv1258c*	Conserved membrane transport protein	INH, RIF, ETB, OFX; TET, ERY; SP	MFS	PMF	5; 89; 200;352; 398;
Stp	*stp* (*Rv2333c*)	Conserved membrane transport protein	TET; SP	MFS	PMF	89; 351
Rv1877	*Rv1877*	Conserved membrane protein	TET, KAN, ERY	MFS	PMF	89; 243
Rv2459	*Rv2459*	Conserved integral membrane transport protein	INH; ETB	MFS	PMF	89; 169
MmpL5	*mmpL5* (*Rv0676c)*	Probable conserved transmembrane transport protein	TET; BDQ	RND	PMF	89; 176; 285
MmpL7	*mmpL7* (*Rv2942*)	Probable conserved transmembrane transport protein	INH	RND	PMF	89; 131; 322
IniBAC	*iniB* (*Rv0341*) *iniA* (*Rv0342*) *iniC* (*Rv0343*)	Isoniazid- inducible protein IniB Isoniazid- inducible protein IniA Isoniazid- inducible protein IniC	INH; ETB	Membrane protein	-	11; 87; 89

ABC, ATP-binding cassette; SMR, small multidrug resistance; MFS, major facilitator superfamily; RND, resistance nodulation; ATP, adenosine triphosphate; PMF, proton motive force; AMP, ampicillin; CAM, chloramphenicol; STR, streptomycin; NVB, novobiocin; TET, tetracycline; VAN, vancomycin; ETB, ethambutol; RIF, rifampicin; INH, isoniazid; FQ, fluoroquinolones; CIP, ciprofloxacin; TPP, tetraphenylphosphonium; EtBr, ethidium bromide; ERY, erythromycin; ACR, acriflavine; AGs, aminoglycosides; OFX, ofloxacin; SP, spectinomycin; KAN, kanamycin; BDQ, bedaquiline.

Some of these putative efflux pumps have been associated with the transport of fluoroquinolones, isoniazid, rifampicin, ethambutol, β-lactams, doxorubicin, aminoglycosides, macrolides, tetracycline, and dyes, among others. Generally, these efflux systems are responsible for low-level resistance to antibiotics, contrasting with the high-level resistance caused by mutations in the genes that code for the primary targets of these antibiotics (117). However, the overexpression of efflux systems can result in a decrease of the intracellular levels of antibiotics, allowing the emergence of a subpopulation presenting a high-level resistance phenotype. The pressure caused by a subinhibitory concentration of antibiotic can result in an increased efflux activity, allowing the selection of spontaneous mutants and therefore determining clinically significant resistance levels (470).

Drug resistance mediated by the activity of efflux pumps is hypothesized to be dependent on the PMF and the availability of ATP within the cell. The contribution of both PMF and ATP dependent efflux systems, on the intrinsic resistance of *M. smegmatis* to isoniazid was first demonstrated in the work by Choudhri *et al*. (83). In that study the accumulation of isoniazid was increased upon addition of the protonophore CCCP, suggesting that a PMF dependent efflux system was involved in the efflux of isoniazid. Oxidative phosphorylation is the main source of energy production in mycobacteria. *M. tuberculosis* is an obligate aerobe pathogen and consequently it depends on oxidative phosphorylation for growth and survival. During oxidative phosphorylation, ATP is produced through the activity of the ATPases, which is coupled to the PMF (289). The PMF is established through the development of the transmembranar proton gradient which occurs due to the movement of electrons through the electron transport chain resulting in the establishment of the membrane potential. The proton gradient generated through oxidative phosphorylation leads to ATP synthesis via the ATP synthase which is responsible for the conversion of the electrochemical potential energy generated by the PMF into chemical energy in the form of ATP (147). Thus, energy metabolism and ATP production through the PMF, which is established by the electron transport chain, significantly contribute to drug susceptibility in *M. tuberculosis* (46). Several drugs whose mechanism of action is known to affect mycobacterial metabolic energy and respiration are now in the pipeline.

Below, we describe the mechanism of action of three new drug candidates with potential to improve tuberculosis therapy that have reached the phase of clinical testing.

The discovery of the highly effective antituberculosis drug diarylquinoline highlights the importance of energy production pathways for *M. tuberculosis*. Diarylquinolines have been identified in a process of screening various compounds for potential antituberculosis activity (18). The lead compound, bedaquiline (also called TMC207, R207910, or compound J), an ATP synthase inhibitor, is the first drug to be approved for treatment of multidrug resistant tuberculosis in decades. Bedaquiline binds to subunit c (AtpE) of mycobacterial ATP synthase enzyme by blocking its action (115; 223). Bedaquiline possess activity against susceptible and multidrug resistant strains (170) both replicating and under hypoxia (224). During hypoxia, diarylquinolines inhibit ATP synthesis activity but even at high concentrations had no significant effect on membrane potential (224). Recently, was demonstrated that upon bedaquiline exposure, the mycobacteria tend to minimize the consumption of cellular ATP and at same time enhance the capacity of ATP-generating pathways, which contributes to maintaining bacterial viability in spite of antibiotic stress (225). It was also showed that mycobacteria grown on lipid-rich media display enhanced bedaquiline mediated killing indicating a role of energy source on mycobacterial susceptibility (225). Since bedaquiline target differs from that of the currently available antituberculosis drugs it is not anticipated the development of cross-resistance (18). This type of compounds inhibit ATP synthase not only in bacteria but also in mitochondria (269; 270), however, it was shown by Haagsma *et al*. (173) that bedaquiline may not elicit ATP synthesis-related toxicity in mammalian cells. Mycobacteria that are resistant to bedaquiline *in vitro* have mutations in the *atpE* gene, which encodes the subunit c of ATP synthase (196; 331). However, resistant strains with no detectable mutations in the complete F0 ATP synthase operon (*atpB*, *atpE*, and *atpF* genes) and the F1 ATP synthase operon (*atpH*, *atpA*, *atpG*, *atpD*, and *atpC* genes) were already detected. Recently, clofazimine-resistant *M. tuberculosis* mutants isolated *in vitro*, were found to be also resistant to bedaquiline. Mutations in the transcriptional regulator *Rv0678*, with concomitant upregulation of the efflux pump, MmpL5, accounted for this cross-resistance (176). Together, these studies reveal the existence of alternative resistance mechanisms to bedaquiline.

Other promising compounds are the nitroimidazopyrans. Nitroimidazofurans, originally investigated as radiosensitizers for use in cancer chemotherapy (4), were found to possess *in vitro* activity against *M. tuber*culosis (22; 301; 475). Nitroimidazopyrans have been derived from the bicyclic nitroimidazofurans and are structurally related to metronidazole (422). The lead compound PA-824 showed potent bactericidal activity against multidrug resistant *M. tuberculosis*. PA-824 and delamanid (OPC-67683) are bicyclic nitroimidazoles (402) in phase II and III of clinical trials, respectively, for the treatment of multidrug resistant tuberculosis (58). Both compounds share the same mechanism of action. Although delamanid seems to be more active than PA-824 (268), PA-824 has been widely used to describe the mechanism of action of this class of compounds. PA-824 is active against *M. tuberculosis* complex (except *M. canettii*) (149) not only against actively replicating but also against non-replicating bacteria. Its mechanism of action involves the inhibition of mycolic acids synthesis (422) and respiratory poisoning (402). PA-824 is a prodrug that requires activation by the deazaflavin (cofactor F420) dependent nitroreductase (Ddn) (256; 268). In turn, F420, whose redox cycling requires the glucose-6-phosphate dehydrogenase Fgd1, is synthesized by FbiA, FbiB, and FbiC (79; 80). Rv3547, or Ddn, converts PA-824 into three primary metabolites; the principal one is des-nitroimidazole (des-nitro) (402). The formation of des-nitro metabolite generates reactive nitrogen species, including nitric oxide (402). Respiratory poisoning through nitric oxide release seems to be fundamental for the anaerobic activity by PA-824 (257; 402). Like cyanide, PA-824 dramatically shifted the predominant isoprenoid quinol/quinone ratio (MK9H2/MK9) in a time and concentration dependent manner. The effect of PA-824 on the respiratory complex under hypoxic non-replicating conditions was also manifested by a rapid drop in intracellular ATP levels. By this manner, PA-824 acts by releasing toxic nitric oxide within mycobacterial cells and nitric oxide possibly reacts with cytochromes/cytochrome oxidase to interfere with the electron flow and ATP homeostasis under non-replicating conditions (257). No cross-resistance with current antituberculosis drugs has been observed (422). Mutations in any of the mycobacterial genes *fgd1*, *fbiA*, *fbiB*, and *fbiC* lead resistance to PA-824 (79; 80; 256; 422). Mutations in the *Rv3547* gene, encoding the Ddn, have been described in PA-824 resistant strains (257; 268). Rv3547 is a protein with 151 amino acid residues with no detectable

sequence homology with any other proteins of known function and was shown to be nonessential (381). Complementation of the mutants with an intact Rv3547 fully restored the ability of the mutants to metabolize PA-824 (256).

The search for new antituberculosis drugs also prompted studies demonstrating that many compounds belonging to other pharmacological groups, such as antihypertensives, antipsychotics, and antispasmodic agents also have influence on the metabolic energy and on the viability of microorganisms (470). The potential use of these drugs in combination with antibiotics can constitute an important alternative as adjuvants in the antituberculosis conventional therapeutic regime and possess the advantage of being cost effective and time-saving. Data with respect to toxicity and pharmacokinetics is already available as they are normally employed in clinical practice and are approved by the FDA. Generally, these compounds are dopamine agonists and calcium channel blockers, blocking D2 receptors in the dopamine pathways (480). They are also called as efflux inhibitors as they can inhibit the efflux of drugs by disruption the energy necessary to maintaining the pumps working.

To date, several compounds have been appointed as potential efflux inhibitors. Among these are the ion channel blockers thioridazine and chlorpromazine and its derivative flupenthixol; haloperidol, and verapamil (Figure I.8) (470). Ion channel blockers like phenothiazines and verapamil have been shown to inhibit the *in vitro* growth of *M. tuberculosis* strains. Of this group, chlorpromazine was the first antipsychotic drug discovered (245). Phenotiazines are divided in three groups: the aminoalkyl compounds, like chlorpromazine; piperidine compounds, like thioridazine and; piperazine compounds, like fluphenazine (62). This class of drugs are known to inhibit the transport of calcium by preventing its binding to calcium dependent enzymes (373). The phenotiazines possess a three-ring structure, in which a sulphur atom and a nitrogen atom are linked to two benzene rings. In thioxanthenes, the nitrogen was substituted by a carbon atom in the central ring (299). Among these compounds, flupentixol is a high-potency thioxanthene with well-established antipsychotic properties (488).

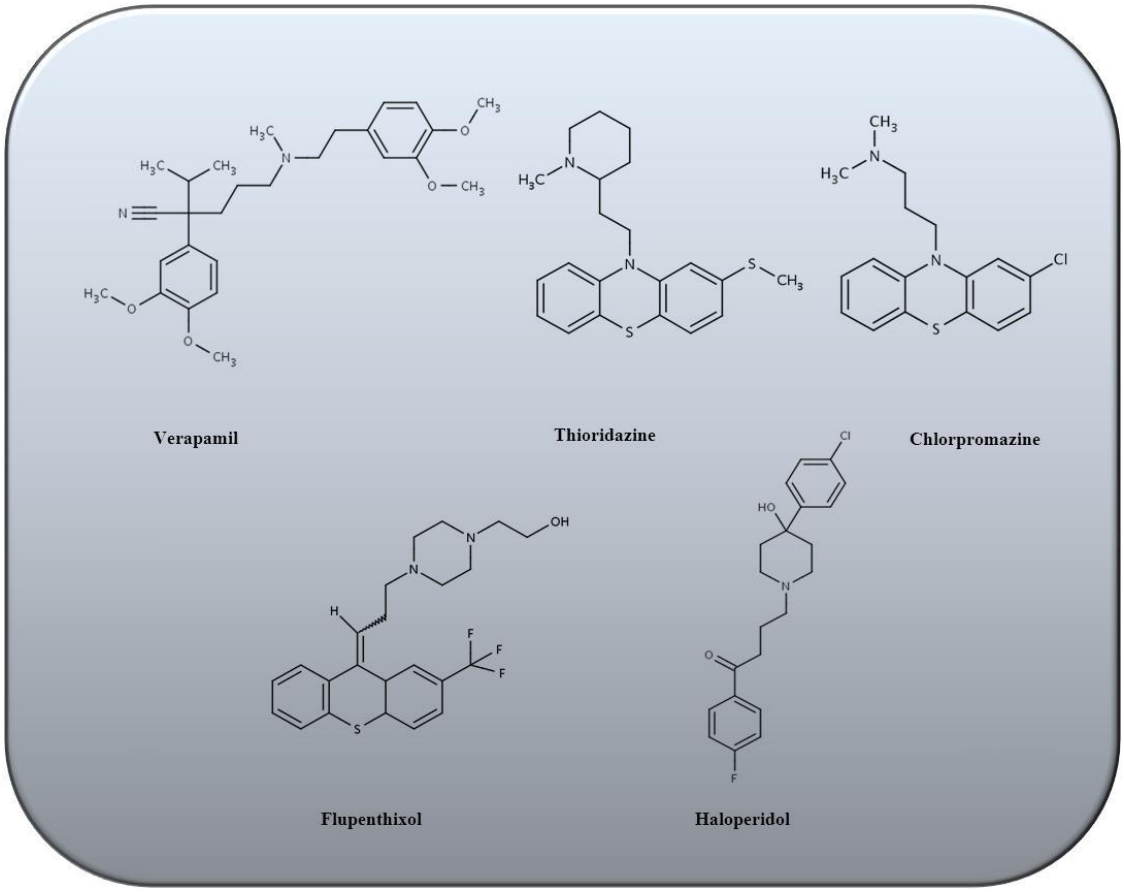

Figure I.8. Structures of the most important ion channel blockers reported to have inhibitory activity against *M. tuberculosis*.

Besides the thioxanthenes, the butyrophenones, structurally (piperazine moiety) and clinically related with phenothiazines, and whose representative is haloperidol, are also used as antipsychotic drugs (41; 62; 88). Thioridazine and chlorpromazine have been shown to have active efflux inhibition properties and to inhibit the *in vitro* growth of *M. tuberculosis* strains alone or in combination with antimycobacterial drugs (14; 250; 361). Thioridazine demonstrates significant activity against multidrug resistant tuberculosis in a murine model (460) and it has been successfully employed to treat extensively drug resistant tuberculosis on the basis of compassionate reasons (1). Recently, was demonstrated that thioridazine disturbs peptidoglycan synthesis in *Staphylococcus aureus* providing insights on the mechanism underlying the synergism between thioridazine and β-lactam antibiotics (445a). Altogether, these data suggests that thioridazine may have dual functions. Due to its toxicity, thioridazine was discontinued for the treatment of mental disorders in June 2005 (500). Verapamil, a phenylalkylamine, is a derivative of opium. *Papaver somniferum*, the opium poppy,

contains several alkaloids that belong to two different classes: phenanthrene, that includes codeine and morphine, and benzylisoquinoline, that includes papaverine (179). Unlike the other alkaloids, papaverine is not an analgesic or narcotic (489). It was initially applied to treat angina, hypertension, and arrhythmias but without success (179). Instead, it was found useful for the relief of intestinal colic (197). This led, in 1959, to the synthesis of a very active synthetic analogue, D365, by Ferdinand Dengel from Knoll AG (Ludwigshafen/Germany). Latter it was called verapamil. Its pharmacological properties as calcium antagonist were first described by Fleckenstein in 1963. In 1972, Schamroth, Krikler, and Garrett were the firsts to link the arrhythmia terminating action of verapamil with the concept of calcium channel blockade (179). Nowadays, verapamil is extensively used for the treatment of various disorders such as angina, hypertension and cardiac arrhythmia (16; 277). Verapamil is also an inhibitor of P-glycoprotein in mammalian cells (143). Verapamil has been shown to be the most potent mycobacterial inhibitor, being able to enhance the inhibitory activity of isoniazid (250; 361) and rifampicin (248) in *M. tuberculosis* clinical strains. Recently, Gupta *et al*. (170) demonstrate that efflux inhibition by verapamil can potentiate the activity of bedaquiline. It has also been shown that the addition of verapamil accelerates the bactericidal and sterilising activities of tuberculosis therapy in the mouse model (171).

The concept of enhancing the utility of the current antituberculosis drugs by employing ion channel blockers is quite appealing. However, their mechanism of action is not completely elucidated. Nowadays, evidence is emerging that their inhibitory functions possibly involve a role in cellular calcium homeostasis. In eukaryotic cells, L-type calcium channels need cell depolarization for activation (509). Cell trauma leads to intracellular calcium overload resulting in membrane depolarization (480). Once membrane depolarization occurs, calcium enters and initiates several damage mechanisms such as mitochondrial dysfunction which leads to a failure of aerobic energy metabolism (respiration dependent on calcium transport) (480). The role of ion channel blockers is to restore the homeostasis at membrane level inhibiting the influx of calcium and retention of dopamine. The activity of ion channel blockers is attributed to their effect on the mitochondrial electron transport chain. In eukaryotic cells, the neuroleptics haloperidol and chlorpromazine inhibit the mitochondrial NADH: ubiquinone oxidoreductase (complex I) (29). Evidence for the involvement of the

electron transport chain on the mode of action of neuroleptic compounds in *M. tuberculosis* comes from the studies performed with the phenotiazines. Phenothiazines are known to inhibit one of the key enzymes of the mycobacterial respiratory chain, the type II NADH: menaquinone oxidoreductase (NDH-2) (476; 484) also called as alternative NADH dehydrogenase (212). *M. tuberculosis* possesses two NADH dehydrogenases: the NDH-1, encoded by the *nuo* operon, which is non-essential in *M. tuberculosis* (353) and, the non-proton-translocating NDH-2, encoded by the *ndh* gene, which is essential for *M. tuberculosis* survival (284). NDH-1 is an equivalent of mitochondrial complex I, while NDH-2 is a single subunit enzyme with NADH/quinol oxidoreductase activity (508). In *M. tuberculosis*, energy generation is mainly performed by NDH-2 (353) whereas NDH-1 is mostly implicated on anti-apoptotic activity of host cells and, not on its original function of generation of energy (461). Rao *et al*. (353) established a link between NDH-2 and PMF through the demonstration that the inhibition of NDH-2 by thioridazine dissipates the membrane potential. Thioridazine and chlorpromazine act as partial uncouplers of oxidative phosphorylation in mitochondria (293) and was recently demonstrated that besides their inhibitory effect on NDH-2 they also act as uncouplers of oxidative phosphorylation in *S. aureus* (383). The increase on intracellular calcium levels is one of the major consequences of the inhibition of the respiratory chain. Reactive oxygen species (ROS) is responsible for the increase of calcium and this calcium increase is linked to the dissipation of membrane potential (279). The collapse of PMF has detrimental effects on the bacterial cell such as increased production of ROS, inhibition of ATP synthesis, and disruption of calcium homeostasis leading to cell death.

The search for novel compounds to be used in combination with antibiotics to treat drug resistant tuberculosis infections has become a major goal of drug discovery programs. The compounds presented here act on the mycobacterial electron transport chain. Besides, some of them also target *M. tuberculosis* efflux pumps through the disruption of energy necessary for their function. The inhibition of these functions will result in the intracellular accumulation of antibiotics and prevention of drug resistance. Furthermore, enhanced killing of the macrophage using ion channel blockers has also been demonstrated and proceeds through a mechanism that promotes the acidification of the phagolysosome wherein the calcium and potassium pumps are inactivated (14). The

above mentioned compounds have been shown to be effective efflux inhibitors with activity against a large range of bacteria. These compounds are ideal candidates that can be tested in combination with conventional antibiotics not only because they promote the retention of the co-administered antibiotics but also because these compounds enhance the killing activity of the macrophages. The observed killing enhancement has been postulated to be due to the inhibition of calcium and potassium transport. These ions are necessary to activate hydrolases required for the subsequent killing of intracellular bacteria. Furthermore, Adams *et al*. (3a) shown that the selective pressure exerted by the macrophage on internalized *M. tuberculosis* can induce the bacteria efflux pumps and thus drug-tolerance. The addition of the calcium channel blocker, verapamil or its metabolites reverses the tolerance to isoniazid and rifampicin (3a; 3b). These data highlight the importance and benefits of combined therapeutic regimens (261a). The delivery of antituberculosis drugs in conjunction with inhibitors of transport of calcium and potassium may provide a novel and effective therapeutic approach that obviates the serious side effects resulting from the current chemotherapeutic applications and provides a new approach for the therapy of multidrug resistant infections.

In conclusion, these compounds seems to be promising antituberculosis drug candidates for the treatment of drug resistant tuberculosis infections. Understanding the dynamic underlying the bacterial antibiotic-induced systems could provide a route for new chemotherapeutic approaches. By this manner, in the particular case of *M. tuberculosis*, the inhibition of its antibiotic intrinsic resistance could render this organism susceptible to many of the currently accessible antibiotics, allowing the recovery of compounds that are already out of the clinical usage rather development new compounds.

References

1. Abbate E, Vescovo M, Natiello M, Cufré M, García A, Montaner P, Ambroggi M, Ritacco V, van Soolingen D. Successful alternative treatment of extensively drug-resistant tuberculosis in Argentina with a combination of linezolid, moxifloxacin and thioridazine. J Antimicrob Chemother. 2012; 67: 473-7.

2. Abe C, Kobayashi I, Mitarai S, Wada M, Kawabe Y, Takashima T, Suzuki K, Sng L, Wang S, Htay H, Ogat H. Biological and molecular characteristics of *Mycobacterium tuberculosis* clinical isolates with low-level resistance to isoniazid in Japan. J Clin Microbiol. 2008; 46: 2263-8.

3. Adékambi T, Drancourt M. Dissection of phylogenetic relationships among 19 rapidly growing *Mycobacterium* species by 16S rRNA, *hsp65*, *sodA*, *recA* and *rpoB* gene sequencing. Int J Syst Evol Microbiol. 2004; 54: 2095-105.

3a. Adams K, Takaki K, Connolly L, Wiedenhoft H, Winglee K, Humbert O, Edelstein P, Cosma C, Ramakrishnan L. Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. Cell; 2011: 145: 39-53.

3b. Adams K, Szumowski J, Ramakrishnan L. Verapamil, and its metabolite norverapamil, inhibit macrophage-induced, bacterial efflux pump-mediated to multiple anti-tubercular drugs. J Infect Dis. 2014; doi: 10.1093/infdis/jiu095.

4. Agrawal K, Bears K, Sehgal R, Brown J, Rist P, Rupp W. Potential radiosensitizing agents. Dinitroimidazoles. J Med Chem. 22; 583-6.

5. Ainsa J, Blokpoel M, Otal I, Young D, De Smet K, Martin C. Molecular cloning and characterization of Tap, a putative multidrug efflux pump present in *Mycobacterium fortuitum* and *Mycobacterium tuberculosis*. J Bacteriol. 1998; 80: 5836-43.

6. Akbergenov R, Shcherbakov D, Matt T, Duscha S, Meyer M, Wilson D, Böttger E. Molecular basis for the selectivity of antituberculosis compounds capreomycin and viomycin. Antimicrob Agents Chemother. 2011; 55: 4712-7.

7. Alangaden G, Kreiswirth B, Aouad A, Khetarpal M, Igno F, Moghazeh S, Manavathu E, Lerner S. Mechanism of resistance to amikacin and kanamycin in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1998; 42: 1295-7.

8. Alcaide F, Pfyffer G, Telenti A. Role of *embB* in natural and acquired resistance to ethambutol in mycobacteria. Antimicrob Agents Chemother. 1997; 41: 2270-3.

9. Alexander D, Ma J, Guthrie J, Blair J, Chedore P, Jamieson F. Gene sequencing for routine verification of pyrazinamide resistance in *Mycobacterium tuberculosis*: a role for *pncA* but not *rpsA*. J Clin Microbiol. 2012; 50: 3726-8.

10. Alexander K, Laver P, Michel A, Williams M, van Helden P, Warren R, van Pittius N. Novel *Mycobacterium tuberculosis* complex pathogen, *M. mungi*. Emerg Infect Dis. 2010; 16: 1296-9.

11. Alland D, Kramnik I, Weisbrod T, Otsubo L, Cerny R, Miller L, Jacobs Jr W, Bloom B. Identification of differentially expressed mRNA in prokaryotic organisms by customized amplification libraries (DECAL): the effect of isoniazid on gene expression in *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA. 1998; 95: 13227-32.

12. Allix-Béguec C, Harmsen D, Weniger T, Supply P, Niemann S. Evaluation and strategy for use of MIRU-VNTR*plus*, a multifunctional database for online analysis of genotyping data and phylogenetic identification of *Mycobacterium tuberculosis* complex isolates. J Clin Microbiol. 2008; 46: 2692-9.

13. Alonso H, Aguilo J, Samper S, Caminero J, Campos-Herrero M, Gicquel B, Brosch R, Martín C, Otal I. Deciphering the role of IS*6110* in a highly transmissible *Mycobacterium tuberculosis* Beijing strain, GC1237. Tuberculosis. 2011; 10.1016/j.tube.2010.12.007.

14. Amaral L, Martins M, Viveiros M. Enhanced killing of intracellular multidrug-resistant *Mycobacterium tuberculosis* by compounds that affect the activity of efflux pumps. J Antimicrob Chemother. 2007; 59: 1237-46.

15. An D, Duyen N, Lan N, Hoa D, Ha D, Kiet V, Thu D, Chau N, Dung N, Sy D, Farrar J, Caws M. Beijing genotype of *Mycobacterium tuberculosis* is significantly associated with high-level fluoroquinolone resistance in Vietnam. Antimicrob Agents Chemother. 2009; 53: 4835-9.

16. Andersen C, Holland I, Jacq A. Verapamil, a Ca^{2+} channel inhibitor acts as a local anesthetic and induces the sigma E dependent extra-cytoplasmic stress response in *Escherichia coli*. Biochim Biophys Acta. 2006; 1758: 1587-95.

17. Anderson M, MacGowan A. Development of the quinolones. J Antimicrob Chemother. 2003; 51: suppl S1 1-11.

18. Andries K, Verhasselt P, Guillemont J, Göhlmann H, Neefs J, Winkler H, Gestel J, Timmerman P, Zhu M, Lee E, Williams P, de Chaffoy D, Huitric E, Hoffner S, Cambau E, Truffot-Pernot C, Lounis N, Jarlier V. A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. Science. 2005; 307: 223-7.

19. Antunes M, Fonseca-Antunes A. The tuberculosis situation in Portugal: a historical perspective to 1994. Euro Surveill. 1996; 1: 199.

20. Aranaz A, Cousins D, Mateos A, Dominguez L. Elevation of *Mycobacterium tuberculosis* subsp. *caprae* Aranaz et al. 1999 to species rank as *Mycobacterium caprae* comb. nov., sp. nov. Int J Syst Evol Microbiol. 2003; 53: 1785-9.

21. Armstrong J, Hart P. Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. J Exp Med. 1971; 134: 713-40.

22. Ashtekar D, Costa-Perira R, Nagrajan K, Vishvanathan N, Bhatt A, Rittel W. *In vitro* and *in vivo* activities of the nitroimidazole CGI 17341 against *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1993; 37: 183-6.

23. Assis S, Santos A, Roberts C. Evidence of hypertrophic osteoarthropathy in individuals from the Coimbra Skeletal Identified Collection (Portugal). Int J Paleopathol. 2011; 1: 155-63.

24. Aubry A, Veziris N, Cambau E, Truffot-Pernot C, Jarlier V, Fisher L. Novel gyrase mutations in quinolone-resistant and hypersusceptible clinical isolates of *Mycobacterium tuberculosis*: functional analysis of mutant enzymes. Antimicrob Agents and Chemother. 2006; 50: 104-12.

25. Aufderheide A, Rodríguez-Martin C. The Cambridge Encyclopedia of Human Paleopathology. Cambridge: Cambridge University Press. 1998.

26. Avent M, Rogers B. Current use of aminoglycosides: indications, pharmacokinetics and monitoring for toxicity. Intern Med J. 2011; 41: 441-9.

27. Baker L, Brown T, Maiden M, Drobniewski F. Silent nucleotide polymorphisms and a phylogeny for *Mycobacterium tuberculosis*. Emerg Infect Dis. 2004; 10: 1568-77.

28. Balganesh M, Kuruppath S, Marcel N, Sharma S, Nair A, Sharma U. Rv1218c, an ABC transporter of *Mycobacterium tuberculosis* with implications in drug discovery. Antimicrob Agents Chemother. 2010; 54: 5167-72.

29. Balijepalli S, Boyd M, Ravindranath V. Inhibition of mitochondrial complex I by haloperidol: the role of thiol oxidation. Neuropharmacology. 1999; 38: 567-77.

30. Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Um K, Wilson T, Collins D, de Lisle G, Jacobs Jr WR. *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. Science. 1994; 263: 227-30.

31. Barlow F. The King's Evil. Engl Hist Rev. 1980; 95: 3-27.

32. Barnard F, Maxwell A. Interaction between DNA gyrase and quinolones: effects of alanine mutations at GyrA subunit residues Ser^{83} and Asp^{87}. Antimicrob Agents Chemother. 2001; 41: 1994-2000.

33. Barry 3rd C, Richard E, Mdluli L, Sampson A, Schroeder B, Slayden R, Yuan Y. Mycolic acids: structure, biosynthesis and physiological functions. Prog Lipid Res. 1998; 37: 143-79.

34. Bartz Q, Ehrlich J, Mold J, Penne MA, Smith RM. Viomycin, a new tuberculostatic antibiotic. Am Rev Tuberc. 1951; 63: 4-6.

35. Beggs M, Eisenach K, Cave M. Mapping of IS*6110* insertion sites in two epidemic strains of *Mycobacterium tuberculosis*. J Clin Microbiol. 2000; 8: 2923-8.

36. Behar S, Martin C, Booty M, Nishimura T, Zhao X, Gan H, Divangahi M, Remold H. Apoptosis is an innate defense function of macrophages against *Mycobacterium tuberculosis*. Mucosal Immunol. 2011; 4: 279-87.

37. Behr M, Wilson M, Gill W, Salamon H, Schoolnik G, Rane S, Small P. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science. 1999; 284: 1520-3.

38. Bell M. Vampires and death in New England, 1784 to 1892. Anthropology and Humanism. 2006; 31: 124-40.

39. Bentrup K, Russell DG. Mycobacterial persistence: adaptation to a changing environment. Trends Microbiol. 2001; 9: 597-605.

40. Benz R. Structure and function of porins from Gram-negative bacteria. Ann Rev Microbiol. 1988; 42: 359-93.

41. Beresford R, Ward A. Haloperidol decanoate. A preliminary review of its pharmacodynamic and pharmacokinetic properties and therapeutic use in psychosis. Drugs. 1987; 33: 31-49.

42. Bernstein J, Lott W, Steinberg B, Yale H. Chemotherapy of experimental tuberculosis. V. Isonicotinic acid hydrazide (nydrazid) and related compounds. Am Rev Tuberc. 1952; 65: 357-64.

43. Bhatt A, Molle V, Besra G, Jacobs Jr, W, Kremer L. The *Mycobacterium tuberculosis* FAS-II condensing enzymes: their role in mycolic acid biosynthesis, acid-fastness, pathogenesis and in future drug development. Mol Microbiol. 2007; 64: 1442-54.

44. Bishop P, Neumann G. The history of the Ziehl-Neelsen stain. Tubercle. 1970; 51: 196-206.

45. Bishop P. Laennec: a great student of tuberculosis. Tubercle. 1981; 62: 129-34.

46. Black P, Warren R, Louw G, van Helden P, Victor T, Kana B. Energy metabolism and drug efflux in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2014; 58: 2491-503.

47. Bloom B, Murray C. Tuberculosis: commentary on a reemergent killer. Science. 1992; 257: 1055-64.

48. Blumberg H, Burman W, Chaisson R, Daley C, Etkind S, Friedman L, Fujiwara P, Grzemska M, Hopewell P, Iseman M, Jasmer R, Koppaka V, Menzies R, O'Brien R, Reves R, Reichman L, Simone P, Starke J, Vernon A. American Thoracic Society, Centers for Disease Control and Prevention and the Infectious Diseases Society: treatment of tuberculosis. Am J Respir Crit Care Med. 2003; 167: 603-62.

49. Bobadilla-del-Valle M, Ponce-de-Leon A, Arenas-Huertero C, Vargas-Alarcon G, Kato-Maeda M, Small P, Couary P, Ruiz-Palacios G, Sifuentes-Osornio J. *rpoB* Gene mutations in rifampin-resistant *Mycobacterium tuberculosis* identified by polymerase chain reaction single-stranded conformational polymorphism. Emerg Infect Dis. 2001; 7: 1010-3.

50. Bőddinghaus B, Rogall T, Flohr T, Blőcker H, Bőttger E. Detection and identification of mycobacteria by amplification of rRNA. J Clin Microbiol. 1990; 28: 1751-9.

51. Boshoff H, Mizrahi V, Barry C. Effects of pyrazinamide on fatty acid synthesis by whole mycobacterial cells and purified fatty acid synthase I. J Bacteriol. 2002; 184: 2167-72.

52. Böttger E. The ins and outs of *Mycobacterium tuberculosis* drug susceptibility testing. Clin Microbiol Infect. 2011; 17: 1128-34.

53. Bozeman L, Burman W, Metchock B, Welch L, Weiner M. Fluoroquinolone susceptibility among *Mycobacterium tuberculosis* isolates from the United States and Canada. Clin Infect Dis. 2005; 40: 386-91.

54. Braibant M, Gilot P, Content J. The ATP binding cassette (ABC) transport systems of *Mycobacterium tuberculosis*. FEMS Microbiol Rev. 2000; 24: 449-67.

55. Breathnach C. Richard Morton's Phthisiologia. J R Soc Med. 1998; 91: 551-2.

56. Brennan P, Nikaido H. The envelope of mycobacteria. Annu Rev Biochem. 1995. 64: 29-63.

57. Brennan P. Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*. Tuberculosis. 2003; 83: 91-7.

58. Brigden G, Nyang'wa B, du Cros P, Varaine F, Hughes J, Rich M, Horsburgh Jr, R, Mitnick C, Nuermberger E, McIlleron H, Phillips P, Balasegarama M. Principles for designing future regimens for multidrug-resistant tuberculosis. Bull World Health Organ. 2014; 92: 68-74.

59. Brosch R, Gordon S, Marmiesse M, Brodin P, Buchrieser C, Eiglmeier K, Garnier T, Gutierrez C, Hewinson G, Kremer K, Parsons L, Pym A, Samper S, van Soolingen D, Cole S. A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proc Natl Acad Sci USA. 2002; 99: 3684-9.

60. Brossier F, Veziris N, Aubry A, Jarlier V, Sougakoff W. Detection by GenoType MTBDR*sl* test of complex mechanisms of resistance to second-line drugs and ethambutol in multidrug-resistant *Mycobacterium tuberculosis* complex isolates. J Clin Microbiol. 2010; 48: 1683-9.

61. Brudey K, Driscoll J, Rigouts L, Prodinger W, Gori G, Al-Hajoj S, Allix C, Aristimuño L, Arora J, Baumanis V, Binder L, Cafrune P, Cataldi A, Cheong S, Diel R, Ellermeier C, Evans J, Fauville-Dufaux M, Ferdinand S, de Viedma D, Garzelli C, Gazzola L, Gomes H, Guttierez M, Hawkey P, van Helden P, Kadival G, Kreiswirth B, Kremer K, Kubin M, Kulkarni S, Liens B, Lillebaek T, Ly H, Martin C, Martin C, Mokrousov I, Narvskaïa O, Ngeow Y, Naumann L, Niemann S, Parwati I, Rahim Z, Rasolofo-Razanamparany V, Rasolonavalona T, Rossetti M, Rüsch-Gerdes S, Sajduda A, Samper S, Shemyakin I, Singh U, Somoskovi A, Skuce R, van Soolingen D, Streicher E, Suffys P, Tortoli E, Tracevska T, Vincent V, Victor T, Warren R, Yap S, Zaman K, Portaels F, Rastogi N, Sola C. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol. 2006; 6: 23.

62. Brunton L, Parker K, Blumenthal D, Buxton I. Goodman & Gilman's Manual of Pharmacology and Therapeutics. The McGraw-Hill Companies, Inc. USA. 2008.

63. Burke H. The pathogenesis of Pott's disease. Trans Am Clin Climatol Assoc. 1947; 59: 122-37.

64. Cabral J, Jackson A, Smith C, Shikotra N, Maxwell A, Liddington R. Structure of the DNA breakage-reunion domain of DNA gyrase. Nature. 1997; 388: 903-6.

65. Caminero J, Sotgiu G, Zumla A, Migliori G. Best drug treatment for multidrug-resistant and extensively drug-resistant tuberculosis. Lancet Infect Dis. 2010; 10: 621-9.

66. Campbell E, Korzheva N, Mustaev A, Murakami K, Nair S, Goldfarb A, Darst S. Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. Cell. 2001; 104: 901-12.

67. Campbell P, Morlock G, Sikes R, Dalton T, Metchock B, Starks A, Hooks D, Cowan L, Plikaytis B, Posey J. Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2011; 55: 2032-41.

68. Camus J, Pryor M, Médigue C, Cole S. Re-annotation of the genome sequence of *Mycobacterium tuberculosis* H37Rv. Microbiology. 2002; 148: 2967-73.

69. Canci A, Minozzi S, Tarli S. New evidence of tuberculous spondylitis from Neolithic Liguria (Italy). Int J Osteoarchaeol. 1996; 6: 497-501.

70. Canetti G, Froman S, Grosset J, Hauduroy P, Langerova M, Mahler H, Meissener G, Mitchison D, Šula L. Mycobacteria: laboratory methods for testing drug sensitivity and resistance. Bull World Health Organ. 1963; 29: 565-78.

71. Centers for Disease and Control. Notices to readers: nucleic acid amplification tests for tuberculosis. MMWR. 1996; 45: 950-2.

72. Centers for Disease and Control. Notice to readers: Update: nucleic acid amplification tests for tuberculosis. MMWR. 2000; 49: 593-4.

73. Chatterjee D, Hunter S, McNeil M, Brennan P. Lipoarabinomannan. Multiglycosylated form of the mycobacterial mannosylphosphatidylinositols. J Biol Chem. 1992; 267: 6228-33.

74. Chatterjee D, Lowell K, Rivoire B, McNeil M, Brennan P. Lipoarabinomannan of *Mycobacterium tuberculosis*. Capping with mannosyl residues in some strains. J Biol Chem. 1992; 267: 6234-9.

75. Chatterjee D. The mycobacterial cell wall: structure, biosynthesis and sites of drug action. Curr Opin Chem Biol. 1997; 1: 568-79.

76. Chedore P, Jamieson F. Routine use of the Gen-Probe MTD2 amplification test for detection of *Mycobacterium tuberculosis* in clinical specimens in a large public health mycobacteriology laboratory. Diagn Microbiol Infect Dis. 1999; 35: 185-91.

77. Chen W, Biswas T, Portera V, Tsodikov O, Garneau-Tsodikova S. Unusual regioversatility of acetyltransferase Eis, a cause of drug resistance in XDR-TB. Proc Natl Acad Sci USA. 2011; 108: 9804-8.

78. Cho E, Shamputa I, Kwak H, Lee J, Lee M, Hwang S, Jeon D, Kim C, Cho S, Via L, Barry 3rd C, Lee J. Utility of the REBA MTB-rifa (R) assay for rapid detection of rifampicin resistant *Mycobacterium tuberculosis*. BMC Infect Dis. 2013; 13: 478.

79. Choi K, Bair T, Bae Y, Daniels L. Use of transposon Tn5367 mutagenesis and a nitroimidazopyran-based selection system to demonstrate a requirement for *fbiA* and *fbiB* in coenzyme F420 biosynthesis by *Mycobacterium bovis* BCG. J Bacteriol. 2001; 183: 7058-66.

80. Choi K, Kendrick N, Daniels L. Demonstration that *fbiC* is required by *Mycobacterium bovis* BCG for coenzyme F(420) and FO biosynthesis. J Bacteriol. 2002; 184: 2420-8.

81. Chopra S, Pai H, Ranganathan A. Expression, purification, and biochemical characterization of *Mycobacterium tuberculosis* aspartate decarboxylase, PanD. Protein Expr Purif. 2002; 25: 533-40.

82. Chorine M. Action del'amide nicotinique sur le bacilles du genre Mycobacterium. Comp rond de l'Acad Des Sci. 1945; 220:150e1.

83. Choudhuri B, Sen S, Chakrabarti P. Isoniazid accumulation in *Mycobacterium smegmatis* is modulated by proton motive force-driven and ATP-dependent extrusion systems. Biochem Biophys Res Commun.1999; 256: 682-4.

84. Choudhuri B, Bhakta S, Barik R, Basu J, Kundu M, Chakrabarti P. Overexpression and functional characterization of an ABC (ATP-binding cassette) transporter encoded by the genes *drrA* and *drrB* of *Mycobacterium tuberculosis*. Biochem J. 2002; 367: 279-85.

85. Claes P, Vanderhaeghe H, Compernolle F. Isolation and identification of minor components of commercial kanamycin. Antimicrob Agents Chemother. 1973; 4: 560-3.

86. Co D, Hogan L, Kim S, Sandor M. Mycobacterial granulomas: keys to a long-lasting host–pathogen relationship. Clin Immunol. 2004; 113: 130-6.

87. Colangeli R, Helb D, Sridharan S, Sun J, Varma-Basil M, Hazbon MH, Harbacheuski R, Megjugorac N, Jacobs WR Jr, Holzenburg A, Sacchettini J, Alland D. The *Mycobacterium tuberculosis iniA* gene is essential for activity of an efflux pump that confers drug tolerance to both isoniazid and ethambutol. Mol Microbiol. 2005; 55: 1829-40.

88. Colasanti B. Antipsycotic drugs. *In* Biological Psychiatry, Bittar E. (ed). Elsevier. 1999.

89. Cole S, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon S, Eiglmeier K, Gas S, Barry, III C, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail M, Rajandream M, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston J, Taylor K, Whitehead S, Barrell B. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature. 1998; 393: 537-44.

90. Comas I, Borrell S, Roetzer A, Rose G, Malla B, Kato-Maeda M, Galagan J, Niemann S, Gagneux S. Whole-genome sequencing of rifampicin-resistant *Mycobacterium tuberculosis* strains identifies compensatory mutations in RNA polymerase genes. Nat Genet. 2012; 44: 106-10.

91. Coros A, DeConno E, Derbyshire K. IS*6110*, a *Mycobacterium tuberculosis* complex-specific insertion sequence, is also present in the genome of *Mycobacterium smegmatis*, suggestive of lateral gene transfer among mycobacterial species. J Bacteriol. 2008; 190: 3408-10.

92. Coscolla M, Gagneux S. Does *M. tuberculosis* genomic diversity explain disease diversity? Drug Discov Today Dis Mech. 2010; 7: e43-e59.

93. Costa E, Ribeiro M, Silva M, Arnold L, Rostirolla D, Cafrune P, Espinoza R, Palaci M, Telles MA, Ritacco V, Suffys P, Lopes M, Campelo C, Miranda S, Kremer K, da Silva P, Fonseca L, Ho J, Kritski A, Rossetti M. Correlations of mutations in *katG*, *oxyR-ahpC* and *inhA* genes and in vitro susceptibility in *Mycobacterium tuberculosis* clinical strains segregated by spoligotype families from tuberculosis prevalent countries in South America. BMC Microbiol. 2009; 9: 39-49.

94. Cousins D, Bastida R, Cataldi A, Quse V, Redrobe S, Dow S, Duignan P, Murray A, Dupont C, Ahmed N, Collins D, Butter W, Dawson D, Rodriguez D, Loureiro J, Romano M, Alito A, Zumarraga M, Bernadelli A. Tuberculosis in seals caused by a novel member of the *Mycobacterium tuberculosis* complex: *Mycobacterium pinnipedii* sp. nov. Int J Syst Evol Microbiol. 2003; 53: 1305-14.

95. Couto I, Machado D, Viveiros M, Rodrigues L, Amaral L. Identification of nontuberculous mycobacteria in clinical samples using molecular methods: a 3-year study. Clin Microbiol Infect. 2010; 16: 1161-4.

96. Crick D, Mahapatra S, Brennan P. Biosynthesys of the arabinogalatan-peptidoglycan complex of *Mycobacterium tuberculosis*. Glycobiology. 2001, 11: 107r-18r.

97. Cronin W, Golub J, Magder L, Baruch N. Lathan M, Mukasa L, Hooper N, Razeq J, Mulcahy D, Benjamin W, Bishai W. Epidemiologic usefulness of spoligotyping for secondary typing of *Mycobacterium tuberculosis* isolates with low copy numbers of IS*6110*. J Clin Microbiol. 2001; 39: 3709-11.

98. Crowle A, Dahl R, Ross E, May M. Evidence that vesicles containing living, virulent *Mycobacterium tuberculosis* or *Mycobacterium avium* in cultured human macrophages are not acidic. Infect Immun. 1991; 59: 1823-31.

99. Crumplin G, Smith J. Nalidixic acid: and antibacterial paradox. Antimicrob Agents Chemother. 1975; 8: 251-61.

100. Cummings D, Ristroph D, Camargo E, Larson S, Wagner Jr, H. Radiometric detection of the metabolic activity of *Mycobacterium tuberculosis*. J Nucl Med. 1975; 16: 1189-91.

101. Cunha E. Paleobiologia das populações medievais portuguesas: os casos de Fão e S. João de Almedina. Tese de Doutoramento. Faculdade de Ciências e Tecnologia, Universidade de Coimbra. Coimbra, Portugal. 1994.

102. Daffe M, Brennan P, McNeil M. Predominant structural features of the cell wall arabinogalactan of *Mycobacterium tuberculosis* as revealed through characterization of oligoglycosyl alditol fragments by gas chromatography/mass spectrometry and by 1H and 13C NMR analyses. J Biol Chem. 1990; 265: 6734-43.

103. D'Amato R, Wallman A, Hochstein L, Colaninno P, Scardamaglia M, Ardila E, Ghouri M, Kim K, Patel R, Miller A. Rapid diagnosis of pulmonary tuberculosis by using Roche AMPLICOR *Mycobacterium tuberculosis* PCR test. J Clin Microbiol. 1995; 33: 1832-4.

104. Daniel T. The history of tuberculosis. Respir Med. 2006; 100: 1862-70.

105. Danilchanka O, Mailaender C, Niederweis M. Identification of a novel multidrug efflux pump of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2008; 52: 2503-11.

106. Dartois V. The path of anti-tuberculosis drugs: from blood to lesions to mycobacterial cells. Nat Rev Microbiol. 2014; 12: 159-67.

107. David H, Jahan M, Jumin A, Grandry J, Lehmaw E. Numerical taxonomy analysis of *Mycobacterium africanum*. Int J Syst Evol Microbiol. 1978; 28: 467-72.

108. David H. Probability distribution of drug-resistant mutants in unselected populations of *Mycobacterium tuberculosis*. Appl Microbiol. 1970; 20: 810-4.

109. David H, Lévy-Frébault V, Thorel M. Méthodes de laboratoire pour mycobactériologie clinique. Institut Pasteur, Paris. 1989.

110. David S, Duarte E, Leite C, Ribeiro J, Maio J, Paixão E, Portugal C, Sancho L, Germano de Sousa J. Implication of the RD^{Rio} *Mycobacterium tuberculosis* sublineage in multidrug resistant tuberculosis in Portugal. Infec Genet Evol. 2012; 12: 1362-7.

111. Davies J, Jones D, Khorana H. A further study of misreading of codons induced by streptomycin and neomycin using ribopolynucleotides containing two nucleotides in alternating sequence as templates. J Mol Biol. 1966; 18: 48-57.

112. Davis A. A historical perspective on tuberculosis and its control. *In* Tuberculosis: a comprehensive international approach, Second edition, L. Reichman and E. Hershfield, ed. Lung Biology in Health and Disease. 2000; 144: 2-47.

113. Davis J, Ramakrishnan L. The role of the granuloma in expansion and dissemination of early tuberculous infection. Cell. 2009; 136: 37-49.

114. De Backer A, Mortele K, De Keulenaer B, Parizel P. Tuberculosis: epidemiology, manifestations, and the value of medical imaging in diagnosis. JBR BTR. 2006; 89: 243-50.

115. de Jonge M, Koymans L, Guillemont J, Koul A, Andries K. A computational model of the inhibition of *Mycobacterium tuberculosis* ATPase by a new drug candidate R207910. Proteins. 2007; 67: 971-80.

116. De Rossi E, Branzoni M, Cantoni R, Milano A, Riccardi G, Ciferri O. *mmr*, a *Mycobacterium tuberculosis* gene conferring resistance to small cationic dyes and inhibitors. J Bacteriol. 1998; 180: 6068-71.

117. De Rossi E, Arrigo P, Bellinzoni M, Silva P, Martin C, Ainsa J, Guglierame P, Riccardi G. The multidrug transporters belonging to major facilitator superfamily in *Mycobacterium tuberculosis*. Mol Med. 2002; 8: 714-24.

118. De Rossi E, Aínsa J, Riccardi G. Role of mycobacterial efflux transporters in drug resistance: an unresolved question. FEMS Microbiol Rev. 2006; 30: 36-52.

119. Demay C, Liens B, Burguière T, Hill V, Couvin D, Millet J, Mokrousov I, Sola C, Zozio T, Rastogi N. SITVITWEB – A publicly available international multimarker database for studying *Mycobacterium tuberculosis* genetic diversity and molecular epidemiology. Infect Genet Evol. 2012; 12: 755-66.

120. Deng L, Mikušová K, Robuck K, Scherman M, Brennan P, McNeil M. Recognition of multiple effects of ethambutol on metabolism of mycobacterial cell envelope. Antimicrob Agents Chemother. 1995; 39: 694-701.

121. Deretic V, Song J, Pagán-Ramos E. Loss of *oxyR* in *Mycobacterium tuberculosis*. Trends in Microbiol. 1997; 5: 367-72.

122. Deretic V, Singh S, Master S, Harris J, Roberts E, Kyei G, Davis A, de Haro S, Naylor J, Lee H, Vergne I. *Mycobacterium tuberculosis* inhibition of phagolysosome biogenesis and autophagy as a host defence mechanism. Cell Microbiol. 2006; 8: 719-27.

123. Dessen A, Quemard A, Blanchard J, Jacobs Jr. WR, Sacchettini J. Crystal structure and function of the isoniazid target of *Mycobacterium tuberculosis*. Science. 1995; 267: 1638-41.

124. DeStasio E, Moazed D, Noller H, Dahlberg A. Mutations in 16S ribosomal RNA disrupt antibiotic-RNA interactions. EMBO J. 1989; 8: 1213-6.

125. Dickinson J, Aber V, Mitchison D. Bactericidal activity of streptomycin, isoniazid, rifampin, ethambutol, and pyrazinamide alone and in combination against *Mycobacterium tuberculosis*. Am Rev Respir Dis. 1977; 116: 627-35.

126. Dinesh N, Sharma S, Balganesh M. Involvement of efflux pumps in the resistance to peptidoglycan synthesis inhibitors in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2013; 57: 1941-43.

127. Direcção-Geral da Saúde. Programa Nacional de Luta Contra a Tuberculose. Ponto da Situação Epidemiológica e de Desempenho de 2002. Direcção-Geral da Saúde, Lisboa, Portugal. 2003.

128. Direcção-Geral da Saúde. Programa Nacional de Luta Contra a Tuberculose. Ponto da Situação Epidemiológica e de Desempenho de 2012. Relatório para o Dia Mundial da

Tuberculose. Avaliação preliminar em Março 2013. Direcção-Geral da Saúde, Lisboa, Portugal. 2013.

129. Direcção-Geral da Saúde. Programa Nacional de Luta Contra a Tuberculose. Relatório preliminar para o Dia Mundial da Tuberculose. Direcção-Geral da Saúde, Lisboa, Portugal. 2014.

130. Doetsch R. Benjamin Marten and His "New theory of consumptions". Microbiol Rev. 1978; 42: 521-8.

131. Domenech P, Reed M, Barry C 3rd. Contribution of the *Mycobacterium tuberculosis* MmpL protein family to virulence and drug resistance. Infect Immun. 2005; 73: 3492-501.

132. Donoghue H, Spigelman M, Zias J, Gernaey-Child A, Minnikin D. *Mycobacterium tuberculosis* complex DNA in calcified pleura from remains 1400 years old. Lett Appl Microbiol. 1998; 27: 265-9.

133. Doran J, Pang Y, Mdluli K, Moran A, Victor T, Stokes R, Mahenthiralingam E, Kreiswirth B, Butt J, Baron G, Treit J, Kerr V, van Helden P, Roberts M, Nano F. *Mycobacterium tuberculosis efpA* encodes an efflux protein of the QacA transporter family. Clin Diagn Lab Immunol. 1997; 4: 23-32.

134. Drlica K, Zhao X. DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiol Mol Biol Rev. 1997; 61: 377-92.

135. Drobniewski F, Nikolayevskyy V, Balabanova Y, Bang D, Papaventsis D. Diagnosis of tuberculosis and drug resistance: what can new tools bring us? Int J Tuberc Lung Dis. 2012; 16: 860-70.

136. Du Q, Dai G, Long Q, Yu X, Dong L, Huang H, Xie J. *Mycobacterium tuberculosis rrs* A1401G mutation correlates with high-level resistance to kanamycin, amikacin, and capreomycin in clinical isolates from mainland China. Diagn Microbiol Infect Dis. 2013; 77: 138-42.

137. du Toit L, Pillay V, Danckwerts M. Tuberculosis chemotherapy: current drug delivery approaches. Respir Res. 2006; 7: 1-18.

138. Du X, Wang W, Kim R, Yakota H, Nguyen H, Kim S. Crystal structure and mechanism of catalysis of a pyrazinamidase from *Pyrococcus horikoshii*. Biochemistry. 2001; 40: 14166-72.

139. Duarte E, Domingos M, Amado A, Cunha M, Botelho A. MIRU-VNTR typing adds discriminatory value to groups of *Mycobacterium bovis* and *Mycobacterium caprae* strains defined by spoligotyping. Vet Microbiol. 2010; 143: 299-306.

140. Dubos R, Dubos J. The white plague: tuberculosis, man, and society. Rutgers University Press. 1952.

141. Duval C. Studies in atypical forms of tubercle bacilli isolated directly from the human tissues in cases of primary cervical adenitis. J Exp Med. 1909; 11: 403-29.

142. El-Naijar M, Al-Shiyab A, Al-Sarie I. Cases of tuberculosis at ‘Ain Ghazal, Jordan. Paléorient. 1997; 22: 123-8.

143. Endicott J, Ling V. The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu Rev Biochem. 1989; 58: 137-71.

144. Euzéby J. List of bacterial names with standing in nomenclature: a folder available on the internet. Int. J. Syst. Bacteriol. 1997; 47: 590-2. (List of Prokaryotic names with Standing in Nomenclature. http://www.bacterio.net – last acess May 2014).

145. Falkinham J. Surrounded by mycobacteria: nontuberculous mycobacteria in the human environment. J Appl Microb. 2009; 107: 356-67.

146. Fang Z, Morrison N, Watt B, Doig C, Forbes K. IS*6110* transposition and evolutionary scenario of the direct repeat locus in a group of closely related *Mycobacterium tuberculosis* strains. J Bacteriol. 1998; 180: 2102-9.

147. Feniouk B, Suzuki T, Yoshida M. Regulatory interplay between proton motive force, ADP, phosphate, and subunit ϵ in bacterial ATP synthase. J Biol Chem. 2007; 282: 764-72.

148. Feuerriegel S, Cox H, Zarkua N, Karimovich H, Braker K, Rüsch-Gerdes S, Niemann S. Sequence analyses of just four genes to detect extensively drug-resistant *Mycobacterium tuberculosis* strains in multidrug resistant tuberculosis patients undergoing treatment. Antimicrob Agents Chemother. 2009; 53: 3353-6.

149. Feuerriegel S, Köser C, Baù D, Rüsch-Gerdes S, Summers D, Archer J, Marti-Renom M, Niemann S. Impact of *fgd1* and *ddn* diversity in *Mycobacterium tuberculosis* complex on *in vitro* susceptibility to PA-824. Antimicrob Agents Chemother. 2011; 55: 5718-22.

150. Feuerriegel S, Köser C, Richter E, Niemann S. *Mycobacterium canettii* is intrinsically resistant to both pyrazinamide and pyrazinoic acid. J Antimicrob Chemother. 2013; 68: 1439-40.

151. Filliol I, Motiwala A, Cavatore M, Qi W, Hazbón M, del Valle M, Fyfe J, García-García L, Rastogi N, Sola C, Zozio T, Guerrero M, León C, Crabtree J, Angiuoli S, Eisenach K, Durmaz R, Joloba M, Rendón A, Sifuentes-Osornio J, de León A, Cave M, Fleischmann R, Whittam T, Alland D. Global phylogeny of *Mycobacterium tuberculosis* based on single nucleotide polymorphism (SNP) analysis: insights into tuberculosis evolution, phylogenetic accuracy of other DNA fingerprinting systems, and recommendations for a minimal standard SNP. J Bacteriol. 2006; 188: 759-72.

152. Finken M, Kirschner P, Meier A, Wrede A, Böttger E. Molecular basis of streptomycin resistance in *Mycobacterium tuberculosis*: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. Mol Microbiol. 1993; 9: 1239-46.

153. Finlay A, Hobby G, Hoschstein F, Lees T, Lenert T, Means J, P'an S, Regna P, Routien J, Sobin B, Tate K, Kane J. Viomycin a new antibiotic active against mycobacteria. Am Rev Tuberc. 1951; 63: 1-3.

154. Firdessa R, Berg S, Hailu E, Schelling E, Gumi B, Erenso G, Gadisa E, Kiros T, Habtamu M, Hussein J, Zinsstag J, Robertson B, Ameni G, Lohan A, Loftus B, Comas I, Gagneux S, Tschopp R, Yamuah L, Hewinson G, Gordon S, Young D, Aseffa A. Mycobacterial lineages causing pulmonary and extrapulmonary tuberculosis, Ethiopia. Emerg Infect Dis. 2013; 19: 460-3.

155. Ford C, Shah R, Maeda M, Gagneux S, Murray M, Cohen T, Johnston J, Gardy J, Lipsitch M, Fortune S. *Mycobacterium tuberculosis* mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. Nat Genet. 2013; 45: 784-90.

156. Formicola V, Milanesi Q, Scarsini C. Evidence of spinal tuberculosis at the beginning of the fourth millennium BC from Arene Candide cave (Liguria, Italy). Am J Phys Anthropol. 1987; 72: 1-6.

157. Frothingham R, Meeker-O'Connell W. Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. Microbiology. 1998; 144: 1189-96.

158. Gagneux S, DeRiemer K, Van T, Kato-Maeda M, de Jong B, Narayanan S, Nicol M, Niemann S, Kremer K, Gutierrez M, Hilt M, Hopewell P, Small P. Variable host–pathogen compatibility in *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA. 2006; 103: 2869-73.

159. Garrett A. O problema da tuberculose em Portugal: traçado d'um plano. Imp. C. Vasconcellos. Porto. 1906

160. Gellert M, Mizuuchi K, O´Dea M, Nash H. DNA gyrase: an enzyme that introduces superhelical turns into DNA. Proc Natl Acad Sci USA. 1976; 73: 3872-6.

161. Gelperina S, Kisich K, Iseman M, Heifets L. The potential advantages of nanoparticle drug delivery systems in chemotherapy of tuberculosis. Am J Respir Crit Care Med. 2005; 172: 1487-90.

162. Gikalo M, Nosova E, Krylova L, Moroz A. The role of *eis* mutations in the development of kanamycin resistance in *Mycobacterium tuberculosis* isolates from the Moscow region. J Antimicrob Chemother. 2012; 67: 2107-9.

163. Gordon S, Brosch R, Billault A, Garnier T, Eiglmeier K, Cole S. Identification of variable regions in the genomes of tubercle bacilli using bacterial artificial chromosome arrays. Mol Microbiol. 1999; 32: 643-55.

164. Goyal M, Saunders N, van Embden J, Young D, Shaw R. Differentiation of *Mycobacterium tuberculosis* isolates by spoligotyping and IS*6110* restriction fragment length polymorphism. J Clin Microbiol. 1997; 35: 647-51.

165. Grange J. The biology of the genus Mycobacterium. J Appl Bacteriol. 1996; 81: 1S-9S.

166. Griffith S. Atypical strains of tubercle bacilli in human tuberculosis. J Hyg. 1941; 41: 272-83.

167. Griffiths G, Nyström B, Sable S, Khuller G. Nanobead-based interventions for the treatment and prevention of tuberculosis. Nat Rev Microbiol. 2010; 8: 827-34.

168. Guo H, Seet Q, Denkin S, Parsons L, Zhang Y. Molecular characterization of isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis* from the USA. J Med Microbiol. 2006; 55: 1527-31.

169. Gupta A, Reddy V, Lavania M, Chauhan D, Venkatesan K, Sharma V, Tyagi A, Katoch V M. *jefA* (Rv2459), a drug efflux gene in *Mycobacterium tuberculosis* confers resistance to isoniazid and ethambutol. Indian J Med Res. 2010; 132: 176-88.

170. Gupta S, Cohen K, Winglee K, Maiga M, Diarra B, Bishai W. 2013. Efflux inhibition with verapamil potentiates bedaquiline in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 58: 574-6.

171. Gupta S, Tyagi S, Almeida D, Maiga M, Ammerman N, Bishai W. Acceleration of tuberculosis treatment by adjunctive therapy with verapamil as an efflux inhibitor. Am J Respir Crit Care Med. 2013; 188: 600-7.

172. Gutacker M, Mathema B, Soini H, Shashkina E, Kreiswirth B, Graviss E, Musser J. Single-nucleotide polymorphism-based population genetic analysis of *Mycobacterium tuberculosis* strains from 4 geographic sites. J Infect Dis. 2006; 193: 121-8.

173. Haagsma S, Abdillahi-Ibrahim R, Wagner M, Krab K, Vergauwen K, Guillemont J, Andries K, Lill H, Koul A, Bald D. Selectivity of TMC207 towards mycobacterial ATP synthase compared with that towards the eukaryotic homologue. Antimicrob Agents Chemother. 2009; 53: 1290-2.

174. Han X, Pham A, Tarrand J, Sood P, Luthra R. Rapid and accurate identification of mycobacteria by sequencing hypervariable regions of the 16S ribosomal RNA gene. Am J Clin Pathol. 2002; 118: 796-801.

175. Hart P, Armstrong J, Brown C, Draper P. Ultrastructural study of the behavior of macrophages toward parasitic mycobacteria. Infect Immun. 1972; 5: 803-7.

176. Hartkoorn R, Uplekar S, Cole S. Cross-resistance between clofazimine and bedaquiline through upregulation of MmpL5 in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2014; 58: 2979-81.

177. Hartmann G, Honikel K, Knüsel F, Nüesch J. The specific inhibition of the DNA-directed RNA synthesis by rifamycin. Biochim Biophys Acta. 1967; 145: 843-4.

178. Hartmann G, Heinrich P, Kollenda M, Skrobranek B, Tropschug M, Weiß W. Molecular mechanism of action of the antibiotic rifampicin. Angewandte Chemie International Edition in English. 1985; 24: 1009-14.

179. Hartnell G, Wilde P. Digital coronary angiography: the new gold standard? Br Heart J. 1991; 66: 196-8.

180. Hazbón M, del Valle M, Guerrero M, Varma-Basil M, Filliol I, Cavatore M, Colangeli R, Safi H, Billman-Jacobe H, Lavender C, Fyfe J, García-García L, Davidow A, Brimacombe M, León C, Porras T, Bose M, Chaves F, Eisenach K, Sifuentes-Osornio J, de León A, Cave M, Alland D. Role of *embB* codon 306 mutations in *Mycobacterium tuberculosis* revisited: a novel association with broad drug resistance and IS*6110* clustering rather than ethambutol resistance. Antimicrob Agents Chemother. 2005; 49: 3794-802.

181. Hazbón M, Brimacombe M, del Valle M, Cavatore M, Guerrero M, Varma-Basil M, Billman-Jacobe H, Lavender C, Fyfe J, García-García L, León C, Bose M, Chaves F, Murray M, Eisenach K, Sifuentes-Osornio J, Cave M, Ponce de León A, Alland D. Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2006; 50: 2640-9.

182. Heep M, Brandstatter B, Rieger U, Lehn N, Richter E, Rüsch-Gerdes S, Niemann S. Frequency of *rpoB* mutations inside and outside the cluster I region in rifampin resistant clinical *Mycobacterium tuberculosis* isolates. J Clin Microbiol. 2001; 39: 107-10.

183. Heifets L, Lindholm-Levy P. Pyrazinamide sterilizing activity *in vitro* against semidormant *Mycobacterium tuberculosis* bacterial populations. Am Rev Respir Dis. 1992; 145: 1223-5.

184. Herr E, Jr, Haney M, Pittenger G, Higgens C. Isolation and characterization of a new peptide antibiotic. Proc Indiana Acad Sci. 1960; 69: 134.

185. Herr E, Jr, Redstone M. Chemical and physical characterization of capreomycin. Ann NY Acad Sci. 1966; 135: 940-6.

186. Herrera L, Jiménez S, Valverde A, García-Aranda M, Sáez-Nieto J. Molecular analysis of rifampicin-resistant *Mycobacterium tuberculosis* isolated in Spain (1996/2001). Description of new mutations in the *rpoB* gene and review of the literature. Int J Antimicrob Agents. 2003; 21: 403-8.

187. Herzog B. History of tuberculosis. Respiration. 1998; 65: 5-15.

188. Heym B, Cole S. Isolation and characterization of isoniazid-resistant mutants of *Mycobacterium smegmatis* and *M. aurum*. Res Microbiol. 1992; 143: 721-30.

189. Heym B, Zhang Y, Poulet S, Young D, Cole S. Characterization of the *katG* gene encoding a catalase-peroxidase required for the isoniazid susceptibility of *Mycobacterium tuberculosis*. J Bacteriol. 1993; 175: 4255-9.

190. Heym B, Saint-Joanis B, Cole S. The molecular basis of isoniazid resistance in *Mycobacterium tuberculosis*. Tuber Lung Dis. 1999; 79: 267-71.

191. Hillemann D, Rüsch-Gerdes S, Richter E. Feasibility of the GenoType MTBDR*sl* assay for fluoroquinolone, amikacin-capreomycin, and ethambutol resistance testing of *Mycobacterium tuberculosis* strains and clinical specimens. J Clin Microbiol. 2009; 47: 1767-72.

192. Hirsh A, Tsolaki A, DeRiemer K, Feldman M, Small P. Stable association between strains of *Mycobacterium tuberculosis* and their human host populations. Proc Natl Acad Sci USA. 2004; 101: 4871-76.

193. Huang H, Scherman M, D'Haeze W, Vereecke D, Holsters M, Crick D, McNeil M. Identification and active expression of the *Mycobacterium tuberculosis* gene encoding 5-phospho-α-D-ribose-1-diphosphate: decaprenyl-phosphate 5-phosphoribosyltransferase, the first enzyme committed to decaprenylphosphoryl-D-arabinose synthesis. J Biol Chem. 2005; 280: 24539-43.

194. Huang W, Chi T, Wu M, Jou R. Performance assessment of the GenoType MTBDR*sl* test and DNA sequencing for detection of second-line and ethambutol drug resistance among patients infected with multidrug-resistant *Mycobacterium tuberculosis*. J Clin Microbiol. 2011; 49: 2502-8.

195. Huitric E, Werngren J, Juréen P, Hoffner S. Resistance levels and *rpoB* gene mutations among *in vitro*-selected rifampin-resistant *Mycobacterium tuberculosis* mutants. Antimicrob Agents Chemother. 2006; 50: 2860-2.

196. Huitric E, Verhasselt P, Koul A, Andries K, Hoffner S, Andersson D. Rates and mechanisms of resistance development in *Mycobacterium tuberculosis* to a novel diarylquinoline ATP synthase inhibitor. Antimicrob Agents Chemother. 2010; 54: 1022-8.

197. Jacques A. Clinical uses of papaverine hydrochloride. Anesthesia and Analgesia. 1950; 29: 22-8.

198. Jain A, Mondal R, Srivastava S, Prasad R, Singh K, Ahuja R. Novel mutations in *embB* gene of ethambutol resistant isolates of *Mycobacterium tuberculosis*: a preliminary report. Indian J Med Res. 2008; 128: 634-9.

199. Jain A, Dhammi I, Jain S, Mishra P. Kyphosis in spinal tuberculosis - prevention and correction. Indian J Orthop. 2010; 44: 127-36.

200. Jiang X, Zhang W, Zhang Y, Gao F, Lu C, Zhang X, Wang H. Assessment of efflux pump gene expression in a clinical isolate *Mycobacterium tuberculosis* by real-time reverse transcription PCR. Microb Drug Resist. 2008; 14: 7-11.

201. Johansen S, Maus C, Plikaytis B, Douthwaite S. Capreomycin binds across the ribosomal subunit interface using tlyA-encoded 2'-O-methylations in 16S and 23S rRNAs. Mol Cell. 2006; 23: 173-82.

202. Johnson R, Streicher E, Louw G, Warren R, van Helden P, Victor T. Drug resistance in *Mycobacterium tuberculosis*. Curr Issues Mol Biol. 2006; 8: 97-112.

203. Jugheli L, Bzekalava N, de Rijk P, Fissette K, Portaels F, Rigouts L. High level of cross-resistance between kanamycin, amikacin, and capreomycin among *Mycobacterium tuberculosis* isolates from Georgia and a close relation with mutations in the *rrs* gene. Antimicrob Agents Chemother. 2009; 53: 5064-8.

204. Kamerbeek J, Schouls L, Kolk A, Van Agterveld M, Van Soolingen D, Bunschoten K, Molhuizen H, Shaw R, van Embden J. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol. 1997; 35: 907-14.

205. Kapur V, Li L, Iordanescu S, Hamrick M, Wanger A, Kreiswirth B, Musser J. Characterization by automated DNA sequencing of mutations in the gene (*rpoB*) encoding the RNA polymerase β subunit in rifampin-resistant *Mycobacterium tuberculosis* strains from New York City and Texas. J Clin Microbiol. 1994; 32: 1095-8.

206. Karamanou M, Androutsos G. The masterful description of pulmonary tuberculosis by Soranus of Ephesus (c. 98–138 A.D.). Am J Respir Crit Care Med. 2012; 186: 571-1.

207. Karlson A. The *in vitro* activity of ethambutol (dextro-2,2 [ethylenediimino]di-1-butanol) against tubercle bacilli and other microorganisms. Am Rev Resp Dis. 1961; 83: 905.

208. Karlson A. Therapeutic effect of ethambutol (dextro-2,2'-[ethylene-diimino]- di-1-butanol) on experimental tuberculosis in guinea pigs. Am Rev Respir Dis. 1961; 84: 902-4.

209. Kato J, Nishimura Y, Imamura R, Niki H, Hiraga S, Suzuki H. New topoisomerase essential for chromosome segregation in *E. coli*. Cell. 1990; 63: 393-404.

210. Kato-Maeda M, Kim E, Flores L, Jarlsberg L, Osmond D, Hopewell P. Differences among sublineages of the East-Asian lineage of *Mycobacterium tuberculosis* in genotypic clustering. Int J Tuberc Lung Dis. 2010; 14: 538-44.

211. Kelly C, Jefferies C, Cryan S. Targeted liposomal drug delivery to monocytes and macrophages. J Drug Deliv. 2011; doi:10.1155/2011/727241.

212. Kerscher S. Diversity and origin of alternative NADH: ubiquinone oxidoreductases. Biochim Biophys Acta. 2000; 1459: 274-83.

213. Kiers A, Klarenbeek A, Mendelts B, Van Soolingen D, Koëter G. Transmission of *Mycobacterium pinnipedii* to humans in a zoo with marine mammals. Int J Tuberc Lung Dis. 2008; 12: 1469-73.

214. Kiet V, Lan N, An D, Dung N, Hoa D, Chau N, Chinh N, Farrar J, Caws M. Evaluation of the MTBDR*sl* test for detection of second-line-drug resistance in *Mycobacterium tuberculosis*. J Clin Microbiol 2010; 48: 2934-9.

215. Kilburn J, Greenberg J. Effect of ethambutol on the viable cell count in *Mycobacterium smegmatis*. Antimicrob Agents Chemother. 1977; 11: 534-40.

216. Kilburn J, Takayama K. Effects of ethambutol on accumulation and secretion of trehalose mycolates and free mycolic acid in *Mycobacterium smegmatis*. Antimicrob Agents Chemother. 1981; 20: 401-4.

217. Koch R. Die aetiologie der tuberkulose. J Mol Med. 1932; 11: 490-2.

218. Koch R. A further communication on a remedy for tuberculosis. Br Med J. 1891; 17: 125-7.

219. Köhler K, Molnár E, Zalai-Gaál I, Osztás A, Bánffy E, Kirinó K, Kiss K, Mende B. A late Neolithic case of Pott's disease from Hungary. Int J Osteoarchaeol. 2012; 10.1002/oa.2254.

220. Konno K, Feldmann F, McDermott W. Pyrazinamide susceptibility and amidase activity of tubercle bacilli. Am Rev Respir Dis. 1967; 95: 461-9.

221. Köser C, Feuerriegel S, Summers D, Archer J, Niemann S. Importance of the genetic diversity within the *Mycobacterium tuberculosis* complex for the development of novel antibiotics and diagnostic tests of drug resistance. Antimicrob Agents Chemother. 2012; 56: 6080-7.

222. Kotra L, Haddad J, Mobashery S. Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. Antimicrob Agents Chemother. 2000; 44: 3249-56.

223. Koul A, Dendouga N, Vergauwen K, Molenberghs B, Vranckx L, Willebrords R, Ristic Z, Lill H, Dorange I, Guillemont J, Bald D, Andries K. Diarylquinolines target subunit c of mycobacterial ATP synthase. Nat Chem Biol. 2007; 3: 323-4.

224. Koul A, Vranckx L, Dendouga N, Balemans W, den Wyngaert I, Vergauwen K, Göhlmann H, Willebrords R, Poncelet A, Guillemont J, Bald D, Andries K. Diarylquinolines are bactericidal for dormant mycobacteria as a result of disturbed ATP homeostasis. J Biol Chem. 2008; 283: 25273-80.

225. Koul A, Vranckx L, Dhar N, Göhlmann H, Özdemir E, Neefs J, Schulz M, Lu P, Mørtz E, McKinney J, Andries K, Bald D. Delayed bactericidal response of *Mycobacterium tuberculosis* to bedaquiline involves remodelling of bacterial metabolism. Nat Commun. 2014; 5: 3369.

226. Kubica G, Kim T, Dunbar F. Designation of strain H37Rv as the neotype of *Mycobacterium tuberculosis*. Int J Syst Bacteriol. 1972; 22: 99-106.

227. Kumar A, Schweizer H. Bacterial resistance to antibiotics: active efflux and reduced uptake. Adv Drug Deliv Rev. 2005; 57: 1486-513.

228. Kushner S, Yeager R, Munroe W, Dessau F. Schwartz W Minutes 11th conference on chemotherapy tuberc. VA., St. Louis, Mo. 1952.

229. Lange C, Pai M, Drobniewski F, Migliori G. Interferon-γ release assays for the diagnosis of active tuberculosis: sensible or silly? Eur Respir J. 2009; 33: 1250-3.

230. Lau R, Ho P, Kao R, Yew W, Lau T, Cheng V, Yuen K, Tsui S, Chen X, Yam W. Molecular characterization of fluoroquinolone resistance in *Mycobacterium tuberculosis*: functional analysis of *gyrA* mutation at position 74. Antimicrob Agents Chemother. 2011; 55: 608-14.

231. Lawn S, Nicol M. Xpert® MTB/RIF assay: development, evaluation and implementation of a new rapid molecular diagnostic for tuberculosis and rifampicin resistance. Future Microbiol. 2011; 6: 1067-82.

232. Lazarevic V, Flynn J. CD8$^+$ T cells in tuberculosis. Am J Respir Crit Care Med. 2002; 166: 1116-21.

233. Lebrun L, Espinasse F, Poveda J, Vincent-Levy-Frebault V. Evaluation of nonradioactive DNA probes for identification of mycobacteria. J Clin Microbiol. 1992; 30: 2476-8.

234. Lee A, Teo A, Wong S. Novel mutations in *ndh* in isoniazid-resistant *Mycobacterium tuberculosis* isolates. Antimicrob Agents Chemother 2001; 45: 2157-9.

235. Lee A, Othman S, Ho Y, Wong S. Novel mutations within the *embB* gene in ethambutol-susceptible clinical isolates of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2004; 48: 4447-9.

236. Lee R, Mikušová K, Brennan P, Besra G. Synthesis of the mycobacterial arabinose donor β-d- arabinofuranosyl- 1 -monophosphoryldecaprenol, development of a basic arabinosyl-transferase assay, and identification of ethambutol as an arabinosyl transferase inhibitor. J Am Chem SOC. 1995; 117: 11829-32.

237. Lemaitre N, Sougakoff W, Truffot-Pernot C, Jarlier V. Characterization of new mutations in pyrazinamide-resistant strains of *Mycobacterium tuberculosis* and identification of conserved regions important for the catalytic activity of the pyrazinamidase *pncA*. Antimicrob Agents Chemother. 1999; 43: 1761-3.

238. Lesher G, Froelich E, Gruett M, Bailey J, Brundage R. 1, 8-Naphthyridine derivatives: a new class of chemotherapeutic agents. J Med Pharm Chem. 1962; 5:1063-5.

239. Leung E, Ho P, Yuen K, Woo W, Lam T, Kao R, Seto W, Yam W. Molecular characterization of isoniazid resistance in *Mycobacterium tuberculosis*: identification of a novel mutation in *inhA*. Antimicrob Agents Chemother 2006; 50: 1075-8.

240. Levin M, Hatfull G. *Mycobacterium smegmatis* RNA polymerase: DNA supercoiling, action of rifampicin and mechanism of rifampicin resistance. Mol Microbiol. 1993; 8: 277-85.

241. Levine C, Hiasa H, Marians K. DNA gyrase and topoisomerase IV: biochemical activities, physiological roles during chromosome replication, and drug sensitivities. Biochim Biophys Acta. 1998; 1400: 29-43.

242. Lew J, Kapopoulou A, Jones L, Cole S. TubercuList - 10 years after. Tuberculosis (Edinb). 2011; 91: 1-7.

243. Li X, Zhang L, Nikaido H. Efflux pump-mediated intrinsic drug resistance in *Mycobacterium smegmatis*. Antimicrob Agents Chemother. 2004; 48: 2415-23.

244. Lopes C, Santos A, Cunha E. A possible case of human tuberculosis on a medieval farm in Corroios (Portugal) [abstract]. *In*: Cockburn E, editor. Paleopathology Association. Papers on paleopathology presented at the 26th Annual Meeting of the Paleopathology Association, Columbus, Ohio. Detroit, MI: p 12. 1999.

245. López-Muñoz F, Alamo C, Cuenca E, Shen W, Clervoy P, Rubio G. History of the discovery and clinical introduction of chlorpromazine. Ann Clin Psychiatry. 2005; 17: 113-35.

246. Louw G, Warren R, Donald P, Murray M, Bosman M, Van Helden P, Young D, Victor T. Frequency and implications of pyrazinamide resistance in managing previously treated tuberculosis patients. Int J Tuberc Lung Dis. 2006; 10: 802-7.

247. Louw G, Warren R, van Pittius N, McEvoy C, van Helden P, Victor T. A balancing act: efflux/influx in mycobacterial drug resistance. Antimicrob Agents Chemother. 2009; 53: 3181-9.

248. Louw G, Warren R, van Pittius N, Leon R, Jimenez A, Hernandez-Pando R, McEvoy C, Grobbelaar M, Murray M, van Helden P, Victor T. Rifampicin reduces susceptibility to ofloxacin in rifampicin-resistant *Mycobacterium tuberculosis* through efflux. Am J Respir Crit Care Med. 2011; 184: 269-76.

249. Lyle J, Hammond P, Wiese W, Nelson N. Assay formats involving acridinium-ester-labeled DNA probes. Clin Chem. 1989; 35: 1588-94.

250. Machado D, Couto I, Perdigão J, Rodrigues L, Baptista P, Portugal I, Veigas B, Amaral L, Viveiros M. Contribution of efflux to the emergence of isoniazid and multidrug resistance in *Mycobacterium tuberculosis*. PLoS One. 2012; 7: e34538.

251. Machado D, Perdigão J, Ramos J, Couto I, Portugal I, Ritter C, Boettger EC, Viveiros M. High-level resistance to isoniazid and ethionamide in multidrug-resistant *Mycobacterium tuberculosis* of the Lisboa family is associated with *inhA* double mutations. J Antimicrob Chemother. 2013; 68: 1728-32.

252. Madison B, Robinson-Dunn B, George I, Gross W, Lipman H, Metchock B, Sloutsky A, Washabaugh G, Mazurek G, Ridderhof J. Multicenter evaluation of ethambutol susceptibility testing of *Mycobacterium tuberculosis* by agar proportion and radiometric methods. J Clin Microbiol. 2002; 40: 3976-9.

253. Mahairas G, Sabo P, Hickey M, Singh D, Stover C. Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. J Bacteriol. 1996; 6: 1274-82.

254. Mäkinen J, Marttila H, Marjamäki M, Viljanen M, Soini H. Comparison of two commercially available DNA line probe assays for detection of multidrug-resistant *Mycobacterium tuberculosis*. J Clin Microbiol. 2006; 44: 350-2.

255. Malik M, Chavda K, Zhao X, Shah N, Hussain S, Kurepina N, Kreiswirth B, Kerns J, Drlica K. Induction of mycobacterial resistance to quinolone class antimicrobials. Antimicrob Agents Chemother. 2012; 56: 3879-87.

256. Manjunatha U, Boshoff H, Dowd C, Zhang L, Albert T, Norton J, Daniels L, Dick T, Pang S, Barry C. Identification of a nitroimidazo-oxazine-specific protein involved in PA-824 resistance in *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA. 2006; 103: 431-6.

257. Manjunatha U, Boshoff H, Barry III C. The mechanism of action of PA-824. Commun Integr Biol. 2009; 2: 215-8.

258. Marinho R. Waiting for success. Acta Med Port. 2013; 26: 74-5.

259. Marrakchi H, Lanéelle G, Quémard A. InhA, a target of the antituberculous drug isoniazid, is involved in a mycobacterial fatty acid elongation system, FAS-II. Microbiology. 2000; 146: 289-96.

260. Marrakchi H, Bardou F, Lanéelle M, Daffé M. A comprehensive overview of mycolic acid structure and biosynthesis. *In* The Mycobacterial Cell Envelope. Daffé M, Reyrat J-M, eds. pp. 41–62, Washington, DC. ASM Press, 2008.

261. Martinez J, Sánchez M, Martinez-Solano L, Hernandez A, Garmendia L, Fajardo A, Alvarez-Ortega C. Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. FEMS Microbiol Rev. 2009; 33: 430-49.

261a. Martins M, Viveiros M, Amaral L. The TB laboratory of the future: the role of the macrophage in the selection of agents that can be used for the successful therapy of an XDR-TB infection. Future Microbiol. 2008; 3: 135-44.

262. Masson M, Molnár E, Donoghue H, Besra G, Minnikin D, Wu H, Lee O, Bull I, Pálfi G. Osteological and biomolecular evidence of a 7000-year-old case of hypertrophic pulmonary osteopathy secondary to tuberculosis from the Neolithic Hungary. PLoS One. 2013; 8: e78252.

263. Masur B. Zur Biologie des Tuberkelbazillus. Centralbl. f. Bakteriol. 1926; 1: 46-52.

264. Mathema B, Kurepina N, Bifani P, Kreiswirth B. Molecular epidemiology of tuberculosis: current insights. Clin Micro Rev. 2006; 19: 658-85.

265. Matos V, Marques C, Lopes C. Severe vertebral collapse in a juvenile from the graveyard (13^{th}/14^{th}–19^{th} centuries) of the São Miguel Church (Castelo Branco, Portugal): differential palaeopathological diagnosis. Int J Osteoarchaeol. 2011; 21: 208-17.

266. Matos V, Santos A. On the trail of pulmonary tuberculosis based on rib lesions: results from the Human Identified Skeletal Collection from the Museu Bocage (Lisbon, Portugal). Am J Phys Anthropol. 2006; 30: 190-200.

267. Matsiota-Bernard P, Vrioni G, Marinis E. Characterization of *rpoB* mutations in rifampin-resistant clinical *Mycobacterium tuberculosis* isolates from Greece. J Clin Microbiol. 1998; 36: 20-3.

268. Matsumoto M, Hashizume H, Tomishige T, Kawasaki M, Tsubouchi H, Sasaki H, Tsubouchi H, Sasaki H, Shimokawa Y, Komatsu M. OPC-67683, a nitro-dihydro-imidazooxazole derivative with promising action against tuberculosis *in vitro* and in mice. PLoS Med. 2006; 3: e466.

269. Matsuno-Yagi A, Hatefi Y. Studies on the mechanism of oxidative phosphorylation. Different effects of F0 inhibitors on unisite and multisite ATP hydrolysis by bovine submitochondrial particles. J Biol Chem. 1993; 268: 1539-45.

270. Matsuno-Yagi A, Hatefi Y. Studies on the mechanism of oxidative phosphorylation. ATP synthesis by submitochondrial particles inhibited at F0 by venturicidin and organotin compounds. J Biol Chem. 1993; 268: 6168-73.

271. Maus C, Plikaytis B, Shinnick T. Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2005; 49: 3192-7.

272. Maus C, Plikaytis B, Shinnick T. Mutation of *tlyA* confers capreomycin resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2005; 49: 571-7.

273. Mays S, Taylor G. A first prehistoric case of tuberculosis from Britain. Int J Osteoarchaeol. 2003; 13: 189-96.

274. McKeegan K, Borges-Walmsley M, Walmsley A. The structure and function of drug pumps: an update. Trends Microbiol. 2003; 11: 21-9.

275. McNeil M, Wallner S, Hunter S, Brennan P. Demonstration that the galactosyl and arabinosyl residues in the cell-wall arabinogalactan of *Mycobacterium leprae* and *Mycobacterium tuberculosis* are furanoid. Carbohydr Res. 1987; 166: 299-308.

276. McNeil M, Daffe M, Brennan P. Evidence for the nature of the link between the arabinogalactan and peptidoglycan of mycobacterial cell walls. J Biol Chem. 1990; 265: 18200-6.

277. McTavish D, Sorkin E. Verapamil. An updated review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in hypertension. Drugs. 1989; 38: 19-76.

278. Means T, Wang S, Lien E, Yoshimura A, Golenbock D, Fenton M. Human toll-like receptors mediate cellular activation by *Mycobacterium tuberculosis*. J Immunol. 1999; 163: 3920-27.

279. Mehta A, Shaha C. Apoptotic death in *Leishmania donovani* promastigotes in response to respiratory chain inhibition. Complex II inhibition results in increased pentamidine cytotoxicity. J Biol Chem. 2003; 279: 11798-813.

280. Meier A, Kirschner P, Bange F, Vogel U, Böttger EC. Genetic alterations in streptomycin-resistant *Mycobacterium tuberculosis*: mapping of mutations conferring resistance. Antimicrob Agents Chemother. 1994; 38: 228-33.

281. Meier A, Sander P, Schaper K, Scholz M, Böttger EC. Correlation of molecular resistance mechanisms and phenotypic resistance levels in streptomycin-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1996; 40: 2452-4.

282. Merle C, Sow O, Gninafon M, Horton J, Lapujade O, Lo M, Mitchinson D, Perronne C, Portaels F, Odhiambo J, Olliaro P, Rustomjee R, Lienhardt C, Fielding K. A pivotal registration phase III, multicenter, randomized tuberculosis controlled trial: design issues and lessons learnt from the gatifloxacin for TB (OFLOTUB) project. Trials. 2012; 13: 61.

283. Mestdagh M, Fonteyne P, Realini L, Rossau R, Jannes G, Mijs W, De Smet K, Portaels F, Van den Eeckhout E. Relationship between pyrazinamide resistance, loss of pyrazinamidase activity, and mutations in the *pncA* locus in multidrug-resistant clinical isolates of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1999; 43: 2317-9.

284. Miesel L, Weisbrod T, Marcinkeviciene J, Bittman R, Jacobs Jr W. NADH dehydrogenase defects confer isoniazid resistance and conditional lethality in *Mycobacterium smegmatis*. J Bacteriol. 1998; 180: 2459-67.

285. Milano A, Pasca M, Provvedi R, Lucarelli A, Manina G, Ribeiro A, Manganelli R, Riccardi G. Azole resistance in *Mycobacterium tuberculosis* is mediated by the MmpS5–MmpL5 efflux system. Tuberculosis. 2009; 89: 84-90.

286. Miller L, Crawford J, Shinnick T. The *rpoB* gene of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1994; 38: 805-11.

287. Minnikin D, Lee O, Wu H, Besra G, Donoghue H. Molecular biomarkers for ancient tuberculosis. *In*: Cardona P-J, editor. Understanding tuberculosis – deciphering the secret life of the bacilli. Rijeka, Croatia: InTech - Open Access Publisher. 2012; 1-36.

288. Miranda M, Breiman A, Allain S, Deknuydt F, Altare F. The tuberculous granuloma: an unsuccessful host defence mechanism providing a safety shelter for the bacteria? Clin Dev Immunol. 2012; doi:10.1155/2012/139127.

289. Mitchel P. Proton current flow in mitochondrial systems. Nature. 1961; 191: 144-8.

290. Mitchison D. Basic mechanisms of chemotherapy. Chest.1979; 76: S771-81.

291. Mitchison D. The action of antituberculosis drugs in short-course chemotherapy. Tubercle. 1985; 66: 219-25.

292. Moazed D, Noller H. Interaction of antibiotics with functional sites in 16S ribosomal RNA. Nature. 1987; 327: 389-94.

293. Modica-Napolitano J, Lagace C, Brennan W, Aprille J. Differential effects of typical and atypical neuroleptics on mitochondrial function *in vitro*. Arch Pharm Res. 2003; 26: 951-9.

294. Modolell J, Vazquez D. The inhibition of ribosomal translocation by viomycin. Eur J Biochem. 1977; 81: 491-7.

295. Mokrousov I, Otten T, Vyshnevskiy B, Narvskaya O. Detection of *embB306* mutations in ethambutol-susceptible clinical isolates of *Mycobacterium tuberculosis* from Northwestern Russia: implications for genotypic resistance testing. J Clin Microbiol. 2002; 40: 3810-3.

296. Monshupanee T, Gregory S, Douthwaite S, Chungjatupornchai W, Dahlberg A. Mutations in conserved helix 69 of 23S rRNA of *Thermus thermophilus* that affect capreomycin resistance but not posttranscriptional modifications. J Bacteriol. 2008; 190: 7754-61.

297. Moran N. Microbial minimalism: genome reduction in bacterial pathogens. Cell. 2002; 108: 583-6.

298. Müller B, Borrell S, Rose G, Gagneux S. The heterogeneous evolution of multidrug-resistant *Mycobacterium tuberculosis.* Trends Genet. 2013; 29: 160-9.

299. Muren J, Bloom B. Thioxanthene psychopharmacological agents. II. 9-(3-aminopropylidene)-N,N-dimethylthioxantene-Z-sulfonamides. J Med Chem. 1970; 13: 17-23.

300. Muto A, Fujihara A, Ito K, Matsuno J, Ushida C, Himeno H. Requirement of transfer-messenger RNA for the growth of *Bacillus subtilis* under stresses. Genes to Cells. 2000; 5: 627-35.

301. Nagarajan K, Shankar R, Rajappa S, Shenoy S, Costa-Pereira R. Nitroimidazoles XXI 2,3-dihydro-6-nitroimidazo [2,1-b] oxazoles with antitubercular activity. Eur J Med Chem. 1989; 24: 631-3.

302. Ng V, Cox J, Sousa A, MacMicking J, McKinney J. Role of KatG catalase-peroxidase in mycobacterial pathogenesis: countering the phagocyte oxidative burst. Mol Microbiol. 2004; 52: 1291-302.

303. Niederweis M, Danilchanka O, Huff J, Hoffmann C, Engelhardt H. Mycobacterial outer membranes: in search of proteins. Trends Microbiol. 2010; 18: 109-16.

304. Niemann S, Harmsen D, Rüsch-Gerdes S, Richter E. Differentiation of clinical *Mycobacterium tuberculosis* complex isolates by *gyrB* DNA sequence polymorphism analysis. J Clin Microbiol. 2000; 38: 3231-4.

305. Niemann S, Rüsch-Gerdes S, Joloba M, Whalen C, Guwatudde D, Ellner J, Eisenach K, Fumokong N, Johnson J, Aisu T, Mugerwa R, Okwera A, Schwander S. *Mycobacterium africanum* subtype II is associated with two distinct genotypes and is a major cause of human tuberculosis in Kampala, Uganda. J Clin Microbiol. 2002; 40: 3398-405.

306. Nikaido H. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science. 1994; 264: 382-8.

307. Nikaido H. Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. Semin Cell Dev Biol. 2001; 12: 215-23.

308. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev. 2003; 67: 593-656.

309. Nodwell J. Novel links between antibiotic resistance and antibiotic production. J Bacteriol. 2007; 189: 3683-5.

310. Nuermberger E, Tyagi S, Tasneen R, Williams K, Almeida D, Rosenthal I, Grosset J. Powerful bactericidal and sterilizing activity of a regimen containing PA-824, moxifloxacin, and pyrazinamide in a murine model of tuberculosis. Antimicrob Agents Chemother. 2008; 53: 1522-4.

311. O'Garra A, Redford P, McNab F, Bloom C, Wilkinson R, Berry M. The immune response in tuberculosis. Annu Rev Immunol. 2013; 31: 475-527.

312. Okamoto S, Tamaru A, Nakajima C, Nishimura K, Tanaka Y, Tokuyama S, Suzuki Y, Ochi K. Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. Mol Microbiol. 2007; 63: 1096-106.

313. Ortner D. Disease and mortality in the early Bronze Age people of Bab edh-Dhra, Jordan. Am J Phys Anthropol. 1979; 51: 589-97.

314. Ortner D. Infectious diseases: tuberculosis and leprosy. *In*: DJ Ortner, Identification of pathological conditions in human skeletal remains. Elsevier Academic Press, San Diego, USA. 2003.

315. Pang Y, Lu J, Wang Y, Song Y, Wang S, Zhao Y. Study of the rifampin monoresistance mechanism in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2013; 5: 893-900.

316. Pantel A, Petrella S, Matrat S, Brossier F, Bastian S, Reitter D, Jarlier V, Mayer C, Aubry A. DNA gyrase inhibition assays are necessary to demonstrate fluoroquinolone resistance secondary to *gyrB* mutations in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2011; 55: 4524-9.

317. Pantel A, Petrella S, Veziris N, Brossier F, Bastian S, Jarlier V, Mayer C, Aubry A. Extending the definition of the *gyrB* quinolone resistance-determining region in *Mycobacterium tuberculosis* DNA gyrase for assessing fluoroquinolone resistance in *M. tuberculosis*. Antimicrob Agents Chemother. 2012; 56: 1990-6.

318. Parker K, Petraitis J. Synthesis of ansamycins: an approach to the naphthoquinone portion of the rifamycins and streptovaricins. Tetrahedron Lett. 1981; 22: 397-400.

319. Parsons L, Salfinger M, Clobridge A, Dormandy J, Mirabello L, Polletta V, Sanic A, Sinyavskiy O, Larsen S, Driscoll J, Zickas G, Taber H. Phenotypic and molecular characterization of M*ycobacterium tuberculosis* isolates resistant to both isoniazid and ethambutol. Antimicrob Agents Chemother. 2005; 49: 2218-25.

320. Parsons L, Somoskövi A, Gutierrez C, Lee E, Paramasivan C, Abimiku A, Spector S, Roscigno G, Nkengasong J. Laboratory diagnosis of tuberculosis in resource-poor countries: challenges and opportunities. Clin Microbiol Rev. 2011; 24: 314-50.

321. Pasca M, Guglierame P, Arcesi F, Bellinzoni M, De Rossi E, Riccardi G. Rv2686c-Rv2687c-Rv2688c, an ABC fluoroquinolone efflux pump in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2004; 48: 3175-8.

322. Pasca M, Guglierame P, De Rossi E, Zara F, Riccardi G. *mmpL7* gene of *Mycobacterium tuberculosis* is responsible for isoniazid efflux in Mycobacterium *smegmatis*. Antimicrob Agents Chemother. 49: 4775-7.

323. Pease A. Some remarks on the diagnosis and treatment of tuberculosis in antiquity. Isis. 1940; 31: 380-93.

324. Perdigão J, Macedo R, João I, Fernandes E, Brum L, Portugal I. Multidrug-resistant tuberculosis in Lisbon, Portugal. A molecular epidemiological perspective. Microb Drug Resist. 2008; 14: 133-43.

325. Perdigão J, Macedo R, Ribeiro A, Brum L, Portugal I. Genetic characterization of the ethambutol resistance-determining region in *Mycobacterium tuberculosis*: prevalence and significance of *embB306* mutations. Int J Antimicrob Agents. 2009; 33: 334-8.

326. Perdigão J, Macedo R, Malaquias A, Ferreira A, Brum L, Portugal I. Genetic analysis of extensively drug-resistant *Mycobacterium tuberculosis* strains in Lisbon, Portugal. J Antimicrob Chemother. 2010; 65: 224-7.

327. Perdigão J, Macedo R, Silva C, Pinto C, Furtado C, Brum L, Portugal I. Tuberculosis drug-resistance in Lisbon, Portugal: a 6-year overview. Clin Microbiol Infect. 2011; 17: 1397-402.

328. Perdigão J, Macedo R, Silva C, Machado D, Couto I, Viveiros M, Jordão L, Portugal I. From multidrug-resistant to extensively drug-resistant tuberculosis in Lisbon, Portugal: the stepwise mode of resistance acquisition. J Antimicrob. Chemother. 2013; 68: 23-33.

329. Perdigão J. Molecular and genomic epidemiology of multidrug resistant Mycobacterium tuberculosis isolates in Lisbon, Portugal. PhD Thesis. Faculdade de Farmácia, Universidade de Lisboa. Lisboa, Portugal; 2013.

330. Pestova E, Millichap J, Noskin G, Peterson L. Intracellular targets of moxifloxacin: a comparison with other fluoroquinolones. J Antimicrob Chemother. 2000; 45: 583-90.

331. Petrella S, Cambau E, Chauffour A, Andries K, Jarlier V, Sougakoff W. Genetic basis for natural and acquired resistance to the diarylquinoline R207910 in mycobacteria. Antimicrob Agents Chemother. 2006; 50: 2853-6.

332. Piddock L. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clin Microbiol Rev. 2006; 19: 382-402.

333. Piersimoni C, Scarparo C. Relevance of commercial amplification methods for direct detection of *Mycobacterium tuberculosis* complex in clinical samples. J Clin Microbiol. 2003; 41: 5355-65.

334. Pina J. A tuberculose na viragem do milénio. Lidel-Edições Técnicas, Lda. Lisboa. 2000.

335. Pitaksajjakul P, Wongwit W, Punprasit W, Eampokalap B, Peacock S, Ramasoota P. Mutations in the *gyrA* and *gyrB* genes of fluoroquinolone-resistant *Mycobacterium tuberculosis* from TB patients in Thailand. Southeast Asian J Trop Med Public Health. 2005; 4: 228-37.

336. Plinke C, Rüch-Gerdes S, Niemann S. Significance of mutations in *embB* codon 306 for prediction of ethambutol resistance in clinical *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2006; 50: 1900-02.

337. Plinke C, Cox H, Zarkua N, Karimovich H, Braker K, Diel R, Rüch-Gerdes S, Feuerriegel S, Niemann S. *embCAB* sequence variation among ethambutol-resistant *Mycobacterium tuberculosis* isolates without *embB306* mutation. Antimicrob Agents and Chemother. 2010; 65: 1359-67.

338. Poissy J, Aubry A, Fernandez C, Lott M, Chauffour A, Jarlier V, Farinotti R, Veziris N. Should moxifloxacin be used for the treatment of extensively drug-resistant tuberculosis? An answer from a murine model. Antimicrob Agents Chemother 2010; 54: 4765-71.

339. Portugal I. Epidemiologia molecular da tuberculose multirresistente em Portugal. PhD Thesis. Faculdade de Farmácia, Universidade de Lisboa. Lisboa, Portugal; 1999.

340. Portugal I, Covas M, Brum L, Viveiros M, Ferrinho P, Moniz-Pereira J, David H. Outbreak of multiple drug-resistant tuberculosis in Lisbon: detection by restriction fragment length polymorphism analysis. Int J Lung Dis. 1999; 3: 202-13.

341. Portugal I, Barreiro L, Moniz-Pereira J, Brum L. *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis* isolates in Portugal. Antimicrob Agents Chemother. 2004; 48: 2736-8.

342. Portugal I, Barreiro L, Vultos T, Macedo R, Furtado C, Antunes A, Brum L. Molecular epidemiology of *Mycobacterium tuberculosis* in Lisbon. Rev Port Pneumol. 2008; 14: 239-59.

343. Pozii G, Meloni M, Iona E, Orrù G, Thoresen O, Ricci M, Oggioni M, Fattorini L, Orefici G. *rpoB* mutations in multidrug resistant strains of *Mycobacterium tuberculosis* isolated in Italy. J Clin Microbiol 1999; 37: 1197-9.

344. Pym A, Domenech P, Honoré N, Song J, Deretic V, Cole S. Regulation of catalase–peroxidase (KatG) expression, isoniazid sensitivity and virulence by *furA* of *Mycobacterium tuberculosis*. Mol Microbiol. 2001; 40: 879-89.

345. Quemard A, Dessen A, Sugantino M, Jacobs, WR, Sacchettini J, Blanchard J. Binding of catalase-peroxidase-activated isoniazid to wild-type and mutant *Mycobacterium tuberculosis* enoyl-ACP reductases. J Am Chem Soc. 1996; 118: 1561-2.

346. Qureshi N, Sathyamoorthy N, Takayama K. Biosynthesis of C30 to C56 fatty acids by an extract of *Mycobacterium tuberculosis* H37Ra. J Bacteriol. 1984; 157: 46-52.

347. Ramaswamy S, Musser J. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: update. Tuber Lung Dis. 1998; 79: 3-29.

348. Ramaswamy S, Amin A, Göksel S, Stager C, Dou S, Sahly H, Moghazeh S, Kreiswirth B, Musser J. Molecular genetic analysis of nucleotide polymorphisms associated with ethambutol resistance in human isolates of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2000; 44: 326-36.

349. Ramaswamy S, Reich R, Dou S, Jasperse L, Pan X, Wanger A, Quitugua T, Graviss E. Single nucleotide polymorphisms in genes associated with isoniazid resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2003; 47: 1241-50.

350. Ramaswamy S, Dou S, Rendon A, Yang Z, Cave M, Graviss E. Genotypic analysis of multidrug-resistant *Mycobacterium tuberculosis* isolates from Monterrey, Mexico. J Medical Microbiology. 2004; 53: 107-13.

351. Ramón-García S, Martín C, De Rossi E, Aínsa J. Contribution of the Rv2333c efflux pump (the Stp protein) from *Mycobacterium tuberculosis* to intrinsic antibiotic resistance in *Mycobacterium bovis* BCG. J Antimicrob Chemother. 2007; 59: 544-7.

352. Ramón-García S, Mick V, Dainese E, Martín C, Thompson C, De Rossi E, Manganelli R, Aínsa J. Functional and genetic characterization of the Tap efflux pump in *Mycobacterium bovis* BCG. Antimicrob Agents Chemother. 2012; 56: 2074-83.

353. Rao, S, Alonso S, Rand L, Dick T, Pethe K. The protonmotive force is required for maintaining ATP homeostasis and viability of hypoxia, nonreplicating *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA. 2008;105: 11945-50.

354. Rawat R, Whitty A, Tonge P. The isoniazid-NAD adduct is a slow, tight-binding inhibitor of InhA, the *Mycobacterium tuberculosis* enoyl reductase: adduct affinity and drug resistance. Proc Natl Acad Sci USA. 2003; 100: 13881-6.

355. Reiling N, Homolka S, Walter K, Brandenburg J, Niwinski L, Ernst M, Herzmann C, Lange, Diel R, Ehlers S, Niemann S. Clade-specific virulence patterns of *Mycobacterium tuberculosis* complex strains in human primary macrophages and aerogenically infected mice. 2013; mBio, 4: e00250-13.

356. Reisner B, Gatson A, Woods G. Use of Gen-Probe AccuProbes to identify *Mycobacterium avium* complex, *Mycobacterium tuberculosis* complex, *Mycobacterium kansasii*, and *Mycobacterium gordonae* directly from BACTEC TB broth cultures. J Clin Microbiol. 1994; 32: 2995-8.

357. Richter E, Weizenegger M, Rüsch-Gerdes S, Niemann S. Evaluation of Genotype MTBC assay for differentiation of clinical *Mycobacterium tuberculosis* complex isolates. J Clin Microbiol. 2003; 41: 2672-5.

358. Rigouts L, Nolasco O, de Rijk P, Nduwamahoro E, Van Deun A, Ramsay A, Arevalo J, Portaels F. Newly developed primers for comprehensive amplification of the *rpoB* gene and detection of rifampin resistance in *Mycobacterium tuberculosis*. J Clinical Microbiol. 2007; 45: 252-4.

359. Ritter C, Lucke K, Sirgel F, Warren R, van Helden P, Böttger E, Bloemberg G. Evaluation of the AID TB resistance line probe assay for rapid detection of genetic alterations associated with drug resistance in *Mycobacterium tuberculosis* strains. J Clin Microbiol. 2014; 52: 940-6.

360. Robin A. Treatment of tuberculosis. Churchill, pp. 1-616. 1913.

361. Rodrigues L, Machado D, Couto I, Amaral L, Viveiros M. Contribution of efflux activity to isoniazid resistance in the *Mycobacterium tuberculosis* complex. Infect Genet Evol. 2011; doi:10.1016/j.meegid.2011.08.009.

362. Rodrigues V, Telles M, Ribeiro M, Cafrune P, Rossetti M, Zaha A. Characterization of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis* in Brazil. Antimicrob Agents Chemother. 2005; 49: 444-6.

363. Rodríguez J, Ruiz M, López M, Royo G. *In vitro* activity of moxifloxacin, levofloxacin, gatifloxacin and linezolid against *Mycobacterium tuberculosis*. Int J Antimicrob Agents. 2002; 20: 464-7.

364. Roring S, Scott A, Brittain D, Walker I, Hewinson G, Neill S, Skuce R. Development of variable-number tandem repeat typing of *Mycobacterium bovis*: comparison of results with those obtained by using existing exact tandem repeats and spoligotyping. J Clin Microbiol. 2002; 40: 2126-33.

365. Roth A, Fischer M, Hamid M, Michalke S, Ludwig W, Mauch H. Novel diagnostic algorithm for identification of mycobacteria using genus-specific amplification of the 16S-23S rRNA gene spacer and restriction endonucleases. J Clin Microbiol. 2000; 38: 1094-104.

366. Rozwarski D, Grant G, Barton D, Jacobs Jr. W, Sacchettini J. Modification of the NADH of the isoniazid target (InhA) from *Mycobacterium tuberculosis*. Science. 1998; 279: 98-102.

367. Runyon E. Anonymous mycobacteria in pulmonary disease. Med Clin North Am. 1959; 43: 273-90.

368. Russell D. Who puts the tubercle in tuberculosis. Nat Rev Microbiol. 2007; 5: 39-47.

369. Ryan B, Dougherty T, Beaulieu D, Chuang J, Dougherty B, Barrett J. Efflux in bacteria: what do we really know about it? Expert Opin Investig Drugs. 2001; 10: 1409-22.

370. Safi H, Sayers B, Hazbon M, Alland D. Transfer of *embB* codon 306 mutations into clinical *Mycobacterium tuberculosis* strains alters susceptibility to ethambutol, isoniazid, and rifampin. Antimicrob Agents Chemother. 2008; 52: 2027-34.

371. Safi H, Fleishmann R, Peterson S, Jones M, Jarrahi B, Alland D. Allelic exchange and mutant selection demonstrate that common clinical *embCAB* gene mutations only modestly increase resistance to ethambutol in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2010; 54: 103-8.

372. Safi H, Amin A, Kim S, Jones M, Holmes M, McNeil M, Peterson S, Chatterjee D, Fleischmann R, Alland D. Evolution of high-level ethambutol-resistant tuberculosis through interacting mutations in decaprenylphosphoryl-β-d-arabinose biosynthetic and utilization pathway genes. Nat Genet. 2013; 45: 1190-7.

373. Salih F, Kaushik N, Sharma P, Choudary G, Murthy P, Venkitasubramanian T. Calmodulin-like activity in mycobacteria. Indian J Biochem Biophys. 1991; 28: 491-5.

374. Sampson S, Warren R, Richardson M, van der Spuy G, van Helden P. Disruption of coding regions by IS*6110* insertion in *Mycobacterium tuberculosis*. Tuberc Lung Dis. 1999; 79: 349-59.

375. Sander P, Meier A, Böttger E. Ribosomal drug resistance in mycobacteria. Res Microbiol. 1996; 147: 59-67.

376. Santos A, Cunha E. Some paleopathological aspects from the medieval necropolis of Granja dos Serrões (Portugal). *In*: López, MM, Sánchez J, editors. La enfermedad en los restos humanos arqueológicos. Cádiz: Universidade de Cádiz Servicio de Publicaciones. pp. 335-339. 1997.

377. Santos A, Roberts C. A picture of tuberculosis in young Portuguese people in the early 20th century: a multidisciplinary study of the skeletal and historical evidence. Am J Phys Anthropol. 2001; 115: 38-49.

378. Santos A, Roberts C. Anatomy of a serial killer: differential diagnosis of tuberculosis based on rib lesions of adult individuals from the Coimbra Identified Skeletal Collection, Portugal. Am J Phys Anthropol. 2006; 130: 38-49.

379. Santos A. O combate à tuberculose uma abordagem demográfico-epidemiológica o hospital de repouso de Lisboa (1882-1975). Master Thesis. University of Lisbon. 2010.

380. Sarin J, Aggarwal S, Chaba R, Varshney G, Chakraborti P. β-subunit of phosphate-specific transporter from *Mycobacterium tuberculosis* is a thermostable ATPase. J Biol Chem. 2001; 276: 44590-7.

381. Sassetti C, Boyd D, Rubin E. Comprehensive identification of conditionally essential genes in mycobacteria. Proc Natl Acad Sci USA. 2001; 98: 12712-7.

382. Schatz A, Bugle E, Waksman S. Streptomycin, a substance exhibiting antibiotic activity against Gram-Positive and Gram-Negative bacteria. Exp Biol Med. 1944; 55: 66-9.

383. Schurig-Briccio L, Yano T, Rubin H, Gennis R. Characterization of the type 2 NADH:menaquinone oxidoreductases from *Staphylococcus aureus* and the bactericidal action of phenothiazines. Biochim Biophys Acta. 2014: org/10.1016/j.bbabio.2014.03.017.

384. Scollard D, Adams L, Gillis T, Krahenbuhl J, Truman R, Williams D. The continuing challenges of Leprosy. Clin Microbiol Rev. 2006; 19: 338-81.

385. Scorpio A, Zhang Y. Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nat Med. 1996; 2: 662-7.

386. Senaratne R, Mobasheri H, Papavinasasundaram K, Jenner P, Lea E, Draper P. Expression of a gene for a porin-like protein of the OmpA family from *Mycobacterium tuberculosis* H37Rv. J Bacteriol. 1998; 180: 3541-7.

387. Sensi P. History of the development of rifampin. Rev Infect Dis. 1983; 5: S402-6.

388. Shcherbakov D, Akbergenov R, Matt T, Sander P, Anderson D, Böttger E. Directed mutagenesis of *Mycobacterium smegmatis* 16S rRNA to reconstruct the *in vivo* evolution of aminoglycoside resistance in *Mycobacterium tuberculosis*. Mol Microbiol. 2010; 77: 830-40.

389. Sheen P, Ferrer P, Gilman R, López-Llano J, Fuentes P, Valencia E, Zimic M. Effect of pyrazinamidase activity on pyrazinamide resistance in *Mycobacterium tuberculosis.* Tuberculosis. 2009; 89: 109-13.

390. Shen X, Shen G, Wu J, Gui X, Li X, Mei J, DeRiemer K, Gao Q. Association between *embB* codon 306 mutations and drug resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2007; 51: 2618-20.

391. Sherman D, Mdluli K, Hickey M, Arain T, Morris S, Barry C, Stover C. Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. Science; 1996; 272: 1641-3.

392. Shi R, Zhang J, Li C, Kazumi Y, Sugawara I. Emergence of ofloxacin resistance in *Mycobacterium tuberculosis* clinical isolates from China as determined by *gyrA* mutation analysis using denaturing high-pressure liquid chromatography and DNA sequencing. J Clin Microbiol. 2006; 44: 4566-8.

393. Shi W, Zhang X, Jiang X, Yuan H, Lee J, Barry C, Wang H, Zhang W, Zhang Y. Pyrazinamide inhibits trans-translation in *Mycobacterium tuberculosis*. Science. 2011; 333: 1630-2.

394. Shiba T, Nomoto S, Wakamiya T. The chemical structure of capreomycin. Experientia. 1976; 32: 1109-11.

395. Siddiqi S, Libonati J, Middlebrook G. Evaluation of a rapid radiometric method for drug susceptibility testing of *Mycobacterium tuberculosis*. J Clin Microbiol. 1981; 13: 908-12.

396. Siddiqi S, Hawkins J, Laszlo A. Inter-laboratory drug susceptibility testing of *Mycobacterium tuberculosis* by radiometric procedure and two conventional methods. J Clin Microbiol. 1985; 22: 919-23.

397. Siddiqi N, Shamim M, Hussain S, Choudhary R, Ahmed N, Prachee, Banerjee S, Savithri G, Alam M, Pathak N, Amin A, Hanief M, Katoch V, Sharma S, Hasnain S. Molecular characterization of multidrug-resistant isolates of *Mycobacterium tuberculosis* from patients in North India. Antimicrob Agents Chemother. 2002; 46: 443-50.

398. Siddiqi N, Das R, Pathak N, Banerjee S, Ahmed N, Katoch V, Hasnain S. *Mycobacterium tuberculosis* isolate with a distinct genomic identity overexpresses a tap-like efflux pump. Infection. 2004; 32: 109-11.

399. Silva P, Bigi F, Santangelo M, Romano M, Martín C, Cataldi A, Aínsa J. Characterization of P55, a multidrug efflux pump in *Mycobacterium bovis* and *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2001; 45: 800-4.

400. Simons S, Mulder A, van Ingen J, Boeree M, van Soolingen D. Role of *rpsA* gene sequencing in diagnosis of pyrazinamide resistance. J Clin microbiol. 2013; 51: 382.

401. Singh R, Manjunatha U, Boshoff H, Ha Y, Niyomrattanakit P, Ledwidge R, Dowd C, Lee Y, Kim P, Singh R, Wiseman B, Deemagarn T, Donald L, Duckworth H, Carpena X, Fita I, Loewen P. Catalase-peroxidases (KatG) exhibit NADH oxidase activity. J Biol Chem. 2004; 279: 43098-106.

402. Singh R, Manjunatha U, Boshoff H, Ha Y, Niyomrattanakit P, Ledwidge R, Dowd C, Lee I, Kim P, Zhang L, Kang S, Keller T, Jirice J, Barry 3rd C. PA-824 kills nonreplicating *Mycobacterium tuberculosis* by intracellular NO release. Science. 2008; 322: 1392-5.

403. Sirgel F, Tait M, Warren R, Streicher E, Böttger E, van Helden P, van Pittius N, Coetzee G, Hoosain E, Chabula-Nxiweni M, Hayes C, Victor T, Trollip A. Mutations in the *rrs* A1401G gene and phenotypic resistance to amikacin and capreomycin in *Mycobacterium tuberculosis*. Microb Drug Resist. 2011; 18: 193-7.

404. Siu G, Zhang Y, Lau T, Lau R, Ho P, Yew W, Tsui S, Cheng V, Yuen K, Yam W. Mutations outside the rifampicin resistance-determining region associated with rifampicin resistance in *Mycobacterium tuberculosis*. J Antimicrob Chemother. 2011; 66: 730-3.

405. Skuce R, McCorry T, McCarroll J, Roring S, Scott A, Brittain D, Hughes S, Hewinson R, Neill S. Discrimination of *Mycobacterium tuberculosis* complex bacteria using novel VNTR-PCR targets. Microbiology. 2002; 148: 519-28.

406. Sledzik P, Bellantoni N. Brief communication: bioarcheological and biocultural evidence for the New England vampire folk belief. Am J Phys Anthropol. 1994; 94: 269-74.

407. Sola C, Filliol I, Legrand E, Lesjean S, Locht C, Supply P, Rastogi N. Genotyping of the *Mycobacterium tuberculosis* complex using MIRUs: association with VNTR and spoligotyping for molecular epidemiology and evolutionary genetics. Infect Genet Evol. 2003; 3: 125-33.

408. Song H, Sandie R, Wang Y, Andrade-Navarro M, Niederweis M. Identification of outer membrane proteins of *Mycobacterium tuberculosis*. Tuberculosis. 2008; 88: 526-44.

409. Sosnik A, Carcaboso Á, Glisoni R, Moretton M, Chiappetta D. New old challenges in tuberculosis: potentially effective nanotechnologies in drug delivery. Adv Drug Deliv Rev. 2010; 62: 547-59.

410. Soto C, Menéndez M, Pérez E, Samper S, Gómez A, García M, Martín C. IS*6110* mediates increased transcription of the *phoP* virulence gene in a multidrug-resistant clinical isolate responsible for tuberculosis outbreaks. J Clin Microbiol. 2004; 42: 212-9.

411. Sreevatsan S, Pan X, Stockbauer K, Williams D, Kreiswirth B, Musser J. Characterization of *rpsL* and *rrs* mutations in streptomycin-resistant *Mycobacterium tuberculosis* isolates from diverse geographic localities. Antimicrob Agents Chemother. 1996; 40: 1024-6.

412. Sreevatsan S, Stockbauer K, Pan X, Kreiswirth B, Moghazeh S, Jacobs Jr W, Telenti A, Musser J. Ethambutol resistance in *Mycobacterium tuberculosis*: critical role of *embB* mutations. Antimicrob Agents Chemother. 1997; 8: 1677-81.

413. Srivastava S, Garg A, Ayyagari A, Nyati K, Dhole T, Dwivedi S. Nucleotide polymorphism associated with ethambutol resistance in clinical isolates of *Mycobacterium tuberculosis*. Current Microbiol. 2006; 53: 401-5.

414. Srivastava S, Ayyagaria A, Dholea T, Nyatia K, Dwivedi S. *emb* nucleotide polymorphisms and the role of *embB306* mutations in *Mycobacterium tuberculosis* resistance to ethambutol. Int J Med Microbiol. 2009; 299: 269-80.

415. Stahl C, Kubetzko S, Kaps I, Seeber S, Engelhardt H, Niederweis M. MspA provides the main hydrophilic pathway through the cell wall of *Mycobacterium smegmatis*. Mol Microbiol. 2001; 40: 451-64.

416. Stanley R, Blaha G, Grodzicki R, Strickler M, Steitz T. The structures of the anti-tuberculosis antibiotics viomycin and capreomycin bound to the 70S ribosome. Nat Struct Mol Biol. 2010; 17: 289-93.

417. Starks A, Gumusboga A, Plikaytis B, Shinnick T, Posey J. Mutations at *embB306* are an important molecular indicator of ethambutol resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2009; 53: 1061-6.

418. Stead W, Eisenach K, Cave M, Beggs M, Templeton G, Thoen C, Bates J. When did *Mycobacterium tuberculosis* infection first occur in The New World? An important question with public health implications. Am J Respir Crit Care Med. 1995; 151: 1267-8.

419. Steingart K, Henry M, Ng V, Hopewell P, Ramsay A, Cunningham J, Urbanczik R, Perkins M, Aziz M, Pai M. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis. 2006; 6: 570-81.

420. Steingart K, Ramsay A, Pai M. Optimizing sputum smear microscopy for the diagnosis of pulmonary tuberculosis. Expert Rev Anti Infect Ther. 2007; 5: 327-31.

421. Stone A, Wilbur A, Buikstra J, Roberts C. Tuberculosis and leprosy in perspective. Yearb Phys Anthropol. 2009; 52: 66-94.

422. Stover C, Warrener P, VanDevanter D, Sherman D, Arain T, Langhorne M, Anderson S, Towell J, Yuan Y, McMurray D, Kreiswirth B, Barry C, Baker W. A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. Nature; 2000: 405: 962-6.

423. Streicher E, Victor T, van der Spuy G, Sola C, Rastogi N, van Helden P, Warren R. Spoligotype signatures in the *Mycobacterium tuberculosis* complex. J Clin Microbiol. 2007; 45: 237-40.

424. Sturgill-Koszycki S, Schlesinger P, Chakraborty P, Haddix P, Collins H, Fok A, Allen R, Gluck S, Heuser J, Russel D. Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. Science 1994; 263: 678–81.

425. Sun Y, Bellamy R, Lee A, Ng S, Ravindran S, Wong S, Locht C, Supply P, Paton, N. Use of mycobacterial interspersed repetitive unit-variable-number tandem repeat typing to examine genetic diversity of *Mycobacterium tuberculosis* in Singapore. J Clinical Microbiol. 2004; 42: 1986-93.

426. Sun Z, Scorpio A, Zhang Y. The *pncA* gene from naturally pyrazinamide-resistant *Mycobacterium avium* encodes pyrazinamidase and confers pyrazinamide susceptibility to resistant *M. tuberculosis* complex organisms. Microbiology. 1997; 143: 3367-73.

427. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rüsch-Gerdes S, Willery E, Savine E, de Haas P, van Deutekom H, Roring S, Bifani P, Kurepina N, Kreiswirth B, Sola C, Rastogi N, Vatin V, Gutierrez M, Fauville M, Niemann S, Skuce R, Kremer K, Locht C, van Soolingen D. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol. 2006; 44: 4498-510.

428. Supply P, Magdalena J, Himpens S, Locht C. Identification of novel intergenic repetitive units in a mycobacterial two-component system operon. Mol Microbiol. 1997; 26: 991-1003.

429. Supply P, Mazars E, Lesjean S, Vincent V, Gicquel B, Locht C. Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. Mol. Microbiol. 2000; 36: 762-71.

430. Supply P, Marceau M, Mangenot S, Roche D, Rouanet C, Khanna V, Majlessi L, Criscuolo A, Tap J, Pawlik A, Fiette L, Orgeur M, Fabre M, Parmentier C, Frigui W, Simeone R, Boritsch E, Debrie A, Willery E, Walker D, Quail M, Ma L, Bouchier C, Salvignol G, Sayes F, Cascioferro A, Seemann T, Barbe V, Locht C, Gutierrez M, Leclerc C, Bentley S, Stinear T, Brisse S, Médigue C, Parkhill J, Cruveiller S, Brosch R. Genomic analysis of smooth tubercle bacilli provides insights into ancestry and pathoadaptation of *Mycobacterium tuberculosis*. Nat Genet. 2013; 45: 172-9.

431. Suzuki Y, Katsukawa C, Tamaru A, Abe C, Makino M, Mizuguchi Y, Taniguchi H. Detection of kanamycin-resistant *Mycobacterium tuberculosis* by identifying mutations in the 16S rRNA gene. J Clin Microbiol. 1998; 36: 1220-5.

432. Takayama K, Wang L, David H. Effect of isoniazid on the *in vivo* mycolic acid synthesis, cell growth, and viability of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1972; 2: 29-35.

433. Takayama K, Armstrong E, Kunugi K, Kilburn J. Inhibition by ethambutol of mycolic acid transfer into the cell wall of *Mycobacterium smegmatis*. Antimicrob Agents Chemother. 1979; 16: 240-2.

434. Takayama K, Kilburn J. Inhibition of synthesis of arabinogalactan by ethambutol in *Mycobacterium smegmatis*. Antimicrob Agents Chemother. 1989; 33: 1493-9.

435. Takiff H, Salazar L, Guerrero C, Philipp W, Huang W, Kreiswirth B, Cole S, Jacobs Jr W, Telenti A. Cloning and nucleotide sequence of *Mycobacterium tuberculosis gyrA* and *gyrB* genes and detection of quinolone resistance mutations. Antimicrob Agents Chemother. 1994; 38: 773-80.

436. Talbot E, Williams D, Frothingham R. PCR identification of *Mycobacterium bovis* BCG. J Clin Microbiol. 1997; 35: 566-9.

437. Taniguchi H, Chang B, Abe C, Nikaido Y, Mizuguchi Y, Yoshida Y. Molecular analysis of kanamycin and viomycin resistance in *Mycobacterium smegmatis* by use of the conjugation system. J Bacteriol. 1997; 179: 4795-801.

438. Tarshis M, Weed W, Jr. Lack of significant *in vitro* sensitivity of *Mycobacterium tuberculosis* to pyrazinamide on three different solid media. Am Rev Tuberc. 1953; 67: 391-5.

439. Taylor T, Patterson C, Hale Y, Safranek W. Routine use of PCR-restriction fragment length polymorphism analysis for identification of mycobacteria growing in liquid media. J Clin Microbiol. 1997; 35: 79-85.

440. Telenti A, Imboden P, Marchesi F, Matter L, Schopfer K, Bodmer T, Lowrie D, Colston M, Cole S. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. Lancet. 1993; 341: 647-50.

441. Telenti A, Philipp W, Sreevatsan S, Bernasconi C, Stockbauer K, Wieles B, Musser J, Jacobs Jr W. The *emb* operon, a gene cluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol. Nat Med. 1997; 3: 567-70.

442. Thibonnier M, Thiberge J, De Reuse H. Trans-translation in *Helicobacter pylori*: essentiality of ribosome rescue and requirement of protein tagging for stress resistance and competence. PLoS One. 2008; 3: e3810.

443. Thierry D, Brisson-Noël A, Vincent-Lévy-Frébault V, Nguyen S, Guesdon J, Gicquel B.. Characterization of a *Mycobacterium tuberculosis* insertion sequence, IS*6110*, and its application in diagnosis. J Clin Microbiol. 1990; 28: 2668-73.

444. Thierry D, Cave M, Eisenach K, Crawford J, Bates J, Gicquel B, Guesdon J. IS*6110*, an IS-like element of *Mycobacterium tuberculosis* complex. Nucl Acids Res. 1990; 18: 188.

445. Thomas J, Baughn C, Wilkinson R, Shepherd R. A new synthetic compound with antituberculous activity in mice: ethambutol (dextro-2, 2'-(ethylenediimino)- di-1-butanol). Am Rev Respir Dis. 1961; 83: 891-3.

445a. Thorsing M, Klitgaard J, Atilano M, Skov M, Kolmos H, Filipe S, Kallipolitis B. Thioridazine induces major changes in global gene expression and cell wall composition in methicillin resistant *Staphylococcus aureus* USA300. PloS One. 2013; 8: e64518.

446. Tortoli E. Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. Clin Microbiol Rev. 2003; 16: 319-54.

447. TransportDB. TransportDB: Transporter protein analysis database. Genomic comparisons of membrane transport systems. 2013. http://www.membranetransport.org. Last access: May 2014.

448. Trifiro S, Bourgault A, Lebel F, René P. Ghost mycobacteria on Gram stain. J Clin Microbiol. 1990; 28: 146-7.

449. Trusov A, Bumgarner R, Valijev R, Chestnova R, Talevski S, Vragoterova C, Neeley E. Comparison of Lumin™ LED fluorescent attachment, fluorescent microscopy and Ziehl-Neelsen for AFB diagnosis. Int J Tuberc Lung Dis. 2009; 13: 836-41.

450. Tufariello J, Chan J, Flynn J. Latent tuberculosis: mechanisms of host and bacillus that contribute to persistent infection. Lancet Infect Dis. 2003; 3: 578-90.

451. Ulrichs T, Kaufmann S. Mycobacterial persistence and immunity. Front Biosci. 2002; 7: 458-69.

452. Ulrichs T, Kaufmann S. New insights into the function of granulomas in human tuberculosis. J Pathol. 2006; 208: 261-9.

453. Umezawa H. Kanamycin: its discovery. Ann NY Acad Sci. 1958; 76: 20-6.

454. van Doorn H, An D, de Jong M, Lan N, Hoa D, Quy H, Chau N, Duy P, Tho D, Chinh N, Farrar J, Caws M. Fluoroquinolone resistance detection in *Mycobacterium tuberculosis* with locked nucleic acid probe real-time PCR. Int J Tuberc Lung Dis. 2008; 12: 736-42.

455. van Embden J, Cave M, Crawford J, Dale J, Eisenach K, Gicquel B, Hermans P, Martin C, McAdam R, Shinnick T, Small P. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J Clin Microbiol. 1993; 31: 406-9.

456. van Embden J, van Gorkom T, Kremer K, Jansen R, van der Zeijst B, Schouls L. Genetic variation and evolutionary origin of the direct repeat locus of *Mycobacterium tuberculosis* complex bacteria. J Bacteriol. 2000; 182: 2393-401.

457. van Ingen J, Rahim Z, Mulder A, Boeree M, Simeone R, Brosch R, van Soolingen D. Characterization of *Mycobacterium orygis* as *M. tuberculosis* complex subspecies. Emerg Infect Dis. 2012; 18: 653-5.

458. van Soolingen D, Hoogenboezem T, de Haas P, Hermans P, Koedam M, Teppema K, Brennan P, Besra G, Portaels F, Top J, Schouls L, van Embden J. A novel pathogenic taxon of the *Mycobacterium tuberculosis* complex, Canetti: characterization of an exceptional isolate from Africa. Int J Syst Bacteriol. 1997; 47: 1236-45.

459. van Soolingen D. Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. J Intern Med. 2001; 249: 1-26.

460. van Soolingen D, Hernandez-Pando H, Orozco H, Aguilar D, Magis-Escurra C, Amaral L, van Ingen J, Boeree M. The antipsychotic thioridazine shows promising therapeutic activity in a mouse model of multidrug-resistant tuberculosis. PLoS One. 2010; 5: e12640.

461. Velmurugan K, Chen B, Miller J, Azogue S, Gurses S, Hsu T, Glickman M, Jacobs Jr W, Porcelli S, Briken V. *Mycobacterium tuberculosis nuoG* is a virulence gene that inhibits apoptosis of infected host cells. PLoS Pathog. 2007; 3: e110.

461a. Veyrier F, Dufort A, Behr M. The rise and fall of the *Mycobacterium tuberculosis* genome. Trends Microbiol. 2011; 19: 154-61.

462. Vieira I. Alguns aspectos das campanhas antituberculosas em Portugal: os congressos da Liga Nacional Contra a Tuberculose (1901-1907). CEM: Cultura, Espaço & Memória: revista do CITCEM, n. º 2, pp. 265-279. 2011.

463. Vilchèze C, Wang F, Arai M, Hazbón M, Colangeli R, Kremer L, Weisbrod T, Alland D, Sacchettini J, Jacobs Jr W. Transfer of a point mutation in *Mycobacterium tuberculosis inhA* resolves the target of isoniazid. Nature Med. 2006; 12: 1027-9.

464. Vilchèze C, Jacobs Jr W. The mechanism of isoniazid killing: clarity through the scope of genetics. Annu Rev Microbiol. 2007; 61: 35-50.

465. Villemin J. Études sur la tuberculose... JB Baillière et Fils. 1868.

466. Viveiros M, Leandro C, Amaral L. Mycobacterial efflux pumps and chemotherapeutic implications. Int J Antimicrob Agents. 2003; 22: 274-8.

467. Viveiros M, Leandro C, Rodrigues L, Almeida J, Bettencourt R, Couto I, Carrilho L, Diogo J, Fonseca A, Lito L, Lopes J, Pacheco T, Pessanha M, Quirim J, Sancho L, Salfinger M, Amaral L. Direct application of the INNO-LiPA Rif.TB line-probe assay for rapid identification of *Mycobacterium tuberculosis* complex strains and detection of rifampin resistance in 360 smear-positive respiratory specimens from an area of high incidence of multidrug-resistant tuberculosis. J Clin Microbiol. 2005; 43: 4880-4.

468. Viveiros M, Dupont M, Rodrigues L, Couto I, Davin-Regli A, Martins M, Pagès J, Amaral L. Antibiotic stress, genetic response and altered permeability of *E. coli*. PLoS One. 2007; 2: e365.

469. Viveiros M, Martins M, Couto I, Rodrigues L, Machado D, Portugal I, Amaral L. Molecular tools for rapid identification and novel effective therapy against MDRTB/XDRTB infections. Expert Rev Anti Infect Ther. 2010; 8: 465-80.

470. Viveiros M, Martins M, Rodrigues L, Machado D, Couto I, Ainsa J, Amaral L. Inhibitors of mycobacterial efflux pumps as potential boosters for anti-tubercular drugs. Expert Rev Anti Infect Ther. 2012; 10: 983-98.

471. Viveiros M, Machado D, Couto I, Amaral L. Improving on the LJ slope – automated liquid culture. *In*: Tuberculosis - Diagnosis and Treatment (Advances in Molecular and Cellular Microbiology Series), T. D. McHugh, Editor. CABI Publishing, Oxfordshire, UK, 304 pp; 2013.

472. Vogler A, Keys C, Nemoto Y, Colman R, Jay Z, Keim P. Effect of repeat copy number on variable-number tandem repeat mutations in *Escherichia coli* O157:H7. J Bacteriol. 2006; 188: 4253-63.

473. Von Groll A, Martin A, Jureen P, Hoffner S, Vandamme P, Portaels F, Palomino J, da Silva P. Fluoroquinolone resistance in *Mycobacterium tuberculosis* and mutations in *gyrA* and *gyrB*. Antimicrob Agents Chemother. 2009; 53: 4498-500.

474. Vyas S, Kannan M, Jain S, Mishra V, Singh P. Design of liposomal aerosols for improved delivery of rifampicin to alveolar macrophages. Int J Pharma. 2004; 269: 37-49.

475. Walsh J, Wang R, Bagan E, Wang C, Wislocki P, Miwa G. Structural alterations that differentially affect the mutagenic and antitrichomonal activities of 5-nitroimidazoles. J Med Chem. 1987; 30: 150-6.

476. Warman A, Rito T, Fisher N, Moss D, Berry N, O´Neill P, Ward S, Giagini G. Antitubercular pharmacodynamics of phenothiazines. J Antimicrob Chemother. 2013; 68: 869-80.

477. Warren P. The evolution of the sanatorium: the first half-century, 1854-1904. Can Bull Med Hist. 2006; 23: 457-76.

478. Warren R, Streicher E, Sampson S, van der Spuy G, Richardson M, Nguyen D, Behr M, Victor T, van Helden P. Microevolution of the direct repeat region of *Mycobacterium tuberculosis*: implications for interpretation of spoligotyping data. J Clin Microbiol. 2002; 40: 4457-65.

479. Warren R, Victor T, Streicher E, Richardson M, van der Spuy G, Johnson R, Chihota V, Locht C, Supply P, van Helden P. Clonal expansion of a globally disseminated lineage of *Mycobacterium tuberculosis* with low IS*6110* copy numbers. J Clin Microbiol. 2004; 42: 5774-82.

480. Waxman S. From neuroscience to neurology neuroscience. Molecular medicine, and the therapeutic transformation of neurology. Elsevier Academic Press. Burlington, USA. 2005.

481. Wayne L, Kubica G. Mycobacteria. *In* Bergey's Manual of Systematic Bacteriology, Vol. 2. Sneath, P.A.H., Mair, N.S., Sharpe, M.E., and Holt, J.G. (eds). Baltimore, MD: The Williams & Wilkins Co. pp. 1436-57. 1986.

482. Wayne L. Dormancy of *Mycobacterium tuberculosis* and latency of disease. Eur J Clin Microbiol Infect Dis. 1994; 13: 908-14.

483. Webber M, Piddock L. The importance of efflux pumps in bacterial antibiotic resistance. J Antimicrob Chemother. 2003; 51: 9-11.

484. Weinstein E, Yano T, Li L, Avarbock D, Avarbock A, Helm D, McColm A, Duncan K, Lonsdale J, Rubin H. Inhibitors of type II NADH:menaquinone oxidoreductase represent a class of antitubercular drugs. Proc Natl Acad Sci USA. 2005; 122: 4548-53.

485. Wells A, Oxon D. Tuberculosis in wild voles. Lancet. 1937; 229: 1221.

486. Wengenack N, Jensen M, Rusnak F, Stern M. *Mycobacterium tuberculosis* KatG is a peroxynitritase. Biochemical and biophysical research communications. 1999; 256: 485-7.

487. Weniger T, Krawczyk J, Supply P, Niemann S, Harmsen D. MIRU-VNTR*plus*: a web tool for polyphasic genotyping of *Mycobacterium tuberculosis* complex bacteria. Nucleic Acids Res. 2010; 38(suppl 2): W326-W331.

488. Wetzel H, Gründer G, Hillert A, Philipp M, Gattaz W, Sauer H, Adler G, Schröder J, Rein W, Benkert O. Amisulpride versus flupentixol in schizophrenia with predominantly positive symptomatology: a double-blind controlled study comparing a selective D2-like antagonist to a mixed D1-/D2-like antagonist. The amisulpride study group. Psychopharmacology. 1998; 137: 223-32.

489. Whipple G. Papaverine as an antiarrhythmic agent. Angiology. 1977; 28: 737-49.

490. Wilkinson R, Shepherd R, Thomas J, Baughn C. Stereospecificity in a new type of synthetic antituberculous agent. (Correspondence). J Am Chem Soc. 1961; 83: 2212-3.

491. Willmott C, Critchlow S, Eperon I, Maxwell A. The complex of DNA gyrase and quinolone drugs with DNA forms a barrier to transcription by RNA polymerase. J Mol Biol. 1994; 242: 351-63.

492. Wolucka B, McNeil M, de Hoffmann E, Chojnacki T, Brennan P. Recognition of the lipid intermediate for arabinogalactan/arabinomannan biosynthesis and its relation to the mode of action of ethambutol on mycobacteria. J Biol Chem. 1994; 269: 23328-35.

493. Wong S, Lee J, Kwak H, Via L, Boshoff H, Barry, III C. Mutations in *gidB* confer low-level streptomycin resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2011; 55: 2515-22.

494. Wong S, Javid B, Addepalli B, Piszczek G, Strader M, Limbach P, Barry, III C. Functional role of methylation of G518 of the 16S rRNA 530 loop by GidB in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2013; 57: 6311-8.

495. Woodley C, Kilburn J, David H, Silcox V. Susceptibility of mycobacteria to rifampin. Antimicrob Agents Chemother. 1972; 2: 245-9.

496. World Health Organization. TB a global emergency. WHO reports on the TB epidemics. WHO/TB/ 94.177. Genebra, Switzerland. World Health Organization. 1994.

497. World Health Organization. The use of essential drugs. WHO technical report series. WHO/TRS/850. Genebra, Switzerland. World Health Organization. 1995.

498. World Health Organization. Guidelines for the management of drug resistant tuberculosis. WHO/TB/96.210. Genebra, Switzerland. World Health Organization. 1996.

499. World Health Organization. Guidelines for the management of drug-resistant tuberculosis. WHO/TB/96.210 (Rev.1). Geneva, Switzerland. World Health Organization. 1997.

500. World Health Organization. WHO Pharmaceuticals Newsletter No. 01. Geneva, Switzerland World Health Organization. 2005.

501. World Health Organization. Guidelines for the programmatic management of drug-resistant tuberculosis. WHO/HTM/TB/2008.402. Geneva, Switzerland. World Health Organization. 2008.

502. World Health Organization. WHO policy statement: Expert Group Report. Molecular line probe assays for rapid screening of patients at risk of multi-drug resistant tuberculosis (MDR-TB). Geneva, Switzerland. World Health Organization. 2008.

503. World Health Organization. Global tuberculosis control: epidemiology, strategy, financing. WHO report 2009. WHO/HTM/TB/2009.411. Genebra, Switzerland. World Health Organization. 2009.

504. World Health Organization. Automated real-time nucleic acid amplification technology for rapid and simultaneous detection of tuberculosis and rifampicin resistance: Xpert MTB/RIF system. Policy statement. WHO/HTM/TB/2011.4. Geneva, Switzerland. World Health Organization. 2011.

505. World Health Organization. Guidelines for the programmatic management of drug-resistant tuberculosis – 2011 update. WHO/HTM/TB/2011.6. Geneva, Switzerland. World Health Organization. 2011.

506. World Health Organization. Global tuberculosis control: WHO report 2013. WHO/HTM/TB/2013.11. Geneva, Switzerland. World Health Organization. 2013.

507. Yamada T, Mizugichi Y, Nierhaus K, Wittmann H. Resistance to viomycin conferred by RNA of either ribosomal subunit. Nature. 1978; 275: 460-1.

508. Yano T, Li L, Weinstein E, Teh J, Rubin H. Steady-state kinetics and inhibitory action of antitubercular phenothiazines on *Mycobacterium tuberculosis* type-II NADH-menaquinone oxidoreductase (NDH-2). J Biol Chem. 2006; 281: 11456-63.

509. Yousef W, Omar A, Morsy M, El-Wahed M, Ghanayem N. The mechanism of action of calcium channel blockers in the treatment of diabetic nephropathy. Int J Diabetes Metabol. 2005; 13: 76-82.

510. Zahrt T, Song J, Siple J, Deretic V. Mycobacterial FurA is a negative regulator of catalase–peroxidase gene *katG*. Mol Microbiol. 2001; 39: 1174-85.

511. Zaunbrecher M, Sikes R, Jr., Metchock B, Shinnick T, Posey J. Overexpression of the chromosomally encoded aminoglycoside acetyltransferase *eis* confers kanamycin resistance in *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA. 2009; 106: 20004-9.

512. Zembower T, Noskin G, Postelnick M, Nguyen C, Peterson L. The utility of aminoglycosides in an era of emerging drug resistance. Int J Antimicrob Agents. 1998; 10: 95-105.

513. Zhang G, Campbell E, Minakhin L, Richter C, Severinov K, Darst S. Crystal structure of *Thermus aquaticus* core RNA Polymerase at 3.3 Å resolution. Cell. 1999; 98: 811-24.

514. Reference deleted.

515. Zhang S, Qi H, Qiu D, Li D, Zhang J, Du C, Wang G, Yang Z, Sun Q. Genotypic analysis of multidrug-resistant *Mycobacterium tuberculosis* isolates recovered from Central China. Biochem Genet. 2007; 45: 281-90.

516. Zhang S, Chen J, Shi W, Liu W, Zhang W, Zhang Y. Mutations in *panD* encoding aspartate decarboxylase are associated with pyrazinamide resistance in *Mycobacterium tuberculosis*. Emerg Microb Infect. 2013; 2: e34.

517. Zhang Y, Heym B, Allen B, Young D, Cole S. The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. Nature. 1992; 358: 591-3.

518. Zhang Y, Young D. Strain variation in the *katG* region of *Mycobacterium tuberculosis*. Mol Microbiol. 1994; 14: 301-8.

519. Zhang Y, Scorpio A, Nikaido H, Sun Z. Role of acid pH and deficient efflux of pyrazinoic acid in unique susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. J Bacteriol. 1999; 181: 2044-9.

520. Zhang Y, Permar S, Sun Z. Conditions that may affect the results of susceptibility testing of *Mycobacterium tuberculosis* to pyrazinamide. J Med Microbiol. 2002; 51: 42-9.

521. Zhang Y, Mitchison D. The curious characteristics of pyrazinamide: a review. Int J Tuberc Lung Dis. 2003; 7: 6-21.

522. Zhang Y, Yew W. Mechanisms of drug resistance in *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis. 2009; 13: 1320-30.

523. Zhao B, Pine R, Domagala J, Drlica K. Fluoroquinolone action against clinical isolates of *Mycobacterium tuberculosis*: effects of a C-8 methoxyl group on survival in liquid media and in human macrophages. Antimicrob Agents Chemother. 1999; 43: 661-6.

524. Zias J. Leprosy and tuberculosis in the Byzantine monasteries of the Judaean Desert. *In*: Ortner, D., Aufderheide AC, editors. Human palaeopathology. Current syntheses and future options. Washington D.C. Smithsonian Institution Press, pp. 197-199. 1991.

525. Zimhony O, Cox J, Welch J, Vilchèze C, Jacobs W. Pyrazinamide inhibits the eukaryotic-like fatty acid synthetase I (FASI) of *Mycobacterium tuberculosis*. Nat Med. 2000; 6: 1043-7.

526. Zink A, Molnár E, Motamedi N, Pálfy G, Marcsik A, Nerlich A. Molecular history of tuberculosis from ancient mummies and skeletons. Int J Osteoarchaeol. 2007; 17: 380-91.

527. Zuber B, Chami M, Houssin C, Dubochet J, Griffiths G, Daffé M. Direct visualization of the outer membrane of mycobacteria and corynebacteria in their native state. J Bacteriol. 2008; 190: 5672-80.

528. Zumla A, Abubakar I, Raviglione M, Hoelscher M, Ditiu L, Mchugh T, Squire S, Cox H, Ford N, McNerney R, Marais B, Grobusch M, Lawn S, Migliori G, Mwaba P, O'Grady J, Pletschette M, Ramsay A, Chakaya J, Schito M, Swaminathan S, Memish Z, Maeurer M, Atun R. Drug-resistant tuberculosis - Current dilemmas, unanswered questions, challenges, and priority needs. J Infect Dis. 2012; 205: S228-40.

Objectives of the thesis

M. tuberculosis strains with multi- or extensively drug resistance are a major threat for human health, worldwide. In order to efficiently treat infections caused by multidrug resistant strains its necessary to know the molecular basis of its resistance, understand how acquired drug resistance develops in a patient under treatment and how it can be controlled and/or prevented. The major objective of this work was to understand the basis of the development of resistance in *M. tuberculosis*, in order to design strategies to circumvent/or prevent its development. This study is divided into three main chapters which focus on the major aspects of *M. tuberculosis* drug resistance: (i) the description of the molecular genetic basis of drug resistance in *M. tuberculosis* clinical strains; (ii) the study on the contribution of efflux to the emergence of drug resistance in *M. tuberculosis*, and (iii) the description of the mechanism of action of putative efflux inhibitors that can be used as adjuvants on tuberculosis therapy.

Particular emphasis was given to:

(i) Characterization of the genetic profile for first- and second-line antituberculosis drugs of multidrug resistance *M. tuberculosis* strains circulating in the Lisbon metropolitan area (Portugal).

(ii) Determination the antibiotic susceptibility pattern to first- and second-line antituberculosis drugs, as well its levels of resistance.

(iii) Epidemiological characterization of the isolates by using MIRU-VNTR typing.

(iv) Analysis of the expression level of genes that code for efflux pumps by qRT-PCR.

(v) Identification of the efflux pumps involved in the development of drug resistance.

(vi) Evaluation of efflux activity by real-time fluorometry.

(vii) Susceptibility testing for the antituberculosis drugs in the presence/absence of selected efflux inhibitors.

(viii) Evaluation of the antimicrobial activity of the efflux inhibitors.

(ix) Investigation on the mechanism of action of the efflux inhibitors namely on their activity either directly on the pump or by depletion of the energy sources.

(x) Study the antimycobacterial activity of the efflux inhibitors *ex vivo* by using the macrophage model.

The enlighten of the dynamics of acquired drug resistance in *M. tuberculosis*, exploring the biological basis of multi- and extensively drug resistant tuberculosis, in order to identify new and alternative combinational therapeutic strategies to prevent and/or treat tuberculosis (in particular drug-resistant forms) is the ultimate objective of this thesis and its future perspectives.

CHAPTER II

Preface to Chapter II

Multidrug resistant tuberculosis is still increasing worldwide. The emergence of multidrug resistant and extensively drug resistant tuberculosis demands accurate and reproducible drug susceptibility testing and the efforts to contain the disease depends on its rapid detection and implementation of effective treatment together with public health interventions. The existence of drug resistant tuberculosis reflects the failure the strategies of implementation of effective tuberculosis control programs and the inefficacy of the health care system to contain the dissemination of the disease. Failure in the antibiotic provision and supply, inadequate prescription, non-adherence to the treatment, inadequate training of health care providers and the association HIV-tuberculosis are some of the contributing factors to the development of drug resistance. The management of multidrug resistant tuberculosis is hindered by the inexistence of standard procedures for detection of drug resistant *M. tuberculosis* strains and second line drug susceptibility testing.

To develop guidelines for the efficient clinical and laboratory diagnosis of all forms of tuberculosis and to optimize the appropriate treatment of drug resistant tuberculosis it is essential to perform accurate drug susceptibility testing and to understand the genetic basis of *M. tuberculosis* drug resistance. With the advances in the molecular biology field, rapid detection of drug resistance has been shown to be an important tool to improve the efficacy of treatment and reduce the spread of these strains.

Although the present official multidrug resistance rates in Portugal are low (0.56% of all new cases detected in 2012 in Portugal), the proportion of extensively drug resistant cases among these is high. In this chapter (Part A and B), we aimed to characterize gene mutations conferring resistance to the first and second line antituberculosis drugs and evaluate its correlation with the phenotypic levels of resistance in *M. tuberculosis* isolates collected from multidrug resistant patients from Lisbon. Based on the gene mutations patterns, its geographic specificity and its correlation with phenotypic levels drug resistance, we hope to contribute with increased knowledge on how the methodologies here used can be applied for the detection of drug resistant *M. tuberculosis* strains and the management of tuberculosis therapy.

Chapter IIA. Exploring the molecular genetic basis of drug-resistant *Mycobacterium tuberculosis* strains in Lisbon, Portugal: correlation between phenotypic and genetic resistance to first and second line antituberculosis drugs

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1. Abstract

The spread of multi- and extensively drug resistant *Mycobacterium tuberculosis* poses a serious threat to the control of tuberculosis. Accurate drug susceptibility testing is essential to reduce the dissemination of the disease. The purpose of this study was to determine the resistance patterns of *M. tuberculosis* strains circulating in Lisbon and its correlation with mutations associated with that resistance. Seventeen multidrug resistant strains isolated from patients residing in this area were characterized by first- and second-line drug quantitative susceptibility testing with the aid of BACTEC MGIT 960 system equipped with the TB eXIST software. Genotypic characterization focused on the analysis of the genes involved in drug resistance with the aid of the line-probe assays Genotype MTBDR*plus* and MTBDRs*l* and/or DNA sequencing. The results show a clear correlation between the presence of a mutation in the target gene and the resistance phenotype. The only exception is related with PAS resistance for which we could not find an explanation so far. The description of the resistance profiles of drug resistant *M. tuberculosis* strains adds new knowledge to the design of more effective tools for the diagnosis of tuberculosis. Together, quantitative drug susceptibility testing and the detection of mutations associated with resistance will allow the early administration of appropriate therapy since strains with low-level resistance may still respond to the treatment despite the presence of the mutation.

2. Introduction

Mycobacterium tuberculosis remains one of the most important human pathogens. Despite the innumerous efforts for its control, tuberculosis is one of the leading causes of death worldwide. According to the World Health Organization, in 2012, an estimated 8.6 million people developed tuberculosis and 1.3 million died from the disease (60). A further concern is the emergence and spread of multidrug resistant strains of *M. tuberculosis*. In 2012, there were an estimated 450 000 new cases of multidrug resistant tuberculosis worldwide, that resulted in 170 000 deaths. The average proportion of multidrug resistant tuberculosis cases with extensively drug resistance was 9.6% (60). The accurate and rapid detection of multi- and extensively drug resistant tuberculosis is imperative for the prompt application of appropriate treatment and isolation measures, and to prevent the transmission of the disease. For these reasons, the delay in the identification of resistant strains threatens the efforts to control the disease. Multidrug resistance is defined as resistance to both isoniazid and rifampicin, and extensively drug resistance is defined as resistance to isoniazid and rifampicin with additional resistance to any fluoroquinolone and at least one of the three second-line injectable drugs: amikacin, capreomycin, or kanamycin. The management of multidrug resistant tuberculosis is expensive, prolonged, and less effective when compared with the treatment for drug susceptible strains. The treatment for extensively drug resistant tuberculosis is even more difficult, and the prognosis is significantly worse (60).

According to the Portuguese National Health Authorities, in 2012 it was estimated an incidence of 21.6 new cases of tuberculosis per 100 000 inhabitants and an average of 0.56% cases of multidrug resistant tuberculosis, of which 20% corresponded to extensively drug resistant tuberculosis. 65% of the multidrug resistant cases where living in the metropolitan area of Lisbon (8). This indicates that although tuberculosis incidence is decreasing from 2011 to 2012 (23 to 21.6/ 100 000 respectively), multidrug resistant rates are increasing. Therefore, the national situation requires attention. In order to contain the spread of these strains it is necessary the development of guidelines for diagnosis and optimization of the treatment of drug resistant tuberculosis with the implementation of accurate drug susceptibility testing for first and second line drugs and a better understanding of the genetic basis of *M. tuberculosis* drug resistance.

It is known that the frequency and specificity of the resistance-associated mutations differ among different geographical areas. A precise understanding of the genetic diversity of *M. tuberculosis* complex strains is required in order to interpret the results of molecular methods for drug susceptibility testing (21). *M. tuberculosis* drug resistance is associated with mutations that occur in specific regions of genes whose products are targeted by the antibiotics. Isoniazid resistance is mainly associated with mutations in the catalase peroxidase (*katG*) gene and/or regulatory region of the *inhA* gene (56). Resistance to rifampicin is linked to mutations in the 81 bp rifampicin resistance determining region (RRDR) of the *rpoB* gene, which encodes the β subunit of RNA polymerase (53). Resistance to fluoroquinolones is associated with mutations in *gyrA* gene, which encodes the DNA gyrase (4), resistance to aminoglycosides (amikacin and kanamycin) and capreomycin is associated with mutations in *rrs* gene that encodes the 16S rRNA (1; 26), resistance to streptomycin is associated with mutations in *rrs* and/or *rpsL* (27), ethambutol resistance is associated with mutations in the *embB* gene, where the mutation in the codon 306 is the most frequent (2). *pncA* mutations are associated with pyrazinamide resistance (46), and *ethA* mutations with ethionamide resistance, along with mutations in *inhA* promoter (28). Despite the characterization of the above genes, some cases of drug resistance among clinical *M. tuberculosis* strains presented with wild-type sequences at the above genes, which indicates the need for further studies. It was described that *gidB* mutations are associated with streptomycin resistance (30; 34) and mutations in *eis* promoter region with amikacin and kanamycin resistance (33; 62).

In this work, we aimed to describe the prevalence and pattern of gene mutations associated with resistance to the first- and second-line drugs, as well as its levels of resistance, in a collection of multi- and extensively drug resistant strains isolated from patients from the Lisbon metropolitan area. By exploring the genetic basis of *M. tuberculosis* drug resistance we hope to contribute to the selection of effective methods for its prompt detection and management.

3. Material and methods

M. tuberculosis strains. Seventeen multidrug resistant strains were included in the study. The isolates were obtained from different patients with active tuberculosis from hospitals of the Lisbon region and correspond to all multidrug resistant strains received in our laboratory during 2009-2011 for identification and first-line susceptibility testing. The selection criterion for this study was only multidrug resistant strains. The strains were grown in Lowenstein-Jensen solid media and MGIT tubes for the BACTEC MGIT 960 liquid culture system (MGIT690; Becton and Dickinson Diagnostic Systems, Sparks, MD, USA). Cultures were identified with *M. tuberculosis* complex probes of the AccuProbe system (GenProbe Inc., San Diego, CA, USA) according to the recommendations of the manufacturer. The *M. tuberculosis* H37Rv ATCC27294^{T} and two clinical strains susceptible to all drugs in study were used as controls.

Antibiotics. Isoniazid, rifampicin, ofloxacin, amikacin, capreomycin, rifabutin, ethionamide, and paraamino-salicylic acid (PAS) were obtained from Sigma-Aldrich (St. Louis, MO, USA); moxifloxacin was obtained from Merck (Whitehouse Station, USA); and linezolid was obtained from Pfizer (New York, USA). All drugs were prepared in sterile deionized water except rifampicin and ethionamide which was prepared in dimethyl sulfoxide (DMSO); rifabutin in 10% methanol, ofloxacin, 10% sodium hydroxide, and PAS, 1% ethanol. The lyophilized drugs (BACTEC^{TM} MGIT^{TM} 960 SIRE and PZA kits; SIRE: streptomycin, isoniazid, rifampicin and ethambutol; PZA: pyrazinamide) used in standard susceptibility testing were purchased from Becton Dickinson and the stock solutions prepared as per manufacturer's instructions.

Susceptibility testing. Drug susceptibility testing for standard first-line drugs was performed with the MGIT960 system, according to the manufacturer's instructions. Drug susceptibility testing for the second-line drugs rifabutin, amikacin, capreomycin, ofloxacin, moxifloxacin, ethionamide, PAS, and linezolid was conducted using the MGIT960 system and the Epicenter V5.80A software equipped with the TB eXIST module (Becton Dickinson) as previously described (47). MGIT tubes were inoculated with 0.8 ml of SIRE supplement (Becton Dickinson), 0.1 ml of antibiotic at the desired concentration and 0.5 ml of the strain suspension. For the preparation of the drug-free growth control tube (proportional control), the strain suspension was diluted 1:100 with

a sterile saline solution and 0.5 ml inoculated into the MGIT tube. For first- and second-line drug susceptibility testing, strains were considered resistant when growth at critical/screening concentration was obtained. Quantitative drug susceptibility testing of the drugs was conducted using the MGIT960 system and the Epicenter V5.80A/TB eXIST module as described above. The interpretation of the results was performed as per Springer *et al*. (47). Briefly, at the time of growth of the proportional control (Growth Units [GU] =400), the comparison between this tube and the tubes containing the drugs(s) was performed. If the GU of the tubes containing the drug were >100, they were considered to be resistant to that concentration. If the GU value were <100 they were considered susceptible. The concentrations used for first- and second-line drug susceptibility testing and for quantitative drug susceptibility testing are described in Table IIA.1.

Detection of mutations associated with resistance. Genomic DNA was extracted using the QIAamp DNA Mini kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's instructions and used for PCR amplification for line probe assays and/or DNA sequencing.

(i) Line probe assays. The most common mutations in *rpoB* and *katG* genes and *inhA* regulatory region were investigated using Genotype MTBDR*plus* (Hain Lifescience GmbH, Nehren, Germany) according to the manufacturer's instructions. The search for the most frequent mutations in *rrs*, *gyrA* and *embB* was performed with Genotype MTBDR*sl* (Hain) according to the manufacturer's instructions.

(ii) DNA sequencing. Genomic analysis of the regions of interest of *rpoB*, *inhA*, *katG*, *ethA*, *pncA*, *rpsL*, *rrs*, *tlyA*, *gidB*, *gyrA*, *gyrB*, *eis*, *embB*, and *thyA* genes (Table IIA.2) was performed by PCR amplification using the primers described in Table IIA.3 followed by DNA sequencing. The PCR reactions were performed with the following amplification profile: initial denaturation at 94ºC for 5 minutes, followed by denaturation at 94ºC for 1 minute, primer dependent annealing temperature for 1 minute and extension at 72ºC for 1 minute during 40 cycles. The final extension occurred at 72ºC for 10 minutes.

Strain typing. Mycobacterial interspersed repetitive unit – variable number of tandem repeats (MIRU-VNTR) genotyping was performed by multiplex PCR amplification of

24 loci, as described by Supply *et al*. (51). The genotype of these strains was analyzed using the MIRU-VNTR*plus* web application (3; 59).

4. Results and discussion

The purpose of this study was to determine the drug resistance patterns of *M. tuberculosis* multidrug resistant strains (n=17) circulating in Lisbon and its correlation with mutations associated with resistance. The results obtained are presented in Table IIA.4.

Resistance to rifampicin is mainly due to point mutations in the *rpoB* gene encoding the β subunit of the RNA polymerase (53). In this study, 17/17 strains were resistant to rifampicin. Sixteen out of 17 strains were resistant to >20 µg/ml and all have the mutation S531L in *rpoB* gene. The remaining strain was resistant at 4 µg/ml and susceptible at 20 µg/ml of rifampicin. This strain possesses the mutation D516V in *rpoB*. For rifabutin, 7/17 strains were resistant to >2 µg/ml, 9 strains resistant at 0.4 µg/ml and susceptible at 2 µg/ml, all of them presenting the mutation S531L in *rpoB*. The remaining strain was resistant at 1 µg/ml and susceptible at 0.4 µg/ml and corresponds to the one that has the mutation D516V in *rpoB*. Cross-resistance between rifampicin and rifabutin was observed for all strains. The results shown that the mutation S531L is associated with high-level resistance to both rifampicin and rifabutin; and that the mutation D516V is associated with intermediate level of resistance to rifampicin and low-level resistance to rifabutin. The mutation S531L was the most common found which is in agreement with other reported studies (5; 7; 19; 57). This mutation was shown to be associated with rifampicin high-level resistance and cross-resistance with rifabutin, as already described (17). Moreover, in this work, the importance of the detection of rifampicin mutations as a surrogate marker for multidrug resistance in the metropolitan area of Lisbon has been once again demonstrated since all strains were also resistant to isoniazid (57).

In contrast, mutations associated with isoniazid resistance have been associated with several targets genes such as *katG*, *inhA*, *kasA* and *ahpC* genes (18; 38). However, the majority of isoniazid resistant isolates carry a mutation at either *katG* gene or in the promoter region of the *inhA* gene (14). The proportion of each mutation varies according to the geographic region. In this study, resistance to isoniazid was shown to

be restricted to mutations in *inhA* and *katG* gene. Seventeen out of 17 strains display high-level resistance to isoniazid. Among these, 3 strains were resistant at 10 µg/ml and 14 were resistant at 1 µg/ml and susceptible at 3 µg/ml of isoniazid. Sixteen out of 17 strains possesses the mutation C-15T in *inhA* promoter region coupled with the mutations S94A (nine strains) or I194T (seven strains) on the structural region of the gene. One strain has the mutation S315T in *katG*, the most commonly found among isoniazid resistant isolates (5; 54).

Mutations within the *ethA* and *inhA* structural genes are associated with high-levels of ethionamide resistance (28). In the present study, 17/17 strains presented high-level resistance to ethionamide (R > 25 µg/ml). The high-level resistance to ethionamide was associated with the presence of mutations in *inhA* gene in 16/17 strains, and for one strain the high-level resistance was caused by a mutation in *ethA* gene. Since mutations in *inhA* not only cause isoniazid resistance, but they also confer cross-resistance to ethionamide, the inclusion of ethionamide in the second-line therapeutic regimen for these strains will be useless.

Resistance to ethambutol is mainly associated with a mutation at codon 306 in *embB* (2). In the present study, 11/17 strains presented resistance to ethambutol. Five out of 11 strains were resistant at 12.5 µg/ml and susceptible at 50 µg/ml, all carrying the mutation M306V in *embB* gene. Six out of 11 strains were resistant at 5 µg/ml and susceptible at 12.5 µg/ml. Of these, two strains presented the mutation M306V whereas no mutation was found for the remaining four strains. Was identified one strain susceptible at critical concentration (5 µg/ml) with the mutation M306V in *embB*. Some studies also describe the occurrence of mutations in *embB*306 of *M. tuberculosis* isolates phenotypically susceptible to ethambutol (13; 31; 49). The identification of mutations in drug susceptible strains can indicate that the breakpoint for ethambutol resistance should be revised. Additional studies are needed in order to find the value at which the phenotypic resistance correlates with genotypic resistance. One strain was found to be resistant at critical concentration of ethambutol without a mutation in the ethambutol resistance determining region (ERDR). This indicates the presence of a mutation in other regions of *embB* or in other genes such as *embC* and *embA* (35; 49). The remaining six strains were susceptible at critical concentration and no mutation was

detected in the ERDR. These results indicated that *embB306* mutation is associated with low- and moderate-level of resistance to ethambutol as previously shown (42; 43).

Resistance to pyrazinamide is mainly associated with mutations in *pncA* gene (45). In this work, resistance at critical concentration (100 µg/ml) was detected in 13/17 strains all of them with associated mutations in *pncA* gene. The mutation V125G was found in 7/10 strains. From the remaining strains, one presents the mutation A-11G in the promoter region, three with a mutation in the initiation codon, M1T, one with an insertion of a cytosine at position 247, and one with a stop codon at position 123 (codon 41). Four strains were susceptible to pyrazinamide. These results indicated that resistance to pyrazinamide among our strains is associated with mutations in *pncA* gene. In parallel, pyrazinamidase activity was determined for the 13 strains tested resistant at critical concentration and no activity was detected in these strains which confirms the results above. Pyrazinamide susceptibility testing is complicated by the fact that pyrazinamide is only active in an acidic media. In this study, two strains were initially tested as resistant to pyrazinamide. PZAse activity was positive and no mutations were found in these strains. The repetition of the pyrazinamide susceptibility testing for the discordant isolates gave susceptibility results to the two strains. Similar results were reported in the study by Chedore *et al*. (6). Pyrazinamide susceptibility testing is affect by i) the inoculum size; ii) the presence of bovine serum albumin on OADC supplement and iii) the age of the cultures (64). All these factors can increase the pH of the media originating false resistance. Therefore, it is recommended that all phenotypic pyrazinamide resistant isolates should be confirmed by *pncA* gene mutation analysis and, if possible, by pyrazinamidase testing.

Mutations associated with streptomycin resistance in *M. tuberculosis* have been described in the genes encoding the 16S rRNA (*rrs*) and ribosomal protein S12 (*rpsL*) (11; 29; 38). Seventeen out of 17 strains were resistant to streptomycin at critical concentration. Ten out of 17 strains were resistant at >20 µg/ml, all with the mutation K43R in *rpsL* gene. Six out of 17 strains were resistance at 4 µg/ml and susceptible at 20 µg/ml and the remaining strain was resistant at 1 µg/ml and susceptible at 4 µg/ml. These seven strains harboured a mutation in the *gidB* gene (A80P). No mutations were found in *rrs*. Mutations in *rpsL* gene correlate with high-level resistance to streptomycin

and are in agreement with other studies (27). The mutations in *gidB* were shown to confer only low-level resistance to streptomycin as previously demonstrated (34).

Resistance to amikacin and capreomycin has been associated with mutations in the 1400 region of the *rrs* gene (26). The mutations most commonly reported as a cause of resistance include the A1401G, C1402T, and G1484T substitutions (1; 26; 32). From the 17 strains evaluated, 12 were resistant to amikacin and capreomycin. Regarding amikacin, 6/12 strains presented low-level resistance, *i.e*., they are resistant at critical concentration (1 µg/ml) and susceptible at 4 µg/ml. These strains do not have any mutation in the region studied of the *rrs* gene. The only mutation found in *rrs* corresponds to a common polymorphism that is not associated with resistance (insertion of a thymine at position 1078) (Table IIA.4). In these six strains was found a mutation in *eis* promoter region (G-10A). This mutation was established to be associated with amikacin low-level resistance in the study by Perdigão *et al*. (33). The remaining 6/12 strains were resistant at >20 µg/ml which corresponds to amikacin high-level resistance and correlates well with the mutation detected in *rrs*, A1401G as described (26). In relation to capreomycin, 6/12 strains with the mutation A1401G in *rrs*, three presented high-level resistance (R >25 µg/ml); and the remaining three presented low-level resistance to capreomycin (resistant at 5 µg/ml and susceptible at 25 µg/ml). All six strains presented also high-level resistance to amikacin indicating that this substitution confers cross-resistance between amikacin (high-level) and capreomycin (low/high-level). In the remaining six strains resistant to capreomycin for which we do not detect a mutation in *rrs* was found an insertion of a GT base pair at position 755/756 of *tlyA* gene. Three out of six strains presented high-level resistance to capreomycin and the remaining three display low-level resistance. The six strains with mutation on *tlyA* exhibit low-level resistance to amikacin. For strain MTB12 with the mutation A1401G, the wild-type sequence was simultaneously detected by the Genotype MTBDR*sl*. This indicates that both wild-type and resistant population are in sufficient number to be detected by the assay. Of notice is that during the susceptibility testing for amikacin and capreomycin the resistant population was selected. This laboratory result anticipates what happens *in vivo* during amikacin/capreomycin therapy. Heteroresistance can result from the emergence of a drug resistant subpopulation or due to the selection of a

different strain in a patient under therapy and was already described for rifampicin, isoniazid, ethambutol and amikacin (16; 41; 63).

Mutations in the quinolone resistance determining region of *gyrA* gene (QRDR) of *gyrA* and, to a lesser extent, *gyrB* genes are responsible for fluoroquinolones resistance (51; 57). The most common mutations in *gyrA* occur in position 94 (D94G or D94A), 90 (A90V), 91 (S91P) and 88 (G88C) (4; 58). In this study, resistance to ofloxacin and moxifloxacin was detected in 10/17 strains. These strains were resistant at 2 µg/ml and susceptible at 10 µg/ml of ofloxacin, and resistant at 0.25 µg/ml and susceptible at 5 µg/ml of moxifloxacin, indicating low-level cross-resistance for the two antibiotics. All the fluoroquinolone resistant strains harbored a mutation in the QRDR of *gyrA* gene, S91P (seven strains) and D94A (three strains). No mutations were found in the QRDR of *gyrB*. From the 17 multidrug resistant strains, nine were found to be extensively drug resistant. This represents 52.9% of the sample.

PAS target the folate pathway (39) and mutations in *thyA* gene, which encodes a thymidylate synthase, have been implicated in clinical PAS resistance (12; 25; 39; 65). In the present work, high-level resistance to PAS (R >64 µg/ml) was detected in four strains. We analyzed the *thyA* gene to search for mutations and found the mutation T202A in the four strains. Feuerriegel *et al*. (10) showed that the T202A polymorphism in *thyA* is not a valid marker for PAS resistance rather a phylogenetic marker for the Latino-American-Mediterranean (LAM) lineage. To evaluate the role of this mutation on PAS resistance we sequence *thyA* gene for the remaining strains. The T202A was also found in 12 strains susceptible to PAS; hence, the involvement of this mutation in PAS resistance is questionable and remains to be clarified. Strain MTB12 possesses a wild type sequence and corresponds to the only Beijing strain among the collection. The remaining 16 strains belong to LAM lineage (see below).

Resistance to linezolid is rarely found among *M. tuberculosis* clinical isolates (15; 40) and when it happens, the resistance is associated with mutations in the *rrl* gene (20; 37; 61) or to non-ribosomal mechanisms (44). In this study, no resistant strains were detected which is in accordance to the previously described. Mutation rate for linezolid is low, 10^{-8}-10^{-9} (15). This can explain the reduced prevalence of resistant mutants due to the defects caused by the drug in the growth rate as already described for *M. smegmatis* (44).

MIRU-VNTR typing of the 17 strains shows that 16/17 strains belong to the LAM genotype and that can be divided in two clusters: cluster Lisboa3 and cluster Q1 (Figure IIA.1). The remaining strain corresponds to a Beijing strain. Both Lisboa3 and Q1 clusters can be distinguished by its mutational pattern regarding the mutations associated with resistance: cluster Lisboa3 comprised the nine strains with the *inhA* S94A, *rpsL* K43R, *tlyA* GT 755/766, *eis* G-10A, and *gyrA* S91P; the seven strains clustered in Q1 possess the following mutational pattern: *inhA* I194T, *embB* M306V, *pncA* V125G, *gidB* A80P, *rrs* A1401G, and *gyrA* D94A (Figure IIA.1).

This study is limited by the size of the sample evaluated. It will be necessary to study more strains including mono- and poly resistant *M. tuberculosis* strains to support this data.

5. Conclusions

To reduce the development and the spread of drug resistance among *M. tuberculosis* strains several control approaches have been developed such as (i) the timely implementation of an effective tuberculosis treatment that desperately needs to be guided by information coming from the performance of accurate drug susceptibility tests for first- and second-line drugs, and (ii) the simultaneous development and implementation of molecular tools for the detection of *M. tuberculosis* and mutations associated with drug resistance. Here, we shown that i) there is a clear correlation between the development of gene mutations in the drug targets and the resistance phenotype of a strain; ii) the chromosomal alterations associated with resistance are greatly conserved; and ii) the mutations can be associated with a specific genotype. Additionally, we were able to establish an association between the mutations and the levels of resistance for the antituberculosis drugs. Concerning the molecular detection of resistance, the Genotype MTBDR*plus* proved to be reliable method for predicting multidrug resistance. However, mutational analyses for *inhA* should be complemented with DNA sequencing. Concerning the detection of strains with extensively drug resistance, our study demonstrates that the Genotype MTBDR*sl* can only cover half of the *M. tuberculosis* strains. Strains with mutations in genes that are associated with low-level resistance for second-line injectable drugs will be missed (e.g. *eis* for amikacin

and *tlyA* for capreomycin). Besides these systems, the recently lunched AID TB resistance assay (GenID, Autoimmun Diagnostika, GmbH, Strassberg, Germany) can be used for the detection of mutations associated with streptomycin resistance; however, low-level streptomycin resistance due to mutations in *gidB* will not be detected. The targets for the remaining antibiotics have to be screened by DNA sequencing.

It is known that the mutational pattern of a strain varies according to geographical regions and our results are in accordance with it. The variability of drug mutations according to the genotype of a strain constitutes a major drawback for the application of molecular assays of the detection of drug resistance in a routine basis. Despite their advances, molecular methods do not identify all phenotypically resistant strains, as we have put in evidence in this work. This fact highlights the limitations of the molecular testing and the need for their complementation with accurate drug susceptibility testing. The implementation of these methods on a routine basis should be done carefully and the best results are those obtained when these methods are combined with the drug susceptibility testing. In Figure IIA.2, a working algorithm is presented showing how drug susceptibility testing can be complemented with the molecular detection of mutations associated with drug resistance.

In conclusion, the description of the resistance profiles of drug resistant *M. tuberculosis* strains circulating in Lisbon adds new knowledge for the design of more effective tools for the diagnosis of tuberculosis and for the treatment of patients infected with drug resistant strains. False resistance cases are a major cause for the incorrect diagnosis of drug resistant *M. tuberculosis* and may lead to unnecessary reduction of the drugs available for the therapeutic regimen. Together, drug susceptibility testing with the simultaneous detection of mutations associated with resistance will allow the early administration of appropriate therapy and strains with low-level resistance may still respond to the adjusted treatment and doses despite the presence of a mutation for drug resistance.

6. Figures

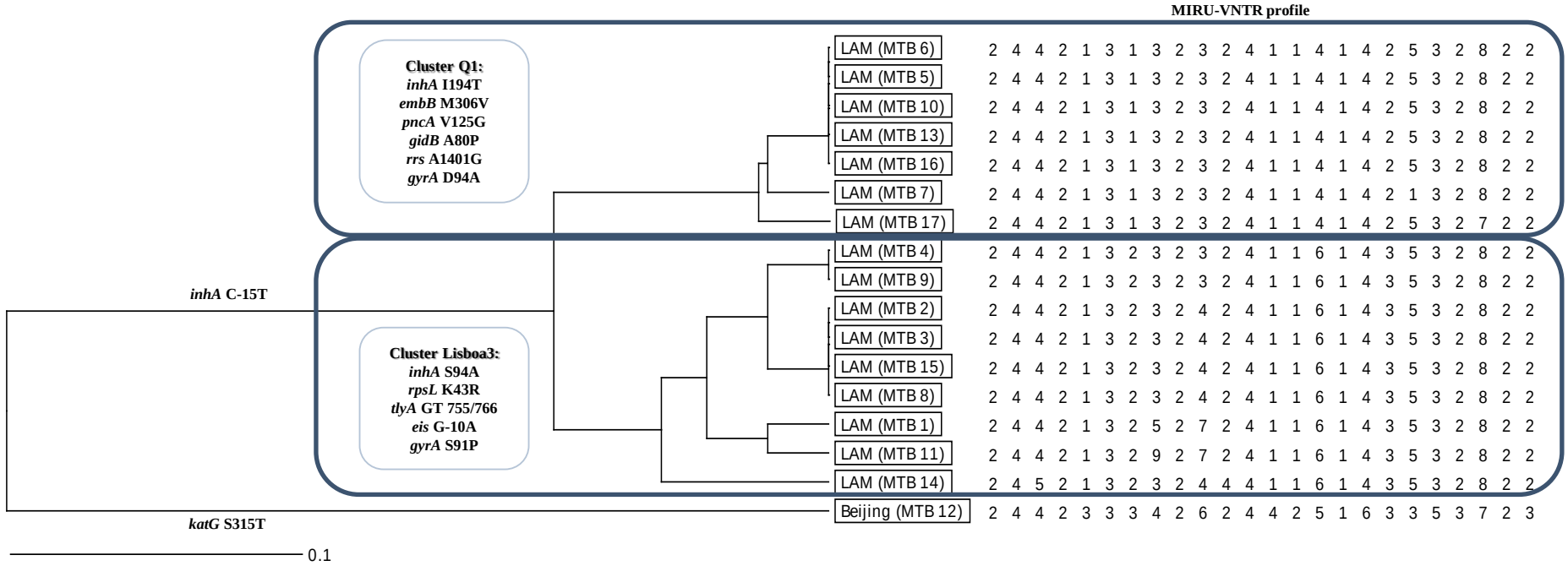

Figure IIA.1. Cladrogram based on 24 loci MIRU-VNTR of the 17 multidrug resistant *M. tuberculosis* isolates from Lisbon, Portugal. The boxes highlights clusters Q1 and Lisboa3. Inside of each box are depicted the mutational pattern for each cluster regarding the mutations associated with resistance. From left to right are shown lineage, strain number and MIRU-VNTR profile. The linkage distance scale is indicated at the bottom. LAM: Latino-American-Mediterranean; MTB: *M. tuberculosis*.

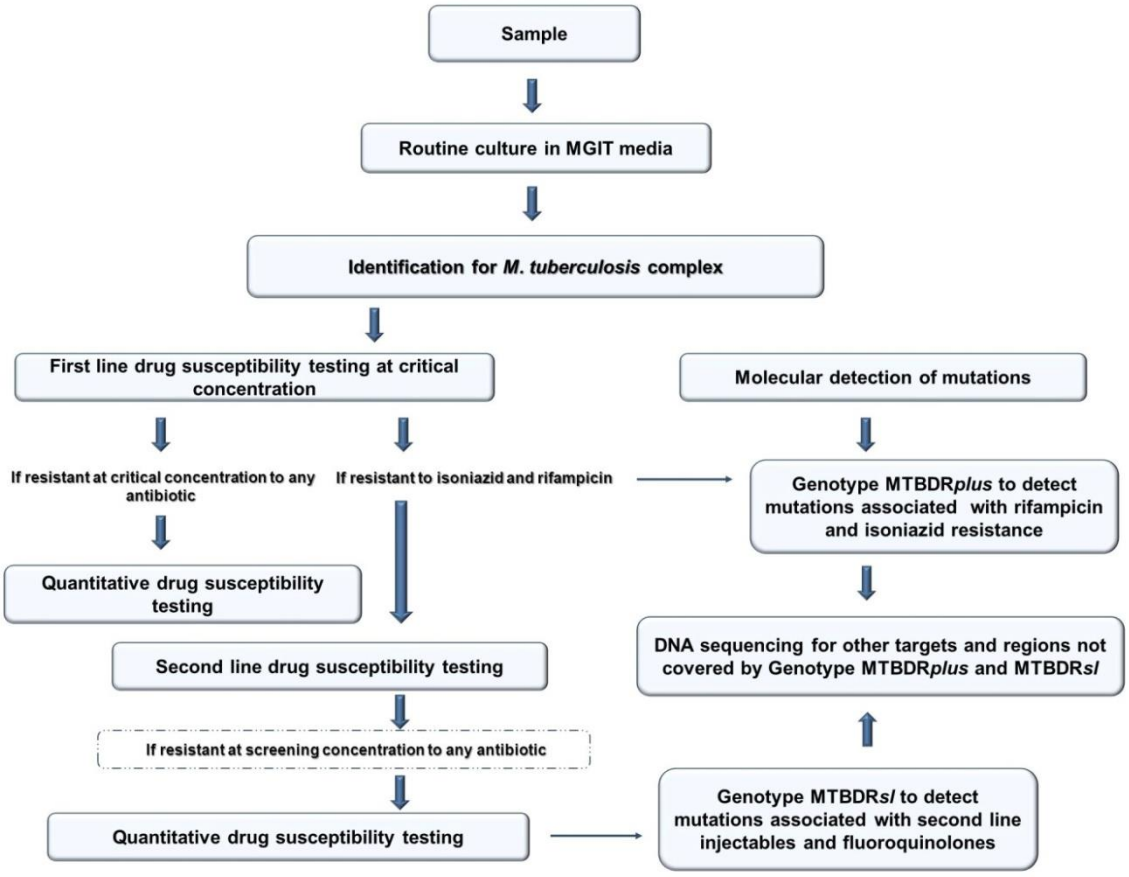

Figure IIA.2. Working algorithm for the application of drug susceptibility testing for first and second line drugs combined with molecular detection of mutations associated with resistance.

7. Tables

Table IIA.1. Concentrations used for first line, second line, and quantitative drug susceptibility testing.

Antibiotics	Critical/screening concentration (µg/ml)	Concentration for quantitative testing (µg/ml)
Isoniazid	0.1	0.4, 1.0, 3.0, 10.0
Rifampicin	1.0	10.0
Ethambutol	5.0	12.5, 50
Pyrazinamide	100.0	-
Streptomycin	1.0	4.0, 20.0
Rifabutin	0.1	0.4, 2.0
Amikacin	1.0	4.0, 20.0
Capreomycin	2.5	5.0, 25.0
Ofloxacin	1.0	2.0, 10.0
Moxifloxacin	0.25	0.5, 2.5, 7.5
Ethionamide	5.0	10.0, 25.0
PAS	4.0	16.0, 64.0
Linezolid	1.0	4.0, 16.0

Table IIA.2. Algorithm used for the molecular analysis.

Antibiotic	Target gene	Additional testing
Rifampicin	*rpoB* RRDR	*rpoB*
Isoniazid	*inhA* prom, *katG* 315	*inhA* structural region
Ethionamide	*inhA*, *ethA*	
Ethambutol	*embB* 306	
Pyrazinamide	*pncA*	pyrazinamidase activity
Streptomycin	*rpsL* (43, 88); *rrs* 530 region	*gidB*
Amikacin	*rrs* 1401 and 1490 region	*e*is
Capreomycin	*rrs*1401 and 1490 region	*tlyA*
Fluoroquinolones	*gyrA* QRDR	*gyrB*
PAS	*thyA*	
Linezolid	*rrl*	

RRDR, rifampicin resistance determining region; QRDR, quinolone resistance determining region.

Table IIA.3. Primers used in this study.

Gene	Primer	Primer sequence (5'-3')	Annealing (^{0}C)	Length (bp)	Ref.
rpoB	rpoB-1	GGG AGC GGA TGA CCA CCC A	60	350	36
	rpoB-2	GCG GTA CGG CGT TTC GAT GAA C			
inhA	inhA-1	CCT CGC TGC CCA GAA AGG GA	64	248	28
	inhA-2	ATC CCC CGG TTT CCT CCG GT			
	inhA-3	AGG TCG CCG GGG TGG TCA GC	60	517	
	inhA-4	AGC GCC TTG GCC ATC GAA GCA			
	inhA-3F	CCA CAT CTC GGC GTA TTC G		501	22
	inhA-5R	TTC CGG TCC GCC GAA CGA CAG			
katG	P4_Fw	CGG ACC ATA ACG GCT TCC TG	62	563	24
	P4_Rv	TTG TCC AAG CTG GCG TTG TC			
	P5_Fw	CGA CAA CGC CAG CTT GGA C		518	
	P5_Rv	CGG TTC CGG TGC CAT ACG			
	P6_Fw	AGC TCG TAT GGC ACC GGA AC		619	
	P6_Rv	TGA CCT CCC ACC CGA CTT GT			
	P7_Fw	ACA AGT CGG GTG GGA GGT C		574	
	P7_Rv	CTG CCG GTC CAC TTC ACC TT			
	P8_Fw	GGG ACC TAC CAG GGC AAG GA		629	
	P8_Rv	CCG GGA GTC AGC AAG TCA CC			
rrs	rrs_420F	TTCACCATCGACGAAGGTCC	62	571	55
	rrs_990R	CTAGACGCGTCCTGTGCATGT			
	rrs_P2	GAG TAC GGC CGC AAG GCT A	66	680	This study
	rrs1539	GGG GCG TTT TCG TGG TGC TCC			1
tlyA	tlyAs	GCA TCG CAC GTC GTC TTT	55	947	9
	tlyAas	GGT CTC GGT GGC TTC GTC			
eis	eisF1	GCC ATG GGA CCG GTA CTT GC	56	601	33
	eisR1	GTA GAT GCC GCC CTC GCT AG			
gidB	gidB_Fw	CGA GAG CGG AGA ATG TTT CA	62	793	This study
	gidB_Rv	CTG GCC CGA CCT TAC GAG			
pncA	pncA_promP1	GCT GGT CAT GTT CGC GAT CG	55	214	46
	pncA_promP2	TCG GCC AGG TAG TCG CTG AT			
	pncA_Fw	AGT CGC CCG AAC GTA TGG TG	62	615	22
	pncA_Rv	CAA CAG TTC ATC CCG GTT CG			
gyrA	gyrA_Fw	ATC GCC GGG TGC TCT ATG	62	321	This study
	gyrA_Rv	GGC CGT CGT AGT TAG GGA TG			
ethA	ethA1	ATC ATC GTC GTC TGA CTA TGG	55	667	28
	ethA5	ACT ACA ACC CCT GGG ACC			
	ethA4	CCT CGA CCT TCC CGT GA	64	692	
	ethA9	CCT CGA GTA CGT CAA GAG CAC			
	ethA8	GGT GGA ACC GGA TAT GCC TG	68	342	
	ethA10	CGT TGA CGG CCT CGA CAT TAC			
embB	embB-F2	AAC CTG CGC CCG CAG ATT GTC	62	526	50
	embB-R2	GGT CTG GCA GGC GCA TCC			
gyrB	gyrB_Fw	GTC GTT GTG AAC AAG GCT GTG	62	413	This study
	gyrB_Rv	GTG GAA ATA TGT TGG CCG TC			
rpsL	rpsL_Fw	GGC CGA CAA ACA GAA CGT	64	504	48
	rpsL_Rv	GTT CAC CAA CTG GGT GAC			
thyA	Rv2764b_Fw	ATC ATC GTG TCG GCC TGG AA	62	506	This study
	Rv2764b_Rv	CTT GAG CCC AGA TCA GCC C			

Fw/F: forward; Rv/R: reverse. S, sense; As, antisense; prom, promoter.

Table IIA.4. Quantitative drug susceptibility testing using the MGIT960 system and the Epicenter TB eXIST software and molecular characterization of resistance associated mutations.

Strain	Isoniazid					*inhA*prom	*inhA*	*katG*	Ethionamide			*ethA*	Rifampicin			Rifabutin			*rpoB*	Ethambutol			*embB*	PZA	*pncA*	*PZAse activity*
	0.1	0.4	1.0	3.0	10.0				2.5	5.0	25.0		1.0	4.0	20.0	0.1	0.4	2.0		5.0	12.5	50.0		100.0		
MTB1	R	R	R	S	S	C-15T	S94A	wt	R	R	R	wt	R	R	R	R	R	S	S531L	S	S	S	wt	S	wt	positive
MTB2	R	R	R	S	S	C-15T	S94A	wt	R	R	R	wt	R	R	R	R	R	R	S531L	S	nd	nd	wt	S	wt	positive
MTB3	R	R	R	S	S	C-15T	S94A	wt	R	R	R	wt	R	R	R	R	R	S	S531L	S	nd	nd	wt	S	wt	positive
MTB4	R	R	R	S	S	C-15T	S94A	wt	R	R	R	wt	R	R	S	R	S	S	D516V	S	nd	nd	wt	R	ins of C at position 247	negative
MTB5	R	R	R	S	S	C-15T	I194T	wt	R	R	R	wt	R	R	R	R	R	R	S531L	R	R	S	M306V	R	V125G	negative
MTB6	R	R	R	S	S	C-15T	I194T	wt	R	R	R	wt	R	R	R	R	R	R	S531L	R	R	S	M306V	R	V125G	negative
MTB7	R	R	R	S	S	C-15T	I194T	wt	R	R	R	wt	R	R	R	R	R	S	S531L	S	nd	nd	M306V	R	V125G	negative
MTB8	R	R	R	S	S	C-15T	S94A	wt	R	R	R	wt	R	R	R	R	R	S	S531L	S	nd	nd	wt	R	M1T	negative
MTB9	R	R	R	R	R	C-15T	S94A	wt	R	R	R	wt	R	R	R	R	R	R	S531L	R	S	S	wt	R	A-11G	negative
MTB10	R	R	R	S	S	C-15T	I194T	wt	R	R	R	wt	R	R	R	R	R	R	S531L	R	S	S	M306V	R	V125G	negative
MTB11	R	R	R	S	S	C-15T	S94A	wt	R	R	R	wt	R	R	R	R	R	S	S531L	R	S	S	wt	R	M1T	negative
MTB12	R	R	R	R	R	wt	wt	S315T	R	R	R	S266R	R	R	R	R	R	R	S531L	R	R	S	M306V	R	TAC_{41}→STOP	negative
MTB13	R	R	R	S	S	C-15T	I194T	wt	R	R	R	wt	R	R	R	R	R	R	S531L	R	R	S	M306V	R	V125G	negative
MTB14	R	R	R	S	S	C-15T	S94A	wt	R	R	R	wt	R	R	R	R	R	S	S531L	R	S	S	wt	R	M1T	negative
MTB15	R	R	R	R	R	C-15T	S94A	wt	R	R	R	wt	R	R	R	R	R	S	S531L	R	S	S	wt	S	wt	positive
MTB16	R	R	R	S	S	C-15T	I194T	wt	R	R	R	wt	R	R	R	R	R	S	S531L	R	S	S	M306V	R	V125G	negative
MTB17	R	R	R	S	S	C-15T	I194T	wt	R	R	R	wt	R	R	R	R	R	S	S531L	R	R	S	M306V	R	V125G	negative
MTB18	S	S	S	S	S	wt	wt	wt	S	S	S	wt	S	S	S	S	S	S	wt	S	S	S	wt	S	wt	positive
MTB19	S	S	S	S	S	wt	wt	wt	S	S	S	wt	S	S	S	S	S	S	wt	S	S	S	wt	S	wt	positive
H37Rv	S	S	S	S	S	wt	wt	wt	S	S	S	wt	S	S	S	S	S	S	wt	S	S	S	wt	S	wt	positive

MTB, *M. tuberculosis*; R, resistant; S, susceptible; prom, promoter; PZA, pyrazinamide; wt, wild type; nd, not done.

Table IIA.4. Quantitative drug susceptibility testing using the MGIT960 system and the Epicenter TB eXIST software and molecular characterization of resistance associated mutations (cont.).

Strain	Streptomycin			500 *rrs* region	*gidB*	*rpsL*	Amikacin			Capreomycin			*1400 rrs region*	*tlyA*	*eis*	PAS			*thyA*	Ofloxacin			Moxifloxacin				*gyrA*	Linezolid		
	1.0	4.0	20.0				1.0	4.0	20.0	2.5	5.0	25.0				4.0	16.0	64.0		1.0	2.0	10.0	0.25	0.5	2.5	7.5		1.0	4.0	16.0
MTB1	R	R	R	nd	nd	K43R	S	S	S	S	nd	nd	wt	wt	nd	S	nd	nd	T202A	R	R	S	R	R	S	S	S91P	S	nd	nd
MTB2	R	R	R	wt	nd	K43R	S	nd	nd	S	S	S	wt	wt	nd	S	nd	nd	T202A	S	nd	nd	S	nd	nd	nd	wt	S	nd	nd
MTB3	R	R	R	nd	nd	K43R	R	S	S	R	R	S	ins T at pos 1078	ins GT at pos 755/756	G-10A	R	R	R	T202A	R	R	S	R	R	S	S	S91P	S	nd	nd
MTB4	R	R	R	wt	nd	K43R	S	nd	nd	S	nd	nd	wt	wt	wt	S	nd	nd	T202A	S	nd	nd	S	nd	nd	nd	wt	S	nd	nd
MTB5	R	R	S	wt	A80P	wt	R	R	R	R	R	S	A1401G	wt	wt	S	nd	nd	T202A	R	R	S	R	R	S	S	D94A	S	nd	nd
MTB6	R	R	S	wt	A80P	wt	R	R	R	R	R	S	A1401G	wt	wt	S	nd	nd	T202A	R	R	S	R	R	S	S	D94A	S	nd	nd
MTB7	R	R	S	wt	A80P	wt	S	nd	nd	S	S	S	wt	wt	wt	S	nd	nd	T202A	S	nd	nd	S	nd	nd	nd	wt	S	nd	nd
MTB8	R	R	R	wt	wt	K43R	R	S	S	R	R	S	ins T at pos 1078	ins GT at pos 755/756	G-10A	S	nd	nd	T202A	R	R	S	R	R	S	S	S91P	S	nd	nd
MTB9	R	R	R	wt	wt	K43R	R	S	S	R	R	S	ins T at pos 1078	ins GT at pos 755/756	G-10A	R	R	R	T202A	R	R	S	R	R	S	S	S91P	S	nd	nd
MTB10	R	S	S	wt	A80P	wt	S	nd	nd	S	S	S	wt	wt	nd	S	nd	nd	T202A	S	nd	nd	S	nd	nd	nd	wt	S	nd	nd
MTB11	R	R	R	nd	nd	K43R	R	S	S	R	R	R	ins T at pos 1078	ins GT at pos 755/756	G-10A	S	nd	nd	T202A	R	R	S	R	R	S	S	S91P	S	nd	nd
MTB12	R	R	R	nd	nd	K43R	R	R	R	R	R	S	wt/A1401G	nd	nd	S	nd	nd	wt	S	nd	nd	S	nd	nd	nd	wt	S	nd	nd
MTB13	R	R	S	wt	A80P	wt	R	R	R	R	R	R	A1401G	wt	wt	S	nd	nd	T202A	S	nd	nd	S	nd	nd	nd	wt	S	nd	wt
MTB14	R	R	R	nd	nd	K43R	R	S	S	R	R	R	ins T at pos 1078	ins GT at pos 755/756	G-10A	R	R	R	T202A	R	R	S	R	R	S	S	S91P	S	nd	nd
MTB15	R	R	R	wt	wt	K43R	R	S	S	R	R	R	ins T at pos 1078	ins GT at pos 755/756	G-10A	R	R	R	T202A	R	R	S	R	R	S	S	S91P	S	nd	nd
MTB16	R	R	S	wt	A80P	wt	R	R	R	R	R	R	A1401G	wt	wt	S	nd	nd	T202A	S	nd	nd	S	nd	nd	nd	wt	S	nd	wt
MTB17	R	R	S	wt	A80P	wt	R	R	R	R	R	R	A1401G	nd	nd	S	nd	nd	T202A	R	R	S	R	R	S	S	D94A	S	nd	nd
MTB18	S	S	S	wt	nd	wt	S	S	S	S	S	S	wt	wt	wt	S	S	S	wt	S	S	S	S	S	S	S	wt	S	S	S
MTB19	S	S	S	wt	nd	wt	S	S	S	S	S	S	wt	wt	wt	S	S	S	wt	S	S	S	S	S	S	S	wt	S	S	S
H37Rv	S	S	S	wt	nd	wt	S	S	S	S	S	S	wt	wt	wt	S	S	S	wt	S	S	S	S	S	S	S	wt	S	S	S

Strains in red are classified as extensively drug resistant. MTB, *M. tuberculosis*; R, resistant; S, susceptible; prom, promoter; PZA, pyrazinamide. Wt, wild type; nd, not done.

8. References

1. Alangaden G, Kreiswirth B, Aouad A, Khetarpal M, Igno F, Moghazeh S, Manavathu E, Lerner S. Mechanism of resistance to amikacin and kanamycin in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1998; 42: 1295-97.

2. Alcaide F, Pfyffer G, Telenti A. Role of *embB* in natural and acquired resistance to ethambutol in mycobacteria. Antimicrob Agents Chemother. 1997; 41: 2270-3.

3. Allix-Béguec C, Harmsen D, Weniger T, Supply P, Niemann S. Evaluation and strategy for use of MIRU-VNTR*plus*, a multifunctional database for online analysis of genotyping data and phylogenetic identification of *Mycobacterium tuberculosis* complex isolates. J Clin Microbiol. 2008; 46: 2692-99.

4. Aubry A, Veziris N, Cambau E, Truffot-Pernot C, Jarlier V, Fisher L. Novel gyrase mutations in quinolone-resistant and-hypersusceptible clinical isolates of *Mycobacterium tuberculosis*: functional analysis of mutant enzymes. Antimicrob Agents Chemother. 2006; 50: 104-12.

5. Bolotin S, Alexander D, Chedore P, Drews S, Jamieson F. Molecular characterization of drug-resistant *Mycobacterium tuberculosis* isolates from Ontario, Canada. J Antimicrob Chemother. 2009; 64: 263-6.

6. Chedore P, Bertucci L, Wolfe J, Sharma M, Jamieson F. Potential for erroneous results indicating resistance when using the Bactec MGIT 960 system for testing susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. J Clin Microbiol. 2010; 48: 300-1.

7. Crudu V, Stratan E, Romancenco E, Allerheiligen V, Hillemann A, Moraru N. First evaluation of an improved assay for molecular genetic detection of tuberculosis as well as rifampin and isoniazid resistances. J Clin Microbiol. 2012; 50: 1264-9.

8. Direcção-Geral da Saúde. Programa Nacional de Luta Contra a Tuberculose. Ponto da Situação Epidemiológica e de Desempenho de 2012. Relatório para o Dia Mundial da Tuberculose. Avaliação preliminar em Março 2013. Direcção-Geral da Saúde, Lisboa, Portugal; 2013.

9. Feuerriegel S, Cox H, Zarkua N, Karimovich H, Braker K, Rüsch-Gerdes S, Niemann S. Sequence analyses of just four genes to detect extensively drug-resistant *Mycobacterium tuberculosis* strains in multidrug-resistant tuberculosis patients undergoing treatment. Antimicrob Agents Chemother. 2009; 53: 3353-6.

10. Feuerriegel S, Köser C, Trübe L, Archer J, Rüsch-Gerdes S, Richter E, Niemann S.. Thr202Ala in *thyA* is a marker for the Latin American Mediterranean lineage of the *Mycobacterium tuberculosis* complex rather than para-aminosalicylic acid resistance. Antimicrob Agents Chemother. 2010; 54: 4794-8.

11. Finken M, Kirschner P, Meier A, Wrede A, Bottger E. Molecular basis of streptomycin resistance in *Mycobacterium tuberculosis*: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. Mol Microbiol. 1993; 9: 1239-46.

12. Fivian-Hughes A, Houghton J, Davis E. *Mycobacterium tuberculosis* thymidylate synthase gene *thyX* is essential and potentially bifunctional, while *thyA* deletion confers resistance to p-aminosalicylic acid. Microbiol. 2012; 158: 308-18.

13. Hazbón M, del Valle M, Guerrero M, Varma-Basil M, Filliol I, Cavatore M, Colangeli R, Safi H, Billman-Jacobe H, Lavender C, Fyfe J, García-García L, Davidow A, Brimacombe M,

León C, Porras T, Bose M, Chaves F, Eisenach K, Sifuentes-Osornio J, de León A, Cave M, Alland D. Role of *embB* codon 306 mutations in *Mycobacterium tuberculosis* revisited: a novel association with broad drug resistance and IS*6110* clustering rather than ethambutol resistance. Antimicrob Agents Chemother. 2005; 49: 3794-802.

14. Hazbón M, Brimacombe M, del Valle M, Cavatore M, Guerrero M, Varma-Basil M, Billman-Jacobe H, Lavender C, Fyfe J, García-García L, León C, Bose M, Chaves F, Murray M, Eisenach K, Sifuentes-Osornio J, Cave M, Ponce de León A, Alland D. Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2006; 50: 2640-9.

15. Hillemann D, Rüsch-Gerdes S, Richter E. *In vitro*-selected linezolid-resistant *Mycobacterium tuberculosis* mutants. Antimicrob Agents Chemother. 2008; 52: 800-1.

16. Hofmann-Thiel S, van Ingen J, Feldmann K, Turaev L, Uzakova G, Murmusaeva G, van Soolingen D, Hoffmann H. Mechanisms of heteroresistance to isoniazid and rifampin of *Mycobacterium tuberculosis* in Tashkent, Uzbekistan. Eur Respir J. 2009; 33: 368-74.

17. Jamieson F, Guthrie J, Neemuchwala A, Lastovetska O, Melano R, Mehaffy C. Profiling of *rpoB* mutations and MICs for rifampin and rifabutin in *Mycobacterium tuberculosis*. J Clin Microbiol. 2014; 52: 2157-62.

18. Johnson R., Streicher E, Louw G, Warren R, van Helden P, Victor T. Drug resistance in *Mycobacterium tuberculosis*. Curr Issues Mol Biol. 2006; 8: 97-112.

19. Khanna A, Raj V, Tarai B, Sood R, Pareek P, Upadhyay D, Sharma P, Rattan A, Saini K, Singh H. Emergence and molecular characterization of extensively drug-resistant *Mycobacterium tuberculosis* clinical isolates from the Delhi Region in India. Antimicrob Agents Chemother. 2010; 54: 4789-93.

20. Kloss P, Xiong L, Shinabarger D, Mankin A. Resistance mutations in 23S rRNA identify the site of action of protein synthesis inhibitor, linezolid, in the ribosomal peptidyl transferase center. J Mol Biol. 1999; 294: 93-101.

21. Köser C, Feuerriegel S, Summers D, Archer J, Niemann S. Importance of the genetic diversity within the *Mycobacterium tuberculosis* complex for the development of novel antibiotics and diagnostic tests of drug resistance. Antimicrob Agents Chemother. 2012; 56: 6080-7.

22. Leung E, Ho P, Yuen K, Woo W, Lam T, Kao R, Seto W, Yam W. Molecular characterization of isoniazid resistance in *Mycobacterium tuberculosis*: identification of a novel mutation in *inhA*. Antimicrob Agents Chemother 2006; 50: 1075-8.

23. Louw G, Warren R, Donald P, Murray M, Bosman M, Van Helden P, Young D, Victor T. Frequency and implications of pyrazinamide resistance in managing previously treated tuberculosis patients. Int J Tuberc Lung Dis. 2006; 10: 802-7.

24. Machado D, Perdigão J, Ramos J, Couto I, Portugal I, Ritter C, Boettger E, Viveiros M. High-level resistance to isoniazid and ethionamide in multidrug-resistant *Mycobacterium tuberculosis* of the Lisboa family is associated with *inhA* double mutations. J Antimicrob Chemother. 2013; 68: 1728-32.

25. Mathys V, Wintjens R, Lefevre P, Bertout J, Singhal A, Kiass M, Kurepina N, Wang X, Mathema B, Baulard A, Kreiswirth B, Bifani P. Molecular genetics of para-aminosalicylic acid resistance in clinical isolates and spontaneous mutants of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2009; 53: 2100-9.

26. Maus C, Plikaytis B, Shinnick T. Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2005; 49: 3192-97.

27. Meier A, Sander P, Schaper K, Scholz M, Böttger E. Correlation of molecular resistance mechanisms and phenotypic resistance levels in streptomycin-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1996; 40: 2452-4.

28. Morlock G, Metchock B, Sikes D, Crawford J, Cooksey R. *ethA*, *inhA*, and *katG* loci of ethionamide-resistant clinical *Mycobacterium tuberculosis* isolates. Antimicrob Agents Chemother 2003; 47: 3799-805.

29. Nair J, Rouse D, Bai G, Morris S. The *rpsL* gene and streptomycin resistance in single and multiple drug-resistant strains of *Mycobacterium tuberculosis*. Mol Microbiol. 1993; 10: 521-7.

30. Okamoto S, Tamaru A, Nakajima K, Tanaka N, Tokuyama S, Suzuki Y, Ochi K. Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. Mol Microbiol. 2007; 63: 1096-106.

31. Perdigão J, Macedo R, Ribeiro A, Brum L, Portugal I. Genetic characterization of the ethambutol resistance-determining region in *Mycobacterium tuberculosis*: prevalence and significance of *embB306* mutations. Int J Antimicrob Agents. 2009; 33: 334-8.

32. Perdigão J, Macedo R, Malaquias A, Ferreira A, Brum L, Portugal I. Genetic analysis of extensively drug-resistant *Mycobacterium tuberculosis* strains in Lisbon, Portugal. J Antimicrob Chemother. 2010; 65: 224-7.

33. Perdigão J, Macedo R, Silva C, Machado D, Couto I, Viveiros M, Jordão L, Portugal I. From multidrug-resistant to extensively drug-resistant tuberculosis in Lisbon, Portugal: the stepwise mode of resistance acquisition. J Antimicrob Chemother. 2013; 68: 27-33

34. Perdigão J, Macedo R, Machado D, Silva C, Jordão L, Couto I, Viveiros M, Portugal I. GidB mutation as a phylogenetic marker for Q1 cluster *Mycobacterium tuberculosis* isolates and intermediate-level streptomycin resistance determinant in Lisbon, Portugal. Clin Microbiol Infect. 2013. doi: 10.1111/1469-0691.12392.

35. Plinke C, Cox H, Zarkua N, Karimovich H, Braker K, Diel R, Rüch-Gerdes S, Feuerriegel S, Niemann S. *embCAB* sequence variation among ethambutol-resistant *Mycobacterium tuberculosis* isolates without *embB306* mutation. Antimicrob Agents and Chemother. 2010; 65: 1359-67.

36. Portugal I, Maia S, Moniz-Pereira J. Discrimination of multidrug-resistant *Mycobacterium tuberculosis* IS*6110* fingerprint subclusters by *rpoB* gene mutation analysis. J Clin Microbiol. 1999; 37: 9: 3022-4.

37. Prystrowsky J, Siddiqui F, Chosay J, Shinabarger D, Millichap J, Peterson L, Noskin G. Resistance to linezolid: characterization of mutations in rRNA and comparison of their occurrences in vancomycin resistant enterococci. Antimicrob Agents Chemother. 2001; 45: 2154-6.

38. Ramaswamy S, Musser J. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tuber Lung Dis. 1998; 79: 3-29.

39. Rengarajan J, Sassetti C, Naroditskaya V, Sloutsky A, Bloom B, Rubin E. The folate pathway is a target for resistance to the drug para-aminosalicylic acid (PAS) in mycobacteria. Mol Microbiol. 2004; 53: 275-82.

40. Richter E, Rüsch-Gerdes S, Hillemann D. First linezolid-resistant clinical isolates of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2007; 51: 1534-6.

41. Rinder H, Mieskes K, Löscher T. Heteroresistance in *Mycobacterium tuberculosis*. Int J Tuber Lung Dis. 2001; 5: 339-45.

42. Safi H, Sayers B, Hazbon M, Alland D. Transfer of *embB* codon 306 mutations into clinical *Mycobacterium tuberculosis* strains alters susceptibility to ethambutol, isoniazid, and rifampin. Antimicrob Agents and Chemother. 2008; 52: 2027-34.

43. Safi H, Fleishmann R, Peterson S, Jones M, Jarrahi B, Alland D. Allelic exchange and mutant selection demonstrate that common clinical *embCAB* gene mutations only modestly increase resistance to ethambutol in *Mycobacterium tuberculosis*. Antimicrob Agents and Chemother. 2010; 54: 103-8.

44. Sander P, Belova L, Kidan Y, Pfister P, Mankin A, Böttger E. Ribosomal and non-ribosomal resistance to oxazolidinones: species-specific idiosyncrasy of ribosomal alterations. Mol Microbiol. 2002; 46: 1295-304.

45. Scorpio A, Zhang Y. Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nat Med. 1996; 2: 662-7.

46. Scorpio A, Lindholm-Levy P, Heifets L, Gilman R, Siddiqi S, Cynamom M, Zhang Y. Characterization of *pncA* mutations in pirazinamide-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1997; 41: 540-3.

47. Springer B, Lucke K, Calligaris-Maibach R, Ritter C, Böttger E. Quantitative drug susceptibility testing of *Mycobacterium tuberculosis* using MGIT960 and the EpiCenter instrumentation. J Clin Microbiol. 2009; 47: 1773-80.

48. Sreevatsan S, Pan X, Stockbauer K, Williams D, Kreiswirth B, Musser J. Characterization of *rpsL* and *rrs* mutations in streptomycin-resistant *Mycobacterium tuberculosis* isolates from diverse geographic localities. Antimicrob Agents Chemother. 1996; 40: 1024-6.

49. Srivastava S, Ayyagaria A, Dholea T, Nyatia K, Dwivedi S. *emb* nucleotide polymorphisms and the role of *embB306* mutations in *Mycobacterium tuberculosis* resistance to ethambutol. Int J Med Microbiol. 2009; 299: 269-80.

50. Starks A, Gumusboga A. Plikaytis B, Shinnick T, Posey J. Mutations at *embB306* are an important molecular indicator of ethambutol resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2009; 53: 1061-66.

5**1.** Supply P, Allix-Béguec C, Lesjean S, Cardoso-Oelemann M, Rüsch-Gerdes S, Willery E, Savine E, de Haas P, van Deutekom H, Roring S, Bifani P, Kurepina N, Kreiswirth B, Sola C, Rastogi N, Vatin V, Gutierrez M, Fauville M, Niemann S, Skuce R, Kremer K, Locht C, van Soolingen D. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol. 2006; 44: 4498-510.

52. Takiff H, Salazar L, Guerrero C, Philipp W, Huang W, Kreiswirth B, Cole S, Jacobs Jr W, Telenti A. Cloning and nucleotide sequence of *Mycobacterium tuberculosis gyrA* and *gyrB* genes and detection of quinolone resistance mutations. Antimicrob Agents Chemother. 1994; 38: 773-80.

53. Telenti A, Imboden P, Marchesi F, Matter L, Schopfer K, Bodmer T, Lowrie D, Colston MJ, Cole S. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. Lancet. 1993; 341: 647-50.

54. Tho D, Lan N, Chau N, Farrar J, Caws M. Multiplex allele-specific polymerase chain reaction for detection of isoniazid resistance in *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis. 2011; 15: 799-803.

55. Via L, Cho S, Hwang S, Bang H, Park S, Kang H, Jeon D, Min S, Oh T, Kim Y, Kim Y, Rajan V, Wong S, Shamputa I, Carroll M, Goldfeder L, Lee S, Holland S, Eum S, Lee H, Barry C. Polymorphisms associated with resistance and cross-resistance to aminoglycosides and capreomycin in *Mycobacterium tuberculosis* isolates from South Korean patients with drug-resistant tuberculosis. J Clin Microbiol. 2010; 48: 402-11.

56. Vilchèze C, Jacobs Jr W. The mechanism of isoniazid killing: clarity through the scope of genetics. Annu Rev Microbiol. 2007; 61: 35-50.

57. Viveiros M, Leandro C, Rodrigues L, Almeida J, Bettencourt R, Couto I, Carrilho L, Diogo J, Fonseca A, Lito L, Lopes J, Pacheco T, Pessanha M, Quirim J, Sancho L, Salfinger M, Amaral L. Direct application of the INNO-LiPA Rif.TB line-probe assay for rapid identification of *Mycobacterium tuberculosis* complex strains and detection of rifampin resistance in 360 smear-positive respiratory specimens from an area of high incidence of multidrug-resistant tuberculosis. J Clin Microbiol. 2005; 43: 4880-4.

58. Von Groll A, Martin A, Jureen P, Hoffner S, Vandamme P, Portaels F, Palomino J, da Silva P. Fluoroquinolone resistance in *Mycobacterium tuberculosis* and mutations in *gyrA* and *gyrB*. Antimicrob Agents Chemother. 2009; 53: 4498-500.

59. Weniger T, Krawczyk J, Supply P, Niemann S, Harmsen D. MIRU-VNTR*plus*: a web tool for polyphasic genotyping of *Mycobacterium tuberculosis* complex bacteria. Nucleic Acids Res. 2010; 38(suppl 2): W326-31.

60. World Health Organization. Global tuberculosis report 2013. WHO/HTM/TB/2013.11. Geneva, Switzerland. World Health Organization; 2013.

61. Xiong L, Kloss P, Douthwaite S, Andersen N, Swaney S, Shinabarger D, Mankin A. Oxazolidinone resistance mutations in 23S rRNA of *Escherichia coli* reveal the central region of domain V as the primary site of drug action. J Bacteriol. 2000; 182: 5325-31.

62. Zaunbrecher M, Sikes R, Metchock B, Shinnick T, Posey J. Overexpression of the chromosomally encoded aminoglycoside acetyltransferase *eis* confers kanamycin resistance in *Mycobacterium tuberculosis*. Proc Natl Acad Sci. 2009; 106: 20004-9.

63. Zhang X, Zhao B, Huang H, Zhu Y, Peng J, Dai G, Jiang G, Liu L, Zhao Y, Jin Q. Co-occurrence of amikacin-resistant and-susceptible *Mycobacterium tuberculosis* isolates in clinical samples from Beijing, China. J Antimicrob Chemother. 2013; 68: 1537-42.

64. Zhang Y, Wade M, Scorpio A, Zhang H, Sun Z. Mode of action of pyrazinamide: disruption of *Mycobacterium tuberculosis* membrane transport and energetics by pyrazinoic acid. J Antimicrob Chemother. 2003; 52: 790-5.

65. Zhang Z, Zhao Y, Li Z, Jia H, Liu Y, Chen X, Liu Z, Du B, Xing A, Ma Y. Mutations in the thymidylate synthase gene is a major mechanism in the para-aminosalicylic acid resistance of *M. tuberculosis*. Chinese J Tuberc Respir Dis. 2007; 30: 683-5.

Chapter IIB. High level resistance to isoniazid and ethionamide in multidrug resistant *Mycobacterium tuberculosis* of the Lisboa family is associated with *inhA* double mutations

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1. Synopsis

Objectives: The purpose of this study was to determine the levels of isoniazid and ethionamide resistance and to identify associated mutations in endemic multidrug resistant (MDR) strains of *M. tuberculosis* from the Lisbon metropolitan area, Portugal.

Methods: Seventeen clinical MDR tuberculosis (TB) strains were characterized by standard and semi-quantitative drug susceptibility testing to assess the level of isoniazid and ethionamide resistance. The genes *katG*, *inhA*, *ethA* and *ndh* were screened for mutations. All strains were genotyped by 24 loci mycobacterial interspersed repetitive unit-variable number of tandem repeats (MIRU-VNTR) analysis.

Results: All strains showed high-level resistance to both isoniazid (>1 mg/L) and ethionamide (>25 mg/L). MIRU-VNTR typing revealed the presence of two main clusters, Lisboa3 and Q1, in 16/17 strains, all of which showed the C-15T mutation in the promoter region of the *inhA* gene. The 16 strains belong to the Latino-American-Mediterranean (LAM) genotype and the other strain belongs to the Beijing genotype. Sequencing of the *inhA* open reading frame revealed that the 16 strains also had mutations in the structural region of the gene, leading to the S94A substitution in 9 strains and the I194T substitution in 7 strains.

Conclusions: The results reveal that the presence of a mutation in the *inhA* regulatory region together with a mutation in the *inhA* coding region can lead to the development of high level isoniazid resistance and cross resistance to ethionamide among the MDR-TB strains circulating in Lisbon. This mutational pattern also hints to a possible involvement of strain-specific factors that could be a feature of the Portuguese MDR-TB strains where the LAM family is the major circulating genotype.

2. Introduction

Isoniazid is one of the most effective drugs for the treatment of tuberculosis (TB). Ethionamide is a second-line drug used in the treatment of multidrug-resistant (MDR) TB. Both compounds are pro-drugs that need activation by different enzymes but share common pathways, which can lead to cross-resistance. The majority of the mutations responsible for high-level isoniazid resistance in *Mycobacterium tuberculosis* are found in the *katG* gene.[1] The second most commonly found mutations are observed in the promoter of *inhA*.[1] These mutations increase InhA expression and confer low-level resistance to isoniazid (0.1<MIC<1.0 mg/L).[2] Mutations in the *inhA* promoter also confer resistance to ethionamide, whose activity against *M. tuberculosis* depends on the activation by EthA.[3] In addition to *inhA* promoter mutations, decreased susceptibility to isoniazid and ethionamide is associated with mutations in the structural region of *inhA* that decrease the affinity to the drug-NAD adduct.[2]

In Portugal, the incidence rate of TB is high and the prevalence of MDR-TB and extensively drug resistant (XDR) TB in the Lisbon region is a cause of major concern.[4] More than 90% of these strains show high-level isoniazid resistance associated with the C-15T *inhA* promoter mutation. This genotype was also related with the high prevalence of ethionamide resistance.[5] To address the apparent incomprehensiveness between the level of isoniazid and ethionamide resistance in these strains and their mutation profile, we analysed the level of resistance towards isoniazid and ethionamide and identified associated mutations in the MDR-TB strains isolated during 2009-2011 at the Mycobacteriology Laboratory of the Instituto de Higiene e Medicina Tropical of the Universidade Nova de Lisboa.

3. Material and methods

Strains. Seventeen clinical strains of *M. tuberculosis* were included in the study (see Table 1). The isolates were obtained from different patients with active TB from hospitals of the Lisbon region and correspond to all MDR-TB strains received in our laboratory during 2009-2011 for identification and first-line susceptibility testing. The selection criterion for this study was only MDR strains. All isolates were identified

using the AccuProbe MTBC test (GenProbe Inc., SanDiego, CA, USA) according to the manufacturer's instructions. The *M. tuberculosis* reference strain H37Rv ATCC27294^{T} was included as the control.

Susceptibility testing. First-line susceptibility testing was carried out with the BACTEC^{TM} MGIT^{TM} 960 (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) for streptomycin, isoniazid, rifampicin, ethambutol and pyrazinamide, according to the manufacturer's instructions. Semi-quantitative drug susceptibility testing of isoniazid and ethionamide was conducted using the MGIT 960 system and the Epicenter V5.80A software equipped with the TB eXIST module (Becton Dickinson), as previously described.[6,7]

Detection of mutations associated with resistance. Genomic DNA was extracted using the QIAamp DNA Mini kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's instructions. The most common mutations in the structural *katG* gene and in the *inhA* regulatory region were investigated using Genotype MTBDR*plus* (Hain Lifescience GmbH, Germany) according to the manufacturer's instructions. Nucleic acid analysis of the complete *inhA*, *katG* and *ethA* genes and an internal fragment of the *ndh* gene (nt 560 to 931) (for a list of the primers and conditions see Table IIB-S1, available as Supplementary data) was performed by DNA sequencing.

Strain typing. Mycobacterial interspersed repetitive unit – variable number of tandem repeats (MIRU-VNTR) genotyping was performed by multiplex PCR amplification of 24 loci, as described by Supply *et al*.[8] The genotype of these strains was analyzed using the MIRU-VNTR*plus* web application[9] and the SITVITWEB database[10] when relevant.

4. Results

The quantitative isoniazid resistance levels were determined for the seventeen MDR-TB isolates and the results are summarized in Table IIB-1. All isolates display isoniazid resistance levels >1 mg/L. According to the Genotype MTBDR*plus* assay, 16 of the 17 strains contain a mutation the *inhA* promoter region (C-15T); one strain (MTB12) has a mutation in the *katG* gene (S315T). The entire *katG* gene was sequenced for a selected

group of strains (MTB2, 4, 5, 9, 10, and 13-17), but no mutation was found. The C-15T mutation is described to confer cross-resistance to ethionamide. All isolates show high-level ethionamide resistance (>25 mg/L) (Table IIB-1). Sequencing of the *inhA* open reading frame (ORF) revealed that all strains in addition to the C-15T mutation in the *inhA* promoter harbour point mutations in the *inhA* structural gene (Table IIB-1). Nine of these isolates carried the S94A mutation. In the remaining seven isolates, we detected the I194T mutation. The single isolate with a mutation in *katG*, in addition carries a mutation previously described in *ethA*, S266R.[11] No mutation was found in the region of the *ndh* gene analyzed.

Analysis of the 17 strains by MIRU-VNTR typing revealed that 16/17 strains represent two clusters, cluster Lisboa3 and cluster Q1 (Figure IIB.1). These 16 strains can be assigned to the Latino-American-Mediterranean (LAM) genotype and the remaining 1 to the Beijing genotype. Cluster Lisboa3 comprised the nine strains with the C-15T/S94A mutations and the seven strains with the C-15T/I194T substitutions were clustered in Q1 (Figure IIB.1).

5. Discussion

In the present study, we determined the levels of resistance to isoniazid and ethionamide for all MDR-TB strains isolated from 2009 to 2011 in our laboratory and we searched for mutations in target genes associated with resistance to isoniazid and ethionamide. We found that 16 strains have cumulative mutations in *inhA* gene: the C-15T mutation in the regulatory region coupled with mutations in the structural region of the gene, leading to the S94A and I194T substitutions. The presence of the mutation C-15T has been reported to confer low-level resistance to isoniazid and cross-resistance to ethionamide.[1] The mutations S94A[2] and I194T[12,13] have also been reported to cause isoniazid resistance and both were associated with low-level resistance to isoniazid.[11,13] However, there are few reports on the combinations C-15T/S94A[3,11] and C-15T/I194T.[12] The double mutation C-15T/S94A has been reported to confer low-level resistance[11], while the C-15T/I194T combination was identified in one isolate with high-level resistance to isoniazid.[12] All 16 strains with a double mutation studied in our work exhibit high-level resistance to both isoniazid and ethionamide. In an attempt to

compare the level of resistance of these strains carrying double mutations with the resistance level of strains carrying only the C-15T mutation, we searched for additional isoniazid-resistant strains in our laboratory isolated during the same period. This C-15T mutation alone was found among non-MDR strains and was correlated with low-level resistance to isoniazid (data not shown).

MIRU-VNTR typing of the strains showed the presence of two main clusters, Lisboa3 and Q1, indicating that the dissemination of these strains is clonal. However, the mutational profile related to drug resistance indicates that the acquisition of drug resistance-associated mutations is non-clonal (data not shown), except for isoniazid and rifampicin. Thus, resistance to isoniazid has been transmitted clonally, as all strains share the same C-15T mutation and the S94A/I194T mutations were acquired subsequent to the C-15T mutation. Sixteen out of 17 strains were found to belong to the LAM genotype, previously shown to be the main genotype circulating in Portugal.[5,14] Fenner *et al*,[15] recently proposed that the genetic background of a strain may influence the level of resistance to isoniazid conveyed by particular drug resistance-conferring mutations. Our results suggest that these two mutations, when combined with the C-15T mutation, can act synergistically to confer high-level resistance in this particular group of strains. All non-MDR strains evaluated during the period of this study, with low-level resistance to isoniazid, belong to the LAM genotype with only one belonging to Lisboa3 (data not shown). The single strain with a KatG S315T alteration is positioned in a completely separated branch and comprises the only Beijing strain found. From this we conclude that strain MTB12 is a foreign strain imported into the Lisbon area, not being part of the endemic circulating MDR clones. Comparison of the 12 loci MIRU with the SITVITWEB showed that MTB12 possess the MIRU international type MIT83, which corresponds to a modern Beijing type.

Given the high-level ethionamide resistance observed in our isolates, the inclusion of ethionamide in second-line treatment regimens will have no benefit and other therapeutic combinations must be considered.[16] Our data point to the need of rapid diagnostic methods for the detection of these strains and strong laboratory support to provide timely and accurate drug resistance information to guide the implementation of appropriate therapy.[4] While our study is limited by the size of the sample evaluated, the

strains investigated account for ≈45% of all the MDR-TB strains isolated in Lisbon during this period.[17-19]

In conclusion, we report that the presence of a mutation in the *inhA* regulatory region together with a mutation in the *inhA* coding region is associated with high-level resistance to both isoniazid and ethionamide among the MDR-TB strains circulating in Lisbon. Furthermore, we demonstrate that MDR-TB cases in Lisbon continue to be caused by a closely related family of strains, identified several years ago as being associated with MDR/XDR-TB.[20] This mutational pattern also suggests the involvement of strain-specific factors that could be a feature of the Portuguese MDR-TB strains with relevance for their appropriate treatment.

6. Figures

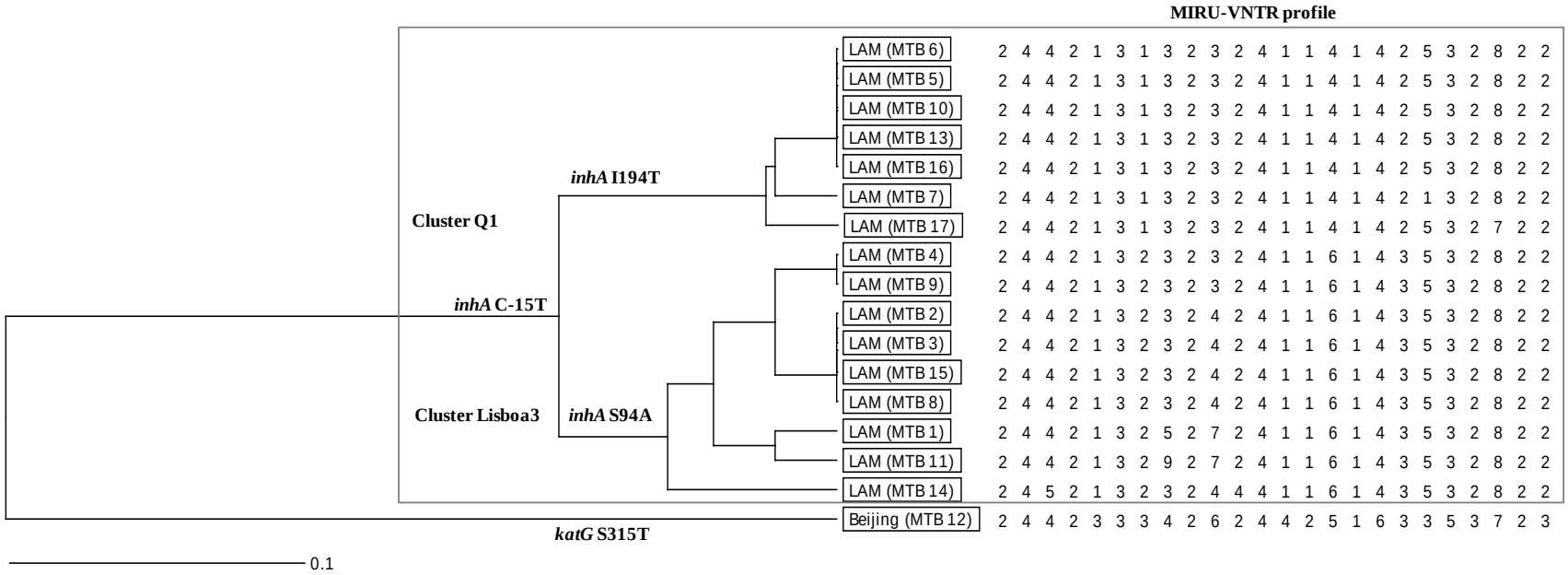

Figure IIB.1. Cladogram based on 24 loci MIRU-VNTR of the 17 MDR *M. tuberculosis* isolates from Lisbon, Portugal. The grey box highlights clusters Q1 and Lisboa3 (both belonging to the LAM genotype). From left to right are shown lineage, strain number and MIRU-VNTR profile. The linkage distance scale is indicated at the bottom. LAM, Latino-American-Mediterranean; MTB, *M. tuberculosis*.

7. Tables

Table IIB.1. Quantitative drug susceptibility profile of isoniazid and ethionamide and associated mutations in the 17 *M. tuberculosis* isolates.

Strain	Standard DST profile	INH qDST (mg/L)					ETH qDST (mg/L)			Gene mutations				
		0.1	0.4	1	3	10	5	10	25	*inhA*prom	*inhA* ORF	*katG*	*ethA*	*ndh*
H37Rv	INH^S, RIF^S, STR^S, ETB^S, PZA^S	S	S	S	S	S	S	S	S	none	none	none	none	none
MTB1	INH^R, RIF^R, STR^R, ETB^S, PZA^R	R	R	R	S	S	R	R	R	C-15T	**S94A**	none	**none**	none
MTB2	INH^R, RIF^R, STR^R, ETB^S, PZA^S	R	R	R	S	S	R	R	R	C-15T	**S94A**	**none**	**none**	none
MTB3	INH^R, RIF^R, STR^R, ETB^S, PZA^S	R	R	R	S	S	R	R	R	C-15T	**S94A**	none	**none**	none
MTB4	INH^R, RIF^R, STR^R, ETB^S, PZA^R	R	R	R	S	S	R	R	R	C-15T	**S94A**	**none**	**none**	none
MTB5	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	S	S	R	R	R	C-15T	**I194T**	**none**	**none**	none
MTB6	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	S	S	R	R	R	C-15T	**I194T**	none	**none**	none
MTB7	INH^R, RIF^R, STR^R, ETB^S, PZA^R	R	R	R	S	S	R	R	R	C-15T	**I194T**	none	**none**	none
MTB8	INH^R, RIF^R, STR^R, ETB^S, PZA^R	R	R	R	S	S	R	R	R	C-15T	**S94A**	none	**none**	none
MTB9	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	R	R	R	R	R	C-15T	**S94A**	**none**	**none**	none
MTB10	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	S	S	R	R	R	C-15T	**I194T**	**none**	**none**	none
MTB11	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	S	S	R	R	R	C-15T	**S94A**	none	**none**	none
MTB12	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	R	R	R	R	R	none	**none**	S315T	**S266R**	none
MTB13	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	I	S	R	R	R	C-15T	**I194T**	**none**	**none**	none
MTB14	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	S	S	R	R	R	C-15T	**S94A**	**none**	**none**	none
MTB15	INH^R, RIF^R, STR^R, ETB^R, PZA^S	R	R	R	S	S	R	R	R	C-15T	**S94A**	**none**	**none**	none
MTB16	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	S	S	R	R	R	C-15T	**I194T**	**none**	**none**	none
MTB17	INH^R, RIF^R, STR^R, ETB^R, PZA^R	R	R	R	S	S	R	R	R	C-15T	**I194T**	**none**	**none**	none

MTB, *M. tuberculosis*; DST, drug susceptibility testing; qDST, quantitative drug susceptibility testing; prom, promoter; INH, isoniazid; ETH, ethionamide; RIF, rifampicin; STR, streptomycin; ETB, ethambutol; PZA, pyrazinamide; R, resistant; I, intermediate; S, susceptible.
Results in bold indicate that the entire gene was sequenced.

6. References

1. Hazbón M, Brimacombe M, del Valle M *et al*. Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2006; 50: 2640-9.

2. Vilchèze C, Wang F, Arai M *et al*. Transfer of a point mutation in *Mycobacterium tuberculosis inhA* resolves the target of isoniazid. *Nat Med* 2006; 12: 1027-9.

3. Morlock G, Metchock B, Sikes D *et al*. *ethA*, *inhA*, and *katG* loci of ethionamide-resistant clinical *Mycobacterium tuberculosis* isolates. *Antimicrob Agents Chemother* 2003; 47: 3799-805.

4. Viveiros M, Martins M, Couto I *et al*. Molecular tools for rapid identification and novel effective therapy against MDRTB/XDRTB infections. *Expert Rev Anti Infect Ther* 2010; 8: 465-80.

5. Perdigão J, Macedo R, João I *et al*. Multidrug-resistant tuberculosis in Lisbon, Portugal: a molecular epidemiological perspective. *Microb Drug Resist* 2008; 14: 133-43.

6. Springer B, Lucke K, Calligaris-Maibach R *et al*. Quantitative drug susceptibility testing of *Mycobacterium tuberculosis* using MGIT960 and the EpiCenter instrumentation. *J Clin Microbiol* 2009; 47: 1773-80.

7. Machado D, Couto I, Perdigão J *et al*. Contribution of efflux to the emergence of isoniazid and multidrug resistance in *Mycobacterium tuberculosis*. *PLoS One* 2012; 7: e34538.

8. Supply P, Allix C, Lesjean S *et al*. Proposal for standardization of optimized mycobacterial interspersed repetitive unit–variable-number tandem repeat typing of *Mycobacterium tuberculosis*. *J Clin Microbiol* 2006; 44: 4498-510.

9. Allix-Béguec C, Harmsen D, Weniger T *et al*. Evaluation and strategy for use of MIRU-VNTRplus, a multifunctional database for online analysis of genotyping data and phylogenetic identification of *Mycobacterium tuberculosis* complex isolates. *J Clin Microbiol* 2008; 46: 2692-99.

10. Demay C, Liens B, Burguiere T *et al*. SITVITWEB–a publicly available international multimarker database for studying *Mycobacterium tuberculosis* genetic diversity and molecular epidemiology. *Infec Genet Evol* 2012; 12: 755-66.

11. Brossier F, Veziris N, Truffot-Pernot C *et al*. Molecular investigation of resistance to the antituberculous drug ethionamide in multidrug-resistant clinical isolates of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2011; 55: 355-60.

12. Leung E, Ho P, Yuen K *et al*. Molecular characterization of isoniazid resistance in *Mycobacterium tuberculosis*: identification of a novel mutation in *inhA*. *Antimicrob Agents Chemother* 2006; 50: 1075-8.

13. Hazbón M, Motiwala AS, Cavatore M *et al*. Convergent evolutionary analysis identifies significant mutations in drug resistance targets of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2008; 52: 3369-76.

14. David S, Duarte EL, Leite CF *et al*. Implication of the RD^{Rio} *Mycobacterium tuberculosis* sublineage in multidrug resistant tuberculosis in Portugal. *Infec Genet Evol* 2012; 12: 1362-7.

15. Fenner L, Egger M, Bodmer T *et al*. Effect of mutation and genetic background on drug resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2012; 3047-53.

16. Falzon D, Jaramillo E, Schünemann H *et al*. WHO guidelines for the programmatic management of drug-resistant tuberculosis: 2011 update. *Eur Respir J* 2011; 38: 516-28.

17. Direcção-Geral da Saúde. Programa Nacional de Luta Contra a Tuberculose. Ponto da Situação Epidemiológica e de Desempenho de 2011. Relatório para o Dia Mundial da Tuberculose. Avaliação preliminar em Março 2012. Direcção-Geral da Saúde, Lisboa, Portugal.

18. Direcção-Geral da Saúde. Programa Nacional de Luta Contra a Tuberculose. Ponto da Situação Epidemiológica e de Desempenho de 2010. Relatório para o Dia Mundial da Tuberculose. Avaliação preliminar em Março 2011. Direcção-Geral da Saúde, Lisboa, Portugal.

19. Direcção-Geral da Saúde. Programa Nacional de Luta Contra a Tuberculose. Ponto da Situação Epidemiológica e de Desempenho de 2009. Relatório para o Dia Mundial da Tuberculose. Avaliação preliminar em Março 2010. Direcção-Geral da Saúde, Lisboa, Portugal.

20. Portugal I, Covas MJ, Brum L *et al.* Outbreak of multiple drug-resistant tuberculosis in Lisbon: detection by restriction fragment length polymorphism analysis. *Int J Tuberc Lung Dis* 1999; 3: 207-13.

8. Supplementary Material and methods

Table IIB.S1. Primers used in this study.

Gene	Primer	Primer Sequence (5'-3')	Annealing temperature (^{0}C)	Amplification product (bp)	Source
inhA	inhA-3	AGG TCG CCG GGG TGG TCA GC	64	517	1
	inhA-4	AGC GCC TTG GCC ATC GAA GCA			
	inhA-3F	CCA CAT CTC GGC GTA TTC G	60	501	2
	inhA-5R	TTC CGG TCC GCC GAA CGA CAG			
ethA	ethA1	ATC ATC GTC GTC TGA CTA TGG	55	667	1
	ethA5	ACT ACA ACC CCT GGG ACC			
	ethA4	CCT CGA CCT TCC CGT GA	64	692	1
	ethA9	CCT CGA GTA CGT CAA GAG CAC			
	ethA8	GGT GGA ACC GGA TAT GCC TG	68	342	1
	ethA10	CGT TGA CGG CCT CGA CAT TAC			
ndh	ndh3S	GAC AGA TCG CCG AGC TGG C	60	372	3
	ndh3AS	TGG ACA GGT CGG GCA GCA C			
katG	P4_Fw	CGG ACC ATA ACG GCT TCC TG	62	563	This study
	P4_Rv	TTG TCC AAG CTG GCG TTG TC			
	P5_Fw	CGA CAA CGC CAG CTT GGA C	62	518	This study
	P5_Rv	CGG TTC CGG TGC CAT ACG			
	P6_Fw	AGC TCG TAT GGC ACC GGA AC	62	619	This study
	P6_Rv	TGA CCT CCC ACC CGA CTT GT			
	P7_Fw	ACA AGT CGG GTG GGA GGT C	62	574	This study
	P7_Rv	CTG CCG GTC CAC TTC ACC TT			
	P8_Fw	GGG ACC TAC CAG GGC AAG GA	62	627	This study
	P8_Rv	CCG GGA GTC AGC AAG TCA CC			

Fw/F: forward; Rv/R: reverse; S: sense; AS: antisense.

The reaction mixtures were prepared for a total reaction volume of 50 µl consisting of 1x Taq buffer (Fermentas, Ontario, Canada), 1.75 mM $MgCl_2$ (1.5 mM for *inhA/katG*), 200 mM of each dNTP, 6 pmol of each primer (20 pmol for *inhA;* 10 pmol for *katG*), 1.5U Taq DNA Polymerase (Fermentas), and 5 µl of chromosomal DNA. The PCR reactions were performed with the following amplification profile: initial denaturation at 94ºC for 5 minutes, followed by denaturation at 94ºC for 1 minute, primer dependent annealing temperature for 1 minute and extension at 72ºC for 1 minute during 40 cycles. The final extension occurred at 72ºC for 10 minutes.

References

1. Morlock G, Metchock B, Sikes D *et al*. *ethA*, *inhA*, and *katG* loci of ethionamide-resistant clinical *Mycobacterium tuberculosis* isolates. *Antimicrob Agents Chemother* 2003; **47**: 3799–805.

2. Leung E, Ho P, Yuen K *et al*. Molecular characterization of isoniazid resistance in *Mycobacterium tuberculosis*: identification of a novel mutation in *inhA*. *Antimicrob Agents Chemother* 2006; **50**: 1075-8.

3. Lee A, Teo A, Wong S. Novel mutations in *ndh* in isoniazid-resistant *Mycobacterium tuberculosis* isolates. Antimicrob Agents Chemother 2001; **45**: 2157-9.

CHAPTER III

Preface to Chapter III

From the results obtained in Chapter II we can conclude that i) there is a clear correlation between the occurrence of gene mutations in the drug targets and the resistance phenotype of a strain and, ii) the chromosomal alterations associated with resistance are greatly conserved. There is, however, little information available on the mechanism behind the development of such resistance and how it emerges in a patient under therapy.

Acquired drug resistance during tuberculosis treatment occurs mainly due to poor compliance resulting in sub-optimal therapeutic conditions that select drug resistant variants. Mutational resistance is responsible for treatment failure and for the emergence of resistance. Preliminary work performed by our group has demonstrated that the exposure of *M. tuberculosis* strains to isoniazid induces the activity of efflux pumps that make the bacteria more resistant to this antibiotic. However, the biological basis of these events remains to be fully characterized. It is now generally accepted that the overall *M. tuberculosis* resistance to any antituberculosis drug is not merely a consequence of the occurrence of gene mutations in the drug target but a balance between the acquisition of mutations and the efflux of antibiotics. Intrinsic drug resistance in mycobacteria is mainly attributed to the reduced permeability of the cell wall that acts synergistically with the activity of efflux pumps present in that wall and the increased expression of genes that code for those efflux pumps. This combined mechanism may constitute the first step for the development of drug resistance in *M. tuberculosis.*

In this chapter, we focus on the understanding of the chain of events leading to the development of resistance in *M. tuberculosis*, with particular emphasis on the differential activity of genes that code for the main *M. tuberculosis* efflux pumps prior to, during and, after the establishment of the resistance phenotype and their interaction with the genetically based resistance. The underlying concept of this study is that increased activity of efflux pumps allows a sustainable resistant population to be maintained in the patient under therapy, from which genetically resistant mutants will emerge with increased frequency. This experimentally produced adaptive phenotypic response should mimic the response of *M. tuberculosis* that infects the patient whose

treatment with those antibiotics is sub-optimal. Testing efflux pumps inhibitors on these strains should reveal new tools to prevent the emergence of acquired resistance.

Chapter III. Contribution of efflux to the emergence of isoniazid and multidrug resistance in *Mycobacterium tuberculosis*

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1. Abstract

Multidrug resistant (MDR) tuberculosis is caused by *Mycobacterium tuberculosis* resistant to isoniazid and rifampicin, the two most effective drugs used in tuberculosis therapy. Here, we investigated the mechanism by which resistance towards isoniazid develops and how overexpression of efflux pumps favors accumulation of mutations in isoniazid targets, thus establishing a MDR phenotype. The study was based on the *in vitro* induction of an isoniazid resistant phenotype by prolonged serial exposure of *M. tuberculosis* strains to the critical concentration of isoniazid employed for determination of drug susceptibility testing in clinical isolates. Results show that susceptible and rifampicin monoresistant strains exposed to this concentration become resistant to isoniazid after three weeks; and that resistance observed for the majority of these strains could be reduced by means of efflux pumps inhibitors. RT-qPCR assessment of efflux pump genes expression showed overexpression of all tested genes. Enhanced real-time efflux of ethidium bromide, a common efflux pump substrate, was also observed, showing a clear relation between overexpression of the genes and increased efflux pump function. Further exposure to isoniazid resulted in the selection and stabilization of spontaneous mutations and deletions in the *katG* gene, alongside with sustained increased efflux activity. Together, results demonstrate the relevance of efflux pumps as one of the factors of isoniazid resistance in *M. tuberculosis*. These results support the hypothesis that activity of efflux pumps allows the maintenance of an isoniazid resistant population in a sub-optimally treated patient from which isoniazid genetically resistant mutants emerge. Therefore, the use of inhibitors of efflux should be considered in the development of new therapeutic strategies for preventing the emergence of MDR-TB during treatment.

2. Introduction

Tuberculosis (TB) remains a serious public health threat around the world, and according to the World Health Organization, nearly two billion people are infected with *Mycobacterium tuberculosis*, with about 8.8 million of new TB cases and 1.3 million deaths in 2010 [1]. Moreover, multidrug resistant tuberculosis (MDR-TB), caused by *M.*

tuberculosis simultaneously resistant to isoniazid and rifampicin, the two most effective anti-bacillary drugs used in TB therapy, represents a challenge to the control of the disease since 650,000 of the TB cases in 2010 are estimated to be MDR-TB cases [1]. Chromosomal gene mutation has been considered the single cause for antibiotic resistance in *M. tuberculosis*, with multidrug resistance arising as a consequence of sequential accumulation of spontaneous mutations in target genes [2]. Resistance to rifampicin is almost always due to point mutations in the *rpoB* gene encoding the β subunit of the RNA polymerase [3]. Furthermore, monoresistance to rifampicin is rare and almost all *M. tuberculosis* strains resistant to rifampicin are also resistant to isoniazid [2,4,5]. Isoniazid is a prodrug that requires activation by the catalase-peroxidase enzyme (KatG) [6] and its molecular target is InhA, a NADH-dependent enoyl acyl carrier protein reductase involved in the synthesis of mycolic acids [7]. The main mechanism of resistance to isoniazid is the occurrence of mutations in its activator, KatG [6,8], whereas mutations in the *inhA* gene represent the second most common mechanism. Together, mutations in these two genes are responsible for approximately 75% of the cases of *M. tuberculosis* resistance to isoniazid in the clinical setting [9]. Resistance to isoniazid has also been associated with mutations in several other genes (*e.g. ndh*, *kasA* and *oxyR–ahpC* intergenic region) [10], but its direct association with resistance is still unclear.

Isoniazid is highly effective against *M. tuberculosis* (bactericidal at low concentrations), the reason why it remains a key component in multiple drug treatment regimens. However, resistant isolates are rapidly generated during monotherapy or inappropriate treatment, and many clinical isolates with no identified mutation have been described [9,11]. As with other bacterial species, these resistant phenotypes also receive significant contributions from membrane transport proteins that prevent the compound from reaching the cellular target [12,13]. The analysis of genome sequences has shown that mycobacteria have multiple putative efflux pumps [14] and to date, several pumps have been identified in various species of mycobacteria in association with low level resistance to various compounds, including isoniazid [15-20].

In general, increased activity of efflux systems is responsible for conferring low-level resistance to antibiotics, contrasting with the high-level resistance caused by mutations in genes encoding for the primary targets of these antibiotics [21]. Increased activity of

efflux systems results in the reduction of intracellular levels of the antibiotic, which may enable the survival of a bacterial subpopulation under constant stress promoted by a sub-lethal level of antibiotic. During this period, mutants with alterations in the genes that favour resistance can be selected, therefore insuring the establishment of an antibiotic resistant population that is clinically significant [22-24]. It is this sub-population of bacteria that may then accumulate mutations with prolonged exposure to a constant concentration of antibiotic [25,26].

Here, we investigated the mechanisms underlying the development of multidrug resistance in *M. tuberculosis* via the constant exposure of several isoniazid susceptible *M. tuberculosis* strains to the critical concentration of isoniazid, 0.1 µg/ml; followed by the evaluation of the effect of efflux inhibitors on the isoniazid minimum inhibitory concentration for the original and isoniazid exposed resistant strains. Analysis of gene expression of six efflux pumps related to isoniazid resistance in *M. tuberculosis* [12,15,19] and its correlation with the cell´s ability to efflux ethidium bromide (a common efflux substrate), provides strong evidence that when challenged with isoniazid, *M. tuberculosis* reacts by a prompt efflux-mediated response. We further demonstrate that this isoniazid induced resistance can be reverted by efflux inhibitors, supporting their role as adjuvants in anti-tuberculosis therapy and prevention of MDR-TB emergence.

3. Results

Exposure to isoniazid

Two *M. tuberculosis* strains susceptible to the first-line antibiotics (including the H37Rv reference strain) and two clinical strains monoresistant to rifampicin were constantly exposed to the critical concentration of isoniazid, 0.1 µg/ml, during an extended period of time – see Figure III.1. Two independent exposure processes were carried out for each strain (exposure process A and B in Figure III.1) to assess the stochastic behaviour of the biological events involved.

The effect of 0.1 µg/ml isoniazid exposure on the minimum inhibitory concentration of isoniazid (INH MIC) is summarised in Table III.1. Briefly, INH MIC increases from an

initial value of 0.05-0.1 µg/ml to as high as 256 µg/ ml (Table III.1). Exposure to isoniazid had no effect on the MIC of rifampicin against all strains (data not shown). Additionally, susceptibility testing for the five 1st line antibiotics (streptomycin, isoniazid, rifampicin, ethambutol and pyrazinamide) applied to all strains at the beginning of the experiments and after the last passage in isoniazid indicated that development of resistance was restricted to isoniazid only (data not shown). We also observed that the time required for growth detection decreased with the number of passages (Table III.1).

Typing by MIRU-VNTR analysis

To confirm the isogenic nature of the exposed and initial parental strains, all strains were subjected to molecular typing by MIRU-VNTR analysis, which confirmed the identity of each culture during the antibiotic exposure processes (Tables III.2 and III.3).

Detection of mutations associated with isoniazid resistance

Cultures corresponding to selected passages of isoniazid exposure were preliminarily monitored for mutations in *katG* and *mabA-inhA* operon by the Genotype MTBDR*plus* system. These strains were later analyzed by DNA sequencing of specific fragments of these same genes – see Table III.2 and III.3.

For strain H37Rv, two different results were obtained for the two independent isoniazid exposure processes. In one of the duplicates, total deletion of *katG* gene was observed at passage #3 ($H37Rv_{INH(a)3}$, GenBank accession number JQ406585). The precise extent and location of this deletion was defined by sequencing and chromosomal primer walking to be located between positions 5'-2150314 and 5'-2159943 of the *M. tuberculosis* H37Rv genome [14]. This deletion, 8084 bp in length, resulted in the complete loss of genes *Rv1903*, *Rv1904*, *aao*, *Rv1906c*, *Rv1907c*, *katG*, *furA*, *Rv1910c*, *lppc* and disruption of *fadB5* (Figure III.2).

Conversely, no alterations were detected on *katG* or any of the other gene targets tested during strain H37Rv second isoniazid exposure process (cf. Tables III.2 and III.3). Interestingly, both isoniazid-exposed cultures evidenced the same levels of resistance at equivalent passages of the exposure process (Table III.1). For the three clinical strains subjected to the same isoniazid exposure process, no alterations were detected, except

for the appearance of a STOP codon in position 1314 of the *katG* gene (codon 438) for strain 359/03. This alteration occurred at passage #2 ($359/03_{INH(a)2}$, accession number JQ316462) of the first exposure process (Table III.2), whereas no alteration was detected in the second exposure process of this same strain (Table III.3). Again, no differences were observed between the isoniazid resistance levels of the two isoniazid-exposed 359/03 cultures (Table III.1).

Effect of EIs on the susceptibility to isoniazid

To test for the involvement of efflux on the increased resistance to isoniazid noticed through the exposure process, we determined the INH MIC in the first passage where this increased resistance was first noticed and in last passage (#26) of the exposure process, in the absence and presence of compounds known to act as efflux inhibitors (EIs). The compounds selected were thioridazine, chlorpromazine and verapamil, for which inhibitory activity against mycobacterial efflux pumps was already demonstrated [16,27, 28]. The effects of these EIs on the INH MICs are summarized in Table III.4. The INH MIC was reduced by thioridazine, chlorpromazine and verapamil to levels equal or below the critical concentration used for the standard susceptibility testing of this antibiotic in the majority of cases. We then assayed the efflux activity of these cultures by a semi-automated fluorometric method [27,29], which uses the broad-range efflux substrate EtBr, in the presence and absence of an EI.

Real-time detection of efflux activity

The assays were performed for all isoniazid non-exposed strains (#0) and at passages #1 and #26 of the two exposure processes (A and B).

The EtBr accumulation assays, used to determine the highest concentration of EtBr that cells can handle without detectable accumulation (see Material and Methods), showed that the clinical strains are able to handle higher EtBr concentrations than H37Rv (0.25-0.5 and 0.125 µg/ml of EtBr, respectively – see values at bold type in legends of Figure III.3A). This means that the concentration at which EtBr influx and efflux reach steady state equilibrium is higher for the clinical strains than for H37Rv, *i.e.* that the former have higher efflux capacity than that of the reference strain [29]. With exposure to isoniazid, this efflux capacity increase, as shown by the flatness of the accumulation curves observed for all cultures at the first step of isoniazid exposure (Fig III.3B). With

continuation of exposure to isoniazid, this efflux activity decreases, as shown by the lower EtBr concentrations needed to reach observable accumulation for cultures at passage #26 (cf. Fig III.3C with III.3B), however not to the original levels shown by the non-exposed cultures (cf. Fig III.3C with III.3A). Interestingly, this decrease of efflux activity with the prolonged exposure to isoniazid is observable for the clinical strains but not for H37Rv. For the sake of space, only the results for exposure process A are shown in Figure III.3, but the same behavior was obtained for the exposure process B, for all the strains.

These assays were then performed using the EtBr concentrations for which influx-efflux are at equilibrium, but now in the presence of verapamil (efflux inhibitor) at sub-inhibitory concentrations (see dotted curves in Figure III.3). Results clearly show that inhibition of efflux occurs, leading to an increased EtBr accumulation within cells. Again, this effect is more pronounced at the first step of isoniazid exposure (passage #1), except for H37Rv, which reinforces the hypothesis that this is the step in which the increase of efflux activity is more significant. Similar results were obtained for thioridazine and chlorpromazine, however with lower inhibitory effect (data not shown). Finally, we evaluated the expression levels of genes coding for the main *M. tuberculosis* efflux pumps for cultures at the different isoniazid exposure steps.

Expression analysis of genes coding for efflux pumps in response to isoniazid

The analysis of the relative expression of efflux pump genes previously identified as transporters of isoniazid was performed for all strains at passages #1 and #26. As detailed in Material and Methods, the expression levels of these genes were determined in the presence of isoniazid and compared to those obtained for the non-exposed culture (#0) [30].

Table III.5 shows that the four strains reacted to the presence of isoniazid by overexpressing the majority of the efflux pump genes tested in a way that is not consistent among the two isoniazid exposures processes to which each strain was submitted. Additionally, and perhaps the most striking observation resulting from expression data, was the absence of a clearly defined pattern of gene expression in response to isoniazid exposure. Nevertheless, a general and strong increase in the expression was observed for the majority of the genes tested, *mmpL7*, *p55*, *efpA*, *mmr*, *Rv1258c* and *Rv2459.*

4. Discussion

In this work, we addressed three questions related to the molecular mechanisms involved in *M. tuberculosis* resistance to isoniazid: **i.** What are the effects of continuous exposure of *M. tuberculosis* strains to the critical concentration of isoniazid? **ii.** What are the biological events involved, in particular, which is the role played by efflux pumps in the emergence of resistance? and, **iii.** Does the final outcome vary when independent exposures are performed for the same strain?

For this purpose, we studied a panel of four *M. tuberculosis* strains, two fully susceptible and two monoresistant to rifampicin, through their independent exposure to a constant concentration of 0.1 µg/ml of isoniazid. During this process, strains became phenotypically resistant with an increase in their INH MIC exceeding 64 µg/ ml, which is considered high-level resistance. The susceptibility towards the other 1st line anti-tuberculosis drugs was not affected by this process, indicating the development of an isoniazid specific resistance mechanism.

In terms of the biological events occurring during exposure to isoniazid (question ii), we observed that for two strains, alterations occurred at the *katG* gene, corresponding to a full deletion in the case of H37Rv and the introduction of a stop codon for strain 359/03. Both alteration occurred early in the exposure process and were maintained for the remaining of the assays. These alterations in *katG* correlated with the loss of catalase activity (data not shown). The mutation found in clinical isolate 359/03 is rarely described in literature; however it fits into the known strategy of KatG functional weakening by introduction of mutations during isoniazid exposure [10,31]. Others have also reported the *in vitro* deletion of total or part of the *katG* gene in strains exposed to isoniazid [31,32], and total/partial deletion of the *katG* gene of isoniazid-resistant clinical isolates has also been previously reported [8,33]. Interestingly, no genetic changes were detected in the second isoniazid-exposure procedure, run in parallel for these same strains, indicating that evolution of the same strain in the same conditions can bring about, in a non-deterministic course, cells that significantly differ both phenotypically and genetically.

Our results clearly show that, in addition to the occurrence of spontaneous mutations, efflux systems play a role in the development of isoniazid resistance. This occurs quite early during exposure to isoniazid and allows cells to survive in the presence of this antibiotic until a mutation conferring high level and stable resistance emerges. Recently, Srivastava *et al*., suggested a model for the development of drug resistance in the *M. tuberculosis* reference strain H37Rv, enabling the rapid emergence of high level resistance to both ethambutol and isoniazid [34]. In this model, it is proposed that induction of an efflux pump which transports two or more drugs is the first step to the emergence of resistance. Our results provide the experimental data that confirm the model proposed by these authors and demonstrate that this mechanism is extendable to clinical isolates.

Moreover, our work provides, for the first time, data captured on a real-time basis for increased efflux activity as the first-line response of *M. tuberculosis* to the critical concentration of isoniazid. This efflux-mediated response was detected for both susceptible and rifampicin monoresistant, reference or clinical strains and provide the cells with a rapid, nonspecific response to a highly noxious agent. As the isoniazid exposure process continues, two different patterns were observed: the susceptible reference strain H37Rv increased its efflux activity, even after deletion of the entire *katG* gene, whereas the clinical strains showed a decreased efflux activity in the last passage of isoniazid exposure. During the entire process, the clinical strains showed a capacity to handle higher EtBr concentrations than H37Rv, an additional evidence of their higher efflux capacity. Overall, the clinical strains appear to be more prompt to respond, via an efflux-mediated pathway, to noxious agents, such as EtBr or isoniazid, whereas H37Rv shows a less prompt, but more stable/prolonged use of efflux as a detoxifying response to these drugs. These results suggest that clinical *M. tuberculosis* strains are primed to efflux noxious compounds, as already observed in other bacteria [35]. The presence of such efflux system(s) and their role in resistance to these drugs was additionally confirmed by the use of efflux inhibitors in both real-time efflux assays and MIC determinations. While in the first assays, these compounds were able to reduce efflux of EtBr, their use in INH MIC determination showed the involvement of efflux on the high level resistance to this antibiotic. For some strains, the reduction on INH MIC by the EIs tested reached levels identical to their susceptible parental counterparts.

This clearly shows that in these cases, high level isoniazid resistance is mainly efflux-driven. From the several inhibitors tested, chlorpromazine and verapamil were the two most effective for inhibiting isoniazid efflux in *M. tuberculosis*, as previously demonstrated for *M. tuberculosis* complex [36].

It is worth noticing that EIs show a more significant effect on the MICs for cultures at first passage compared to their effect on the MICs of the last passage (#26). For strain 401/06, submitted to exposure process A, despite the fact that no evident genetic alteration was detected, the high INH MIC obtained after isoniazid exposure could not be reduced by any of the EIs tested (Table III.4). This result suggests that, for this culture, the isoniazid high level resistance detectable at first passage is already mutation-driven. Interestingly strain 267/05, without detectable mutations is the one with the highest level of expression of genes for efflux further supporting that overexpression of efflux-pumps can sustain isoniazid resistance to levels as high as those achieved by the canonical mutations.

To identify the efflux system(s) involved in this first-line response to isoniazid, we selected a set of genes coding for efflux pumps reported to be involved in the transport of noxious substances, including isoniazid [12,15,37]. The detection by RT-qPCR of highly increased expression of these genes following isoniazid exposure, further evidences that an efflux-mediated response provides an early stress response that creates opportunity for other resistance mechanisms to arise.

Although we detected a general and marked increase of efflux pumps genes, most of which being significantly overexpressed, we also noted the absence of a clearly defined pattern of specific gene expression in response to isoniazid exposure. Efflux pumps seem to be promiscuous in their activity as we cannot associate extrusion of isoniazid to a specific gene. Similar results were obtained by others regarding the extrusion of rifampicin [38]. As described in Materials and Methods, the RT-qPCR data were analyzed considering a cut-off value of fourfold as corresponding to significant overexpression [39]. This can be considered a stringent cut-off and somehow limit our analysis [40], since the levels of gene expression obtained, in comparison to the non-exposed condition, lied in the majority of the cases, above two/three fold. Nevertheless, even considering the more stringent value of four, a clear and general ability to trigger

efflux pump genes overexpression in response to isoniazid presence was observed along the exposure processes, for all strains. The genes for which a more consistent isoniazid-mediated response was observed, were the genes involved in the transport and synthesis of mycolic acids, *mmpL7* and *efpA* respectively [41,42], and *p55*, considered to be involved in isoniazid transport [17,20,38,43,44]. Again, our study complements other earlier findings [15,34,36], who suggested the involvement of these genes in the resistance to isoniazid, by providing experimental data showing that susceptible reference strain and clinical strains use these pumps as an immediate response to the presence of isoniazid concentrations that are considered to be inhibitory.

Finally, concerning the third question raised in this work - does the final outcome vary when independent exposures are performed for the same strain, we have found that each strain may differ at the final outcome of the process of its exposure to the isoniazid critical concentration, in terms of the resistance mechanism it may adopt (mutations in different target genes, etc), although no differences were observed at the resistance level, which was always well above 64 µg/ml of isoniazid. Nevertheless, they all respond in a similar way at the first steps of this process and that is through isoniazid efflux, which may constitute an early stress response of bacteria against environmental noxious agents such as appears to be the case for isoniazid. After this first, efflux-mediated response, evolution may take different non-deterministic paths conducting to high level resistance. Collectively, these observations support the experimental strategy followed in this work that highlighted alternative pathways by which the same *M. tuberculosis* strain responds to 0.1 µg/ml isoniazid, all resulting in the same high resistance level.

In conclusion, constant exposure of *M. tuberculosis* to the commonly used critical concentration of isoniazid causes susceptible strains to become highly resistant to this key anti-tuberculosis drug. The same procedure applied to strains initially monoresistant to rifampin results in the development of multidrug resistance as defined by the WHO, *i.e*, resistance to isoniazid and rifampicin. To our knowledge, this is the first presentation of an *in vitro* process that mimics the development of multidrug resistant *M. tuberculosis* strains, which correlates with the anticipated development of MDR-TB in a patient treated for prolonged periods with a constant dose of isoniazid, as needed

for effective therapy. Therefore, the results obtained in this work emphasize the need for revising isoniazid critical concentration and reinforce the importance of multiple drug therapy in all anti-tuberculosis regimens [45,46]. Furthermore, efflux inhibitors like the ones tested in this work represent relevant alternatives in the search for new effective compounds and new therapeutic strategies for preventing the emergence of and possibly in the treatment of MDR-TB.

5. Materials and Methods

M. tuberculosis strains

The strains studied included two *M. tuberculosis* strains susceptible to the first-line antibiotics, the reference strain H37Rv ATCC27294^{T} and a clinical isolate 401/06, plus; two clinical isolates 359/03 and 267/05, both monoresistant to rifampicin, harboring the most common *rpoB* mutation in clinical isolates, S531L, all from the culture collection of Grupo de Micobactérias, Unidade de Microbiologia Médica, Instituto de Higiene e Medicina Tropical (IHMT, UNL).

Cultures, susceptibility testing, minimum inhibitory concentration (MIC) determination and antibiotic exposure process was conducted using the BACTEC^{TM} MGIT^{TM} 960 system (BACTEC 960) and the Epicenter V5.53A software equipped with the TB eXIST module (Becton Dickinson Diagnostic Systems, Sparks, MD, USA).

Antimicrobial agents

The lyophilized drugs (BACTEC^{TM} MGIT^{TM} 960 SIRE and PZA kits; SIRE: streptomycin, isoniazid, rifampicin and ethambutol; PZA: pyrazinamide) used in the standard susceptibility testing and in the exposure process to isoniazid and rifampicin were purchased from Becton Dickinson and the stock solutions prepared as per the manufacturer's instructions. Isoniazid for MIC determination and efflux inhibitors verapamil, thioridazine and chlorpromazine, as well as the efflux substrate ethidium bromide (EtBr), were purchased from Sigma-Aldrich (St. Louis, MO, USA). All drugs were prepared in sterile deionized water.

Exposure process to the critical concentration of isoniazid

Each strain was exposed to isoniazid (0.1 μg/ml) in duplicate (Figure III.1 – Schematic example for isoniazid exposure of strain H37Rv ATCC27294^{T}). This concentration is defined as the lowest concentration necessary to inhibit 99% of the wild-type strains of *M. tuberculosis* that were never in contact with this antibiotic, and is the critical concentration used for the BACTEC^{TM} MGIT^{TM} 960 SIRE AST procedure [47,48]. Briefly, the exposure process for strains susceptible to isoniazid began with the preparation of MGIT tubes containing SIRE supplement (Becton Dickinson) and 0.1 μg/ml of isoniazid. These tubes were then inoculated with 0.5 ml of the initial culture and subsequently incubated at 37ºC in the BACTEC 960 system until full growth was evident. For each strain, this process was done in duplicate – see Figure III.1 for example.

For convenience, the various passages of the strains are identified as follows: strain number, antibiotic, exposure process (A or B) and the number of the passage. For example, H37Rv$_{INH\ (a)26}$ refers to strain H37Rv exposed to isoniazid, exposure process A, passage 26 – see Figure III.1 for example.

MIC determination and antibiotic susceptibility testing

(i) MIC determination: The MICs of the antibiotics and efflux inhibitors (EIs) were performed in accordance to the procedures issued by the manufacturer of the BACTEC 960 system revised by Springer *et al*. [48,49] for the parental strains at the initial process and periodically for each of the progeny cultures (Figure III.1). The concentrations used were as follows: isoniazid: 0.025 to 256 μg/ml; verapamil: 30 to 512 μg/ml; thioridazine and chlorpromazine: 7.5 to 60 μg/ml; EtBr: 0.25 to 4 μg/ml. At the time of testing, two-fold serial dilutions were prepared to achieve the desired concentrations. Each drug-containing tube was inoculated with 0.8 ml of SIRE supplement, 0.1 ml of each drug in the appropriated concentration and 0.5 ml of the culture. For the preparation of the drug-free growth control tube (proportional control), the culture was diluted 1:100 with a sterile saline solution and 0.5 ml transferred into a new MGIT tube. Additionally, a second drug-free growth control, inoculated with 0.5 ml of the undiluted suspension of the strain, was prepared and served as absolute control for inoculums errors. The tubes were inserted in the BACTEC 960 system and growth

monitored with the TB eXIST module. The interpretation of the results was performed as proposed by Springer *et al*. [49].

(ii) Isoniazid MIC determination in the presence of EIs: The MICs of isoniazid (INH MIC) in combination with the EIs were performed in the first passage where the increased resistance was first noticed and in the last passage (#26) of the serial exposure process to isoniazid – Figure III.1. The EIs were used at a concentration corresponding to ½ of the respective MIC. This concentration was selected since it has no effect on the growth of the strains following the protocol described above.

(iii) Susceptibility testing in the presence and absence of EIs: For standard susceptibility testing against isoniazid, rifampicin, pyrazinamide, streptomycin and ethambutol, the readings were automatically interpreted by the BACTEC 960 system and reported as either susceptible or resistant. The preparation of the drug containing tubes and controls was done as described above. For the susceptibility testing for isoniazid in the presence of the EIs, the tubes containing 0.1 µg/ml of isoniazid were inoculated with the EI at ½ of the MIC.

Genotypic characterization of the strains

(i) DNA extraction: Genomic DNA was extracted using the QIAamp DNA mini kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's instructions.

(**ii) Screening of mutations:** The most common mutations in *rpoB*, *katG* and the *mabA-inhA* operon were screened during the exposure process, using the system Genotype MTBDR*plus* (Hain Lifescience GmbH, Nehren, Germany) according to the manufacturer's instructions.

(iii) DNA sequencing: The analysis of internal fragments of the genes associated with isoniazid resistance, *katG* and the *mabA-inhA* operon, was performed according to Perdigão *et al*. [50].

(iv) MIRU-VNTR analysis: MIRU-VNTR genotyping was performed for each strain and at defined passages of the exposure process by multiplex PCR amplification of 24 MIRU–VNTR loci, as described by Supply *et al*. [51].

Quantification of expression of genes coding for efflux pumps by RT-qPCR

(i) RNA extraction: Total RNA was isolated from the cells using the RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. Briefly, from a culture with

100-200 GU (about 10^6-10^8 cells/ml), 1 ml aliquot was removed and centrifuged at 13 000 rpm during 10 minutes. Then, 500 µl supernatant was removed and 1 ml of RNA*protect Bacteria reagent* (QIAGEN) added. An enzymatic lysis step was carried out with lysozyme at 3 µg/ml (Sigma) for 10 minutes, followed by lysis in an ultrasonic bath at 35 kHz (Gen-Probe, California, USA) during 15 minutes. The RNA was then purified using the RNeasy kit (QIAGEN) and treated with RNase-free DNase I (QIAGEN) during 2 hours and 15 minutes by on-column digestion at room temperature to reduce the presence of contaminating DNA. All RNA samples were aliquoted and frozen at -20ºC until required. **(ii) RT-qPCR assay:** The relative expression level of the genes that code for the main membrane efflux transporters in *M. tuberculosis* (*mmpL7*, *p55*, *efpA*, *mmr*, *Rv1258c* and *Rv2459*) were analyzed by RT-qPCR in the first passage where the increased resistance was first noticed and in last passage (#26) of the exposure process to isoniazid – Figure III.1. The normalization of the data was done using the *M. tuberculosis* 16S rDNA for each experiment. The forward and reversed primers employed are described in Table III.6**.** The RT-qPCR procedure was performed in a Rotor-Gene™ 3000 thermocycler and followed the protocol recommended for use with the QuantiTect SYBR Green RT-PCR Kit (QIAGEN). The determination of the relative mRNA expression level was performed using the comparative quantification cycle (*Cq*) method [40]. The relative expression of the six efflux pump genes analyzed was assessed by comparison of the relative quantity of the respective mRNA in the presence of isoniazid to the non-exposed culture, following the same technical approach previously published [30]. Each culture was assayed in triplicate using total RNA obtained from three independent cultures. A level of relative expression equal to 1 indicates that the expression level was identical to the unexposed strain. Genes showing expression levels equal or above four, when compared with the unexposed strain, were considered to be overexpressed [39].

Semi-automated fluorometric method

This method allows the real-time fluorometric detection of the accumulation and extrusion of EtBr, using the Rotor-Gene 3000™ thermocycler (Corbett Research, Sidney, Australia) [29,52]. The assays were performed based on the protocol previously described [28,29,52] with modifications due to the growth features of this

microorganism, mainly the slow generation time and the minimization of cell clumps. Increased biosafety measures were taken to prevent the production and dispersal of aerosols with infective particles since we were dealing with a Level 3 pathogen. The semi-automated fluorometric method was applied to the initial strains and to the isoniazid exposed strains, at the first passage where the increased resistance was first noticed and in the last passage (#26) of the adaptation process to isoniazid independently of the genetic background of each adapted culture. The strains were grown in 100 ml of Middlebrook 7H9 medium (DIFCO, Madrid, Spain) in Erlenmeyer flasks containing 10% OADC enrichment (Becton Dickinson) and 0.05% Tween 80. All cultures were incubated at 37ºC, without stirring, until they reached an approximate optical density at 600 nm (OD_{600}) of 0.8 (mid-log phase). After the cultures reached the desired OD_{600}, 25 ml cultures were centrifuged at 2700 g during 3 minutes at 25ºC. After this, the supernatant were discarded, the pellet washed, resuspended in PBS and centrifuged as before. This procedure was performed twice. For accumulation assays, the washed cells were re-suspended in PBS and the OD_{600} adjusted to 0.8. In order to determine the lowest concentration of EtBr that causes accumulation, 50 µl of the bacterial suspension was added to 0.2 ml PCR tubes containing different concentrations of EtBr that ranged from 0.0625 to 5 µg/ml and glucose at a final concentration of 0.4%. The final OD_{600} of the bacterial suspension in the assay was 0.4. The assays were conducted at 37ºC in a Rotor-Gene 3000^{TM}, and the fluorescence of EtBr was measured (530/585 nm) at the end of each cycle of 60 seconds, for 60 minutes. After determining the higher concentration of EtBr that do not causes accumulation, the effect of the EIs verapamil, thioridazine and chlorpromazine on the accumulation of EtBr was evaluated. These assays were performed like described above with each EI at ½ of the MIC, EtBr at the higher concentration that do not cause accumulation (determined for each strain and adapted cultures), 37ºC and with glucose.

6. Figures

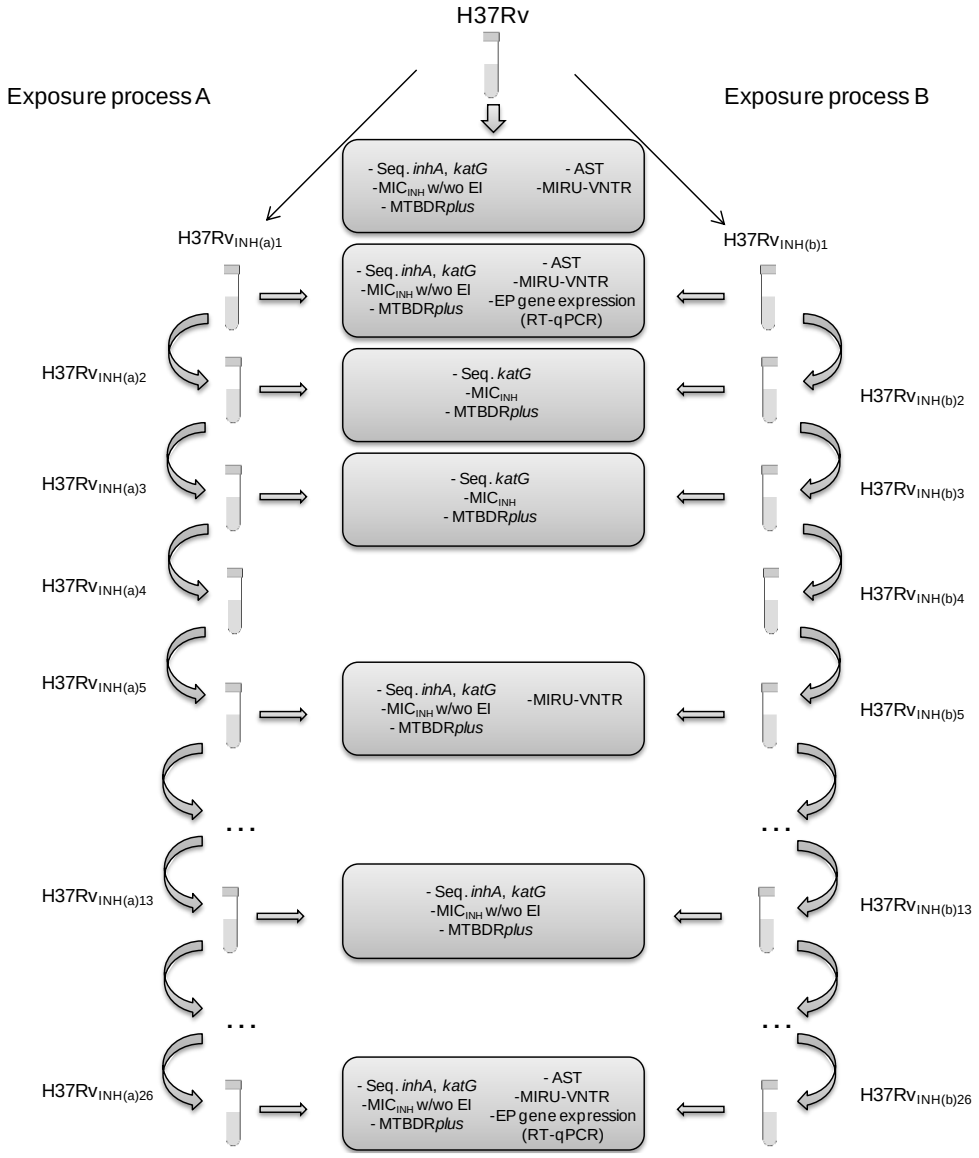

Figure III.1. Schematic representation of exposure of strain H37Rv to 0.1 µg/ml INH using the BACTEC™ MGIT™ 960 and characterization assays performed at selected points. For each strain, exposure to INH was done in duplicate, in two independent assays - processes A and B. INH(a): exposure process A; INH(b): exposure process B; INH: isoniazid; EI: efflux inhibitor. Vertical arrows represent transfer to new MGIT tubes containing 0.1 µg/ml INH. Seq: nucleotide sequence determination for specific fragments of the genes involved in the resistance to INH; AST: susceptibility testing to all first line antibiotics. MIC_{INH}: minimum inhibitory concentration determination of isoniazid. Note: This same procedure, here depicted as an example, was carried out for isoniazid exposure of each strain involved in this study.

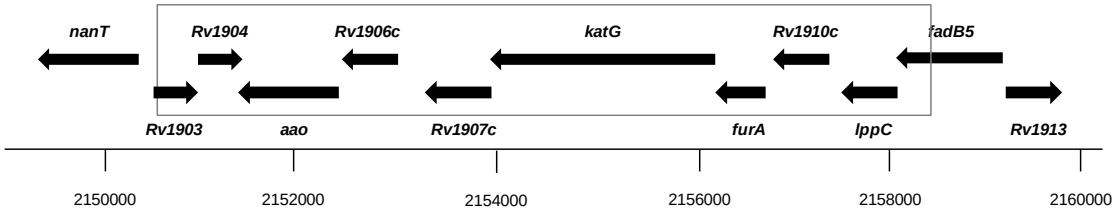

Figure III.2. Map of the region deleted in the *M. tuberculosis* H37Rv reference strain as a result of the exposure to isoniazid. The region analyzed spans from positions 5'-2150314 to 5'-2159943 of the *M. tuberculosis* H37Rv genome sequence [14], adapted from Tuberculist, 2010, http://tuberculist.epfl.ch/. The area delimited corresponds to the fragment deleted in strain $H37Rv_{INH(a)3}$.

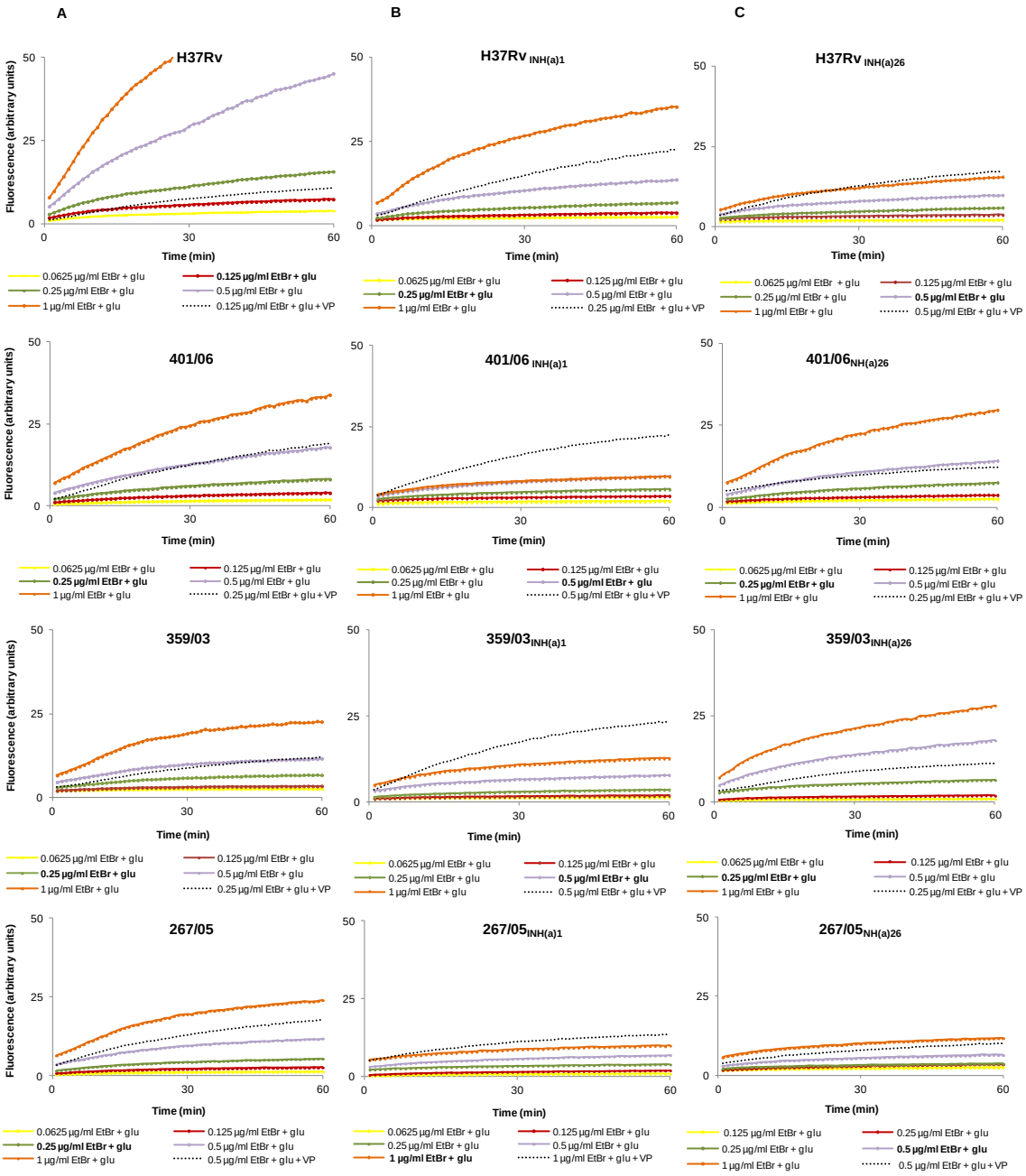

Figure III.3. Accumulation of EtBr by the *M. tuberculosis* strains tested. The figure shows the accumulation of EtBr by the strains from adaptation process A as an example. The values at bold type correspond to the higher concentration of EtBr that cells can handle without detectable accumulation. The dotted line corresponds to the assay run using the EtBr concentrations for which influx-efflux are at equilibrium, in the presence of the EI verapamil, at sub-inhibitory concentrations. Panel (A): Parental strains (passage #0); Panel (B) strains after first passage with INH and Panel (C); strains after 26 passages with INH. INH: isoniazid.

7. Tables

Table III.1. The effect of serial passages in a constant concentration of isoniazid (0.1 µg/ml) on the INH MIC and the number of days required for detection of growth.

	INH MIC (µg/ml) (days required for detection of growth)						
	#0	**#1**	**#2**	**#3**	**#5**	**#13**	**#26**
Fully susceptible strains							
H37Rv INH (a)	0.05 (-)	**128 (19)**	128 (5)	128 (4)	128 (4)	128 (4)	**128 (4)**
H37Rv INH (b)	0.05 (-)	**128 (14)**	128 (8)	128 (5)	128 (3)	128 (3)	**128 (3)**
401/06 INH (a)	0.1 (-)	**256 (20)**	256 (6)	256 (4)	256 (4)	256 (4)	**256 (3)**
401/06 INH (b)	0.1 (-)	**64 (32)**	256 (14)	256 (18)	256 (7)	256 (4)	**256 (3)**
Rifampicin monoresistant strains							
267/05 INH (a)	0.1 (-)	**128 (15)**	128 (7)	128 (4)	128 (6)	128 (3)	**128 (3)**
267/05 INH (b)	0.1 (-)	**128 (17)**	128 (5)	128 (4)	128 (7)	128 (4)	**128 (3)**
359/03 INH (a)	0.05 (-)	**256 (18)**	256 (13)	256 (6)	256 (4)	256 (4)	**256 (3)**
359/03 INH (b)	0.05 (-)	**128 (17)**	256 (16)	256 (3)	256 (3)	256 (8)	**256 (3)**

Legend: INH: isoniazid; RIF: rifampicin; INH (a)/(b): adaptation processes to isoniazid A and B, respectively.

Table III.2. Genotypic characterization of the strains and derived cultures exposed to isoniazid (adaptation process A).

	Genotype MTBDR*plus*			**DNA Sequencing**		
Strain/Passage	***rpoB***	***mabA-inhA***	***katG***	***mabA-inhA***	***katG***	**MIRU-VNTR profile**
H37Rv	Wt	wt	wt	wt	Wt	H37Rv
H37Rv INH (a)1	Wt	wt	wt	wt	wt	H37Rv
H37Rv INH (a)2	Wt	wt	wt	wt	wt	
H37Rv INH (a)3	Wt	wt	**Δ *katG***	wt	**Δ *katG***	
H37Rv INH (a)5	Wt	wt	**Δ *katG***	wt	**Δ *katG***	H37Rv
H37Rv INH (a)13	Wt	wt	**Δ *katG***	wt	**Δ *katG***	
H37Rv INH (a)26	Wt	wt	**Δ *katG***	wt	**Δ *katG***	H37Rv
401/06	Wt	wt	wt	wt	wt	A
401/06 INH (a)5	Wt	wt	wt	wt	wt	A
401/06 INH (a)13	Wt	wt	wt	wt	wt	
401/06 INH (a)26	Wt	wt	wt	wt	wt	A
267/05	S531L	wt	wt	wt	wt	B
267/05 INH (a)5	S531L	wt	wt	wt	wt	B
267/05 INH (a)13	S531L	wt	wt	wt	wt	
267/05 INH (a)26	S531L	wt	wt	wt	wt	B
359/03	S531L	wt	wt	wt	wt	C
359/03 INH (a)1	S531L	wt	wt	wt	wt	C
359/03 INH (a)2	S531L	wt	wt	wt	**TGG_{438}→STOP**	
359/03 INH (a)5	S531L	wt	wt	wt	**TGG_{438}→STOP**	C
359/03 INH (a)13	S531L	wt	wt	wt	**TGG_{438}→STOP**	
359/03 INH (a)26	S531L	wt	wt	wt	**TGG_{438}→STOP**	C

Legend: INH: isoniazid; RIF: rifampicin; wt: wild type; Δ: deletion of *katG* gene; S: serine; L: leucine. MIRU-VNTR profile A: 2,4,2,2,3,4,2,3,2,3,2,4,2,2,6,1,6,3,1,3,1,7,2,2; profile B: 2,4,4,2,3,4,3,3,2,4,2,4,2,2,6,1,5,3,1,3,1,5,2,2; profile C: 2,1,4,2,1,3,2,3,2,2,2,5,1,2,6,1,6,3,3,3,2,4,2,2.

Table III.3. Genotypic characterization of the strains and derived cultures adapted to isoniazid (adaptation process B).

	Genotype MTBDR*plus*			**DNA Sequencing**		
Strain/Passage	***rpoB***	***mabA-inhA***	***katG***	***mabA-inhA***	***katG***	**MIRU-VNTR profile**
H37Rv INH (b)5	Wt	wt	wt	wt	wt	H37Rv
H37Rv INH (b)13	Wt	wt	wt	wt	wt	
H37Rv INH (b)26	Wt	wt	wt	wt	wt	H37Rv
401/06 INH (b)5	Wt	wt	wt	wt	wt	A
401/06 INH (b)13	Wt	wt	wt	wt	wt	
401/06 INH (b)26	Wt	wt	wt	wt	wt	A
267/05 INH (b)5	S531L	wt	wt	wt	wt	B
267/05 INH (b)13	S531L	wt	wt	wt	wt	
267/05 INH (b)26	S531L	wt	wt	wt	wt	B
359/03 INH (b)5	S531L	wt	wt	wt	wt	C
359/03 INH (b)13	S531L	wt	wt	wt	wt	
359/03 INH (b)26	S531L	wt	wt	wt	wt	C

Legend: INH: isoniazid; RIF: rifampicin; wt: wild type; S: serine; L: leucine. MIRU-VNTR profile A: 2,4,2,2,3,4,2,3,2,3,2,4,2,2,6,1,6,3,1,3,1,7,2,2; profile B: 2,4,4,2,3,4,3,3,2,4,2,4,2,2,6,1,5,3,1,3,1,5,2,2; profile C: 2,1,4,2,1,3,2,3,2,2,2,5,1,2,6,1,6,3,3,3,2,4,2,2.

Table III.4. MIC determination and susceptibility testing for the strains exposed to isoniazid in the presence and absence of efflux inhibitors.

	INH MIC (µg/ml) (Susceptibility testing for INH)										
	#0			**#1**				**#26**			
Strain	**+TZ**	**+CPZ**	**+ VP**	**no EI**	**+TZ**	**+CPZ**	**+ VP**	**no EI**	**+TZ**	**+CPZ**	**+ VP**
H37Rv INH (a)	0.05 (S)	0.05 (S)	0.05 (S)	128 (R)	128 (R)	**0.05 (S)**	**0.06 (S)**	128 (R)	128 (R)	128 (R)	128 (R)
H37Rv INH (b)	0.05 (S)	0.05 (S)	0.05 (S)	128 (R)	128 (R)	**0.1 (S)**	**0.1 (S)**	128 (R)	128 (R)	**0.1 (S)**	**0.1 (S)**
401/06 INH (a)	0.1 (S)	0.1 (S)	0.1 (S)	256 (R)	256 (R)	128 (R)	256 (R)	256 (R)	256 (R)	128 (R)	256 (R)
401/06 INH (b)	0.1 (S)	0.1 (S)	0.1 (S)	64 (R)	**0.1(S)**	**0.1 (S)**	**0.1 (S)**	256 (R)	256 (R)	64 (R)	256 (R)
267/05 INH (a)	0.1 (S)	0.1 (S)	0.1 (S)	128 (R)	**0.1 (S)**	**0.1 (S)**	128 (R)	128 (R)	**0.1 (S)**	**0.1 (S)**	128 (R)
267/05 INH (b)	0.1 (S)	0.1 (S)	0.1 (S)	128 (R)	**0.1 (S)**	**0.1 (S)**	128 (R)	128 (R)	**0.1 (S)**	**0.1 (S)**	128 (R)
359/03 INH (a)	0.05 (S)	0.05 (S)	0.05 (S)	256 (R)	128 (R)	**0.1 (S)**	**0.03 (S)**	256 (R)	128 (R)	**0.1 (S)**	**0.03 (S)**
359/03 INH (b)	0.05 (S)	0.05 (S)	0.05 (S)	128 (R)	**0.1 (S)**	**0.1 (S)**	**0.1 (S)**	256 (R)	128 (R)	**0.1 (S)**	**0.1 (S)**

Legend: INH: isoniazid; INH (a): exposure process A; INH (b): exposure process B. S: susceptible; R: resistant. Values in bold correspond to full reversion of the INH resistance phenotype. EIs were used at ½ of their MIC. MICs for the EIs (passage 1): thioridazine (TZ): H37Rv and 359/03: 15 µg/ml, 401/06 and 267/05: 30 µg/ml; chlorpromazine (CPZ): 30 µg/ml for all strains; verapamil (VP): 256 µg/ml for all strains. MICs for the EIs (passage 26): TZ: H37Rv and 359/03: 15 µg/ml, 401/06(a)26: 15 µg/ml; 401/06(b)26: 30 µg/ml; 267/05: 30 µg/ml; CPZ: MIC of 30 µg/ml for all strains except H37Rv INH(a)26: 15 µg/ml; VP: 256 µg/ml for all strains except H37Rv INH(a)26 and 401/06: 128 µg/ml.

Table III.5. Average quantification of the relative expression level, by RT-qPCR, of the genes that code for efflux pumps in *M. tuberculosis* in the two independent isoniazid exposure processes.

	Relative expression level ± SD					
	mmpl7	***Rv1258c***	***p55***	***efpA***	***mmr***	***Rv2459***
Fully susceptible strains						
H37Rv INH(a)1	**8.00 ± 2.38**	**16.00 ± 1.16**	**13.00 ± 2.23**	**9.85 ± 1.41**	**16.65 ± 2.44**	**25.99 ± 2.56**
H37Rv INH(a)26	0.44 ± 0.12	1.99 ± 0.66	0.82 ± 0.15	0.34 ± 0.28	0.44 ± 0.16	0.50 ± 0.02
H37Rv INH(b)1	**10.56 ± 3.48**	**15.26 ± 0.46**	**6.96 ± 1.36**	**8.00 ± 2.98**	**9.95 ± 2.03**	**22.63 ± 1.56**
H37Rv INH(b)26	**4.57 ± 0.25**	2.53 ± 0.03	2.70 ± 0.43	**4.41 ± 0.21**	1.54 ± 0.29	2.31 ± 0.13
401/06 INH(a)1	**34.30 ± 1.24**	**22.63 ± 0.47**	**18.38 ± 1.94**	**16.00 ± 1.32**	**24.25 ± 2.34**	**9.19 ± 0.11**
401/06 INH(a)26	**17.15 ± 0.23**	**14.93 ± 2.30**	**9.85 ± 0.99**	**6.96 ± 1.57**	**9.19 ± 1.76**	**27.86 ± 1.3**
401/06 INH(b)1	1.53 ± 0.29	1.77 ± 0.75	**3.47 ± 1.16**	**6.50 ± 1.30**	1.47 ± 0.07	0.20 ± 0.05
401/06 INH(b)26	**4.16 ± 0.66**	**7.80 ± 4.96**	**11.31 ± 1.11**	**8.57 ± 2.66**	2.29 ± 0.77	2.64 ± 0.39
Rifampicin monoresistant strains						
267/05 INH(a)1	**6.06 ± 0.53**	**4.29 ± 0.24**	**5.28 ± 0.48**	**9.85 ± 0.17**	3.73 ± 0.13	**6.50 ± 1.03**
267/05 INH(a)26	**115.56 ± 59.44**	**53.85 ± 2.64**	**110.80 ± 66.17**	**341.05 ± 66.43**	**59.06 ± 53.66**	**32.00 ± 1.98**
267/05 INH(b)1	**5.01 ± 1.10**	2.00 ± 0.21	3.10 ± 0.43	**3.90 ± 0.28**	2.62 ± 0.54	1.69 ± 0.36
267/05 INH(b)26	**17.15 ± 1.24**	3.04 ± 0.29	**12.13 ± 0.67**	**22.63 ± 3.09**	**3.89 ± 0.57**	0.64 ± 0.09
359/03 INH(a)1	**4.00 ± 0.03**	1.62 ± 0.54	2.08 ± 1.06	1.87 ± 0.00	2.03 ± 0.86	0.76 ± 0.09
359/03 INH(a)26	**13.96 ± 1.36**	**9.01 ± 2.17**	**8.53 ± 2.86**	**10.66 ± 2.07**	**11.46 ± 4.9**	**32.00 ± 2.01**
359/03 INH(b)1	**9.85 ± 2.35**	**5.66 ± 1.24**	**4.00 ± 0.06**	**5.11 ± 2.62**	**6.06 ± 0.54**	0.25 ± 0.03
359/03 INH(b)26	**25.99 ± 1.33**	**29.86 ± 3.29**	**29.86 ± 2.56**	2.30 ± 0.83	**10.56 ± 1.36**	2.30 ± 0.12

Legend: (a)1: exposition process A, first passage; (b)1: exposition process B, first passage; (a)26: exposition process A, passage twenty six; (b)26: exposition process B, passage twenty six. The relative expression of the efflux pump genes was assessed by comparison of the relative quantity of the respective mRNA in the presence of isoniazid to the non-exposed strain. Each culture was assayed in triplicate using total RNA obtained from three independent cultures. A level of relative expression equal to 1 indicates that the expression level was identical to the strain that was being compared. Genes showing expression levels equal or above four, when compared to the non-exposed strain, were considered to be overexpressed and are shown in bold.

Table III.6. Sequences of the primers used in the RT-qPCR assays.

Gene	Primer Sequence (5'-3')	Amplification product (bp)	Reference
*mmpL7*_Fw	TAC CCA AGC TGG AAA CAA	214	[36]
*mmpL7*_Rv	CCG TCA GAA TAG AGG AAC CAG	214	[36]
*p55*_Fw	AGT GGG AAA TAA GCC AGT AA	198	[36]
*p55*_Rv	TGG TTG ATG TCG AGC TGT	198	[36]
*efpA*_Fw	ATG GTA ATG CCT GAC ATC C	131	[36]
*efpA*_Rv	CTA CGG GAA ACC AAC AAA G	131	[36]
*mmr*_Fw	AAC CAG CCT GCT CAA AAG	221	[36]
*mmr*_Rv	CAA CCA CCT TCA TCA CAG A	221	[36]
Rv1258c_Fw	AGT TAT AGA TCG GCT GGA TG	268	[36]
Rv1258c_Rv	GTG CTG TTC CCG AAA TAC	268	[36]
Rv2459_Fw	CAT CTT CAT GGT GTT CGT G	232	This study
Rv2459_Rv	CGG TAG CAC ACA GAC AAT AG	232	This study
16S_Fw	CAA GGC TAA AAC TCA AAG GA	197	[36]
16S_Rv	GGA CTT AAC CCA ACA TCT CA	197	[36]

FW: forward; RV: reverse.

8. References

1. World Health Organization. 2011. Global tuberculosis control: WHO report 2011. WHO/HTM/TB/2011.16. World Health Organization, Geneva, Switzerland.

2. Somoskövi A, Parsons LM, Salfinger M (2001) The molecular basis of resistance to isoniazid, rifampin, and pyrazinamide in *Mycobacterium tuberculosis*. Respir Res 2: 164-168.

3. Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, et al. (1993) Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. Lancet 341: 647-650.

4. Viveiros M, Leandro C, Rodrigues L, Almeida J, Bettencourt R, et al. (2005) Direct application of the INNO-LiPA Rif.TB line-probe assay for rapid identification of *Mycobacterium tuberculosis* complex strains and detection of rifampin resistance in 360 smear-positive respiratory specimens from an area of high incidence of multidrug-resistant tuberculosis. J Clin Microbiol 43: 4880-4884.

5. World Health Organization. 2008. Anti-tuberculosis drug resistance in the world. Fourth Global Report. The WHO/IUATLD Global Project on Anti-tuberculosis Drug Resistance Surveillance. 2002–2007. WHO/HTM/TB/2008.394. World Health Organization, Geneva, Switzerland.

6. Zhang Y, Heym B, Allen B, Young D, Cole S (1992) The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. Nature 358: 591-593.

7. Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Um KS, et al. (1994) *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. Science 263: 227-230.

8. Heym B, Alzari PM, Honore N, Cole ST (1995) Missense mutations in the catalase-peroxidase gene, *katG*, are associated with isoniazid resistance in *Mycobacterium tuberculosis*. Mol Biol 15: 235-245.

9. Guo H, Seet Q, Denkin S, Parsons L, Zhang Y, et al. **(**2006) Molecular characterization of isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis* from the USA. J Med Microbiol 55: 1527-1531.

10. Vilchèze C, Jacobs Jr WR (2007) The mechanism of isoniazid killing: clarity through the scope of genetics. Annu Rev Microbiol 61: 35-50.

11. Hazbón MH, Brimacombe M, del Valle MB, Cavatore M, Guerrero MI, et al. (2006) Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 50: 2640-2649.

12. Louw GE, Warren RM, van Pittius NC, McEvoy CR, van Helden PD, et al. (2009) A balancing act: efflux/influx in mycobacterial drug resistance. Antimicrob Agents Chemother 53: 3181-3189.

13. Viveiros M, Leandro C, Amaral L (2003) Mycobacterial efflux pumps and chemotherapeutic implications. Int J Antimicrob Agents 22: 274-278.

14. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, et al. (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393: 537-544.

15. Gupta AK, Katoch VM, Chauhan DS, Sharma R, Singh M, et al. (2010) Microarray analysis of efflux pump genes in multidrug-resistant *Mycobacterium tuberculosis* during stress induced by common anti-tuberculous drugs. Microb Drug Resist 16: 21-28.

16. Rodrigues L, Aínsa JA, Amaral L, Viveiros M (2011) Inhibition of drug efflux in mycobacteria with phenothiazines and other putative efflux inhibitors. Recent Pat Antiinfect Drug Discov 6: 118-127.

17. Silva PE, Bigi F, Santangelo MP, Romano MI, Martín C, et al. (2001) Characterization of P55, a multidrug efflux pump in *Mycobacterium bovis* and *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 45: 800-804.

18. Doran JL, Pang Y, Mdluli KE, Moran AJ, Victor TC, et al. (1997) *Mycobacterium tuberculosis efpA* encodes an efflux protein of the QacA transporter family. Clin Diagn Lab Immunol 4: 23-32.

19. Gupta AK, Reddy VP, Lavania M, Chauhan DS, Venkatesan K, et al. (2010) *jefA* (Rv2459), a drug efflux gene in *Mycobacterium tuberculosis* confers resistance to isoniazid and ethambutol. Indian J Med Res 132: 176-188.

20. Pasca MR, Guglierame P, De Rossi E, Zara F, Riccardi G (2005) *mmpL7* gene of *Mycobacterium tuberculosis* is responsible for isoniazid efflux in *Mycobacterium smegmatis*. Antimicrob Agents Chemother 49: 4775-4777.

21. De Rossi E, Arrigo P, Bellinzoni M, Silva PA, Martin C, et al. (2002) The multidrug transporters belonging to major facilitator superfamily in *Mycobacterium tuberculosis*. Mol Med 8: 714-724.

22. Martinez JL, Baquero F (2000) Mutation frequencies and antibiotic resistance. Antimicrob Agents Chemother 44: 1771-1777.

23. Webber MA, Piddock LJ (2003) The importance of efflux pumps in bacterial antibiotic resistance. J Antimicrob Chemother 51: 9-11.

24. Viveiros M, Portugal I, Bettencourt R, Victor TC, Jordaan AM, et al. (2002) Isoniazid-induced transient high-level resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 46: 2804-2810.

25. Pasipanodya JG, Gumbo T (2011) A new evolutionary and pharmacokinetic-pharmacodynamic scenario for rapid emergence of resistance to single and multiple anti-tuberculosis drugs. Curr Opin Pharmacol 11: 457-463.

26. Martins A, Iversen C, Rodrigues L, Spengler G, Ramos J, et al. (2009) An AcrAB-mediated multidrug-resistant phenotype is maintained following restoration of wild-type activities by efflux pump genes and their regulators. Int J Antimicrob Agents 34: 602-604.

27. Rodrigues L, Wagner D, Viveiros M, Sampaio D, Couto I, et al. (2008) Thioridazine and chlorpromazine inhibition of ethidium bromide efflux in *Mycobacterium avium* and *Mycobacterium smegmatis*. J Antimicrob Chemother 61: 1076-1082.

28. Rodrigues L, Sampaio D, Couto I, Machado D, Kern WV, et al. (2009) The role of efflux pumps in macrolide resistance in *Mycobacterium avium* complex. Int J Antimicrob Agents 34: 529-533.

29. Paixão L, Rodrigues L, Couto I, Martins M, Fernandes P, et al. (2009) Fluorometric determination of ethidium bromide efflux kinetics in *Escherichia coli*. J Biol Eng 3:18.

30. Viveiros M, Dupont M, Rodrigues L, Couto I, Davin-Regli A, et al (2007) Antibiotic stress, genetic response and altered permeability of *E. coli*. PLoS ONE 2(4): e365.

31. de Steenwinkel JE, de Knegt GJ, Kate MT, van Belkum A, Verbrugh HA, et al. (2010) Time-kill kinetics of anti-tuberculosis drugs, and emergence of resistance, in relation to metabolic activity of *Mycobacterium tuberculosis*. J Antimicrob Chemother 65: 2582-2589.

32. Bergval IL, Schuitema ARJ, Klatser PR, Anthony RM **(**2009) Resistant mutants of *Mycobacterium tuberculosis* selected *in vitro* do not reflect the *in vivo* mechanism of isoniazid resistance. J Antimicrob Chemother 64: 515-523.

33. Vijdea R, Stegger M, Sosnovskaja A, Andersen ÅB, Thomsen VØ, et al. (2008) Multidrug-resistant tuberculosis: rapid detection of resistance to rifampin and high or low levels of isoniazid in clinical specimens and isolates. Eur J Clin Microbiol Infect Dis 27: 1079-1086.

34. Srivastava S, Musuka S, Sherman C, Meek C, Leff R, et al. (2010) Efflux-pump-derived multiple drug resistance to ethambutol monotherapy in *Mycobacterium tuberculosis* and the pharmacokinetics and pharmacodynamics of ethanbutol. J Infect Dis 201: 1225-1231.

35. Costa SS, Falcão C, Viveiros M, Machado D, Martins M, et al. (2011) Exploring the contribution of efflux on the resistance to fluoroquinolones in clinical isolates of *Staphylococcus aureus*. BMC Microbiol 11: 241.

36. Rodrigues L, Machado D, Couto I, Amaral L, Viveiros M (2011) Contribution of efflux activity to isoniazid resistance in the *Mycobacterium tuberculosis* complex. Infect Genet Evol doi:10.1016/j.meegid.2011.08.009.

37. da Silva PEA, Groll AV, Martin A, and Palomino JC (2011) Efflux as a mechanism for drug resistance in *Mycobacterium tuberculosis*. FEMS Immunol Med Microbiol 63: 1-9.

38. Louw GE, Warren RM, van Pittius NC, Leon R, Jimenez A, et al. (2011) Rifampicin reduces susceptibility to ofloxacin in rifampicin resistant *Mycobacterium tuberculosis* through efflux. Am J Respir Crit Care Med 184: 269-276.

39. DeMarco CE, Cushing LA, Frempong-Manso E, Seo SM, Jaravaza TA, et al. (2007) Efflux-related resistance to norfloxacin, dyes and biocides in bloodstream isolates of *Staphylococcus aureus*. Antimicrob Agents Chemother 51: 3235-3239.

40. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt method. Methods 25: 402-408.

41. Portevin D, de Sousa-D'Auria C, Houssin C, Grimaldi C, Daffé MCM, et al. (2004) A polyketide synthase catalyses the last condensation step of mycolic acid biosynthesis in mycobacteria and related organisms. Proc Natl Acad Sci USA 101: 314-319.

42. Cox JS, Chen B, McNeil M, Jabobs Jr WR (1999) Complex lipid determines tissue replication of *Mycobacterium tuberculosis* in mice. Nature 52: 2503-2511.

43. Bianco MV, Blanco FC, Imperiale B, Forrellad MA, Rocha RV, et al. (2011) Role of P27-P55 operon from *Mycobacterium tuberculosis* in the resistance to toxic compounds. BMC Infect Dis. 11:195.

44. Wilson M, DeRisi J, Kristensen H-H, Imboden P, Rane S, et al. (1999) Exploring drug induced alterations in gene expression in *Mycobacterium tuberculosis* by microarray hybridization. Proc Natl Acad Sci USA 96: 12833-12838.

45. Böttger EC (2011) The ins and outs of *Mycobacterium tuberculosis* drug susceptibility testing. Clin Microbiol Infect 17: 1128-1134.

46. Zumla A, Atun R, Maeurer M, Mwaba P, Ma Z, et al. (2011) Viewpoint: Scientific dogmas, paradoxes and mysteries of latent *Mycobacterium tuberculosis* infection. Trop Med Int Health 16: 79-83.

47. Inderlied CB, Nash KA (1996) Antimycobacterial agents: *in vitro* susceptibility testing, spectra of activity, mechanisms of action and resistance, and assays for activity in biologic fluids, In: V. Lorian, Antibiotics in laboratory medicine, 4th ed. Williams and Wilkins, Baltimore, USA. pp 127-175.

48. Siddiqi S, Rüsch-Gerdes S (2006) MGIT960 Procedure Manual for BACTEC™ MGIT 960™ TB System (Also applicable for Manual MGIT). Mycobacteria growth indicator tube (MGIT) culture and drug susceptibility demonstration projects. Foundation for innovative new diagnostics Ed.

49. Springer B, Lucke K, Calligaris-Maibach R, Ritter C, Böttger EC (2009) Quantitative drug susceptibility testing of *Mycobacterium tuberculosis* using MGIT960 and the EpiCenter instrumentation. J Clin Microbiol 47: 1773-1780.

50. Perdigão J, Macedo R, João I, Fernandes E, Brum L, et al. (2008) Multidrug-resistant tuberculosis in Lisbon, Portugal: a molecular epidemiological perspective. Microb Drug Resist 14: 133-143.

51. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rüsch-Gerdes S, et al. (2006) Proposal for standardization of optimized mycobacterial interspersed repetitive unit–variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol 44: 4498-4510.

52. Viveiros M, Rodrigues L, Martins M, Couto I, Spengler G, et al. (2010) Evaluation of efflux activity of bacteria by a semi-automated fluorometric system. Methods Mol. Biol. 642: 159-172.

CHAPTER IV

Preface to Chapter IV

In the previous chapter, we have presented a model on how multidrug resistance develops in tuberculosis patients, the role of efflux pumps on the development of isoniazid resistance once exposed to the critical concentration of this antituberculosis drug and how these events determine the basis of acquired multidrug resistance. The demonstration of the involvement of efflux pumps on the development of drug resistance make these proteins interesting targets for the discovery of novel antituberculosis drugs and, being the efflux an important contributor to mycobacterial drug resistance, as we have previously demonstrated, the identification and characterization of mycobacterial efflux inhibitors is an approach that can be advantageous for the development of new effective anti-tuberculosis therapies.

The use of compounds that have the ability to inhibit mycobacterial efflux pumps promoting the retention of co-administered antibiotics that are subject to efflux will obviously improve the efficacy and will extend the clinical utility of the existing antibiotics. To date, several compounds have been appointed as putative efflux inhibitors *in vitro*. Previously, we have shown that isoniazid phenotypic resistance can be reduced by the use of verapamil, thioridazine and chlorpromazine. Furthermore, these compounds were also shown to enhance the killing of intracellular mycobacteria. They are used in clinical practice as antipsychotics and/or antihypertensive; however, despite several efforts employed on their characterization, the mechanism of action of these compounds remains largely unknown.

In this chapter we aim to contribute to the clarification of the mechanism of action of these putative efflux inhibitors, against drug resistant *M. tuberculosis*. For that purpose, we have designed and applied several experimental procedures to investigate the antimycobacterial properties of a panel of compounds, both *in vitro* and in human monocyte-derived macrophages. We hypothesized that their inhibitory activity involves the disruption cell energy, which will trigger a cascade of events that culminates with the death of the bacteria. The concept behind this study highlights the value of these inhibitors as adjuvants of tuberculosis therapy and in particular drug resistant

tuberculosis therapeutics, and reveals the basis for the identification of novel agents to fight tuberculosis and new therapeutic strategies to fight all forms of tuberculosis.

Chapter IV. Ion channel blockers act as antimicrobial agents and enhancers of macrophage killing activity against drug resistant *Mycobacterium tuberculosis*

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1. Abstract

Here we investigate the antimycobacterial properties of the ion channel blockers verapamil, thioridazine, chlorpromazine, flupenthixol and haloperidol, *in vitro* and in infected human monocyte-derived macrophages. All compounds exhibited synergistic inhibitory activities when combined with the main first-line antituberculosis drugs, isoniazid and rifampicin, and were able to inhibit active efflux, demonstrating their role as efflux inhibitors. Gene expression analysis showed that efflux genes were overexpressed in response to antibiotic exposure, *in vitro* and *ex vivo*, indicating that *M. tuberculosis* drug resistance within macrophages is also mediated by the expression of efflux pumps that can be inhibited by these inhibitors. These compounds displayed a rapid and high killing activity for *M. tuberculosis* that can be correlated with a decrease in intracellular ATP levels. The compounds lead to a decrease in the intracellular mycobacterial load that resulted from their ability to induce phagosome acidification that results in the expression of lysosomal hydrolases. Altogether, we demonstrate that these inhibitors display a dual antimycobacterial inhibitory effect as they target both the bacteria and the host cell. On the bacteria side we hypothesized that these compounds inhibit the mycobacterial respiratory chain which leads to the dissipation of membrane potential, depletion of ATP, reactive oxygen species generation and cell death. Disruption of the proton motive force (PMF), due to inhibition of the respiratory chain, results in the inhibition of PMF-dependent efflux systems in *M. tuberculosis*, therefore promoting the retention of antibiotics subject to active efflux. Concerning the host cell, the phagosomal acidification stimulated by these compounds synergize with several components of the host immune response, restricting *M. tuberculosis* intracellular growth. In conclusion, we provide direct, experimental evidence that these ion channel blockers act as antimicrobial agents and enhancers of macrophage killing activity. This work further highlights the value of these compounds as adjuvants of drug resistant tuberculosis chemotherapy.

2. Author summary

Mycobacterium tuberculosis, the causative agent of tuberculosis, claims millions of human lives every year. Given the ability of *M. tuberculosis* to survive as intracellular

pathogen within phagocytic cells and its propensity to develop resistance to the existing antituberculosis drugs, its treatment requires new approaches. The main objectives of ongoing antituberculosis drug discovery programs are the identification of compounds that have a new mode of action with potential to shorten the duration of chemotherapy. Here we show the potential use of some clinically approved drugs, with antimycobacterial and immunomodulatory properties, as adjuvants for the conventional antituberculosis therapeutic regimen. The ion channel blockers verapamil, thioridazine, chlorpromazine, flupenthixol and haloperidol, demonstrated to be potent inhibitory agents against *M. tuberculosis* both *in vitro* and *ex vivo*. We propose that their inhibitory activity involves the disruption of cell energy which triggers a cascade of events on the bacteria and on the host that, combined, culminates with bacterial death, providing a new insight into the mechanism of the pathogen–host interaction in tuberculosis. The discovery of such antituberculosis properties in drugs already in clinical practice, will contribute to identify new compounds for drug development and therapeutic interventions, shortening the bench to bedside timeline of drug discovery.

3. Introduction

Mycobacterium tuberculosis is a difficult pathogen, mostly due to its impenetrable cell wall coupled with a long generation time, a plastic metabolism and a remarkable ability to establish persistent and opportunistic infections. Consequently, the therapeutic regimen for tuberculosis requires a prolonged antibiotic treatment through, at least, six months in order to attain a favourable clinical outcome (37). Nevertheless, antibiotic resistance arises frequently. Although infections with drug-susceptible *M. tuberculosis* strains can be effectively treated with the current regimen of first-line antituberculosis drugs, the increase of antibiotic resistant *M. tuberculosis* strains represents an ongoing threat to the control of the disease. According to the World Health Organization, in 2012, there were an estimated 450 000 new cases of multidrug resistant tuberculosis (MDRTB, simultaneous resistance to isoniazid and rifampicin) worldwide. The average proportion of MDRTB cases with extensively drug resistance (XDRTB, MDR plus resistance to fluoroquinolones, aminoglycosides and cyclic peptides) was 9.6% (67). Regrettably, these numbers indicate that, despite all efforts, antibiotic resistance in tuberculosis continues to increase.

Resistance to antituberculosis drugs is assumed to be a consequence of the development of spontaneous mutations in defined genes or its promoter regions and the resulting resistance levels can be influenced by the type of alteration in the target gene (8). However, in a proportion of strains, the resistance to a certain drug cannot be solely explained by classical gene mutations, which suggests the presence of additional mechanisms of resistance (31; 32). In mycobacteria, the physiological resistance is generally attributed to the low permeability of the cell wall, which may limit the entry of antibiotics. Besides this reduced permeability, efflux systems also promote the development of resistance by the extrusion of molecules entering the cell prior to their access to the intended target (30).

The contribution of efflux mechanisms to antibiotic resistance in *M. tuberculosis* has been highlighted in a number of studies over the last years. In these studies, several putative efflux pumps of different classes have been described and various have been shown to be involved in the transport of different compounds (61). Besides, it has been demonstrated that the overexpression of efflux pumps contributes to resistance allowing the bacteria to survive for a longer period of time in the presence of sub-inhibitory concentrations of antibiotics, until mutations emerge (32). Concomitantly, it was demonstrated that the resistance level to a given antibiotic can be significantly reduced by an efflux inhibitor despite the presence of a mutation, highlighting the contribution of active efflux to the overall resistance level of a drug resistant strain of *M. tuberculosis* (31; 32).

The addition of an efflux inhibitor to tuberculosis chemotherapy can enhance antimycobacterial killing, prevent the emergence of drug resistance (2; 32; 49) and reduce the duration of tuberculosis treatment (2; 24). To date, several compounds have been appointed as potential efflux inhibitors. These compounds are ion channel blockers and comprised the phenotiazines thioridazine and chlorpromazine and its derivative flupenthixol, a high-potency thioxanthene; haloperidol, a butyrophenone structurally and clinically related with phenothiazines, and verapamil, a phenylalkylamine (61). These compounds are approved by the Food and Drug Administration (FDA) to be used in the treatment of several mental disorders or hypertension, with well characterized pharmacological and toxicological profiles.

Ion channel blockers like thioridazine and verapamil have been shown to have active efflux inhibition properties and to inhibit the *in vitro* growth of *M. tuberculosis* strains alone or in combination with antimycobacterial drugs (5; 6; 11; 32). Thioridazine demonstrated significant activity against MDRTB in a murine model (57) and it has been successfully employed to treat XDRTB patients on the basis of compassionate reasons (1). Verapamil has been shown to be the most potent mycobacterial inhibitor to date, being able to enhance the inhibitory activity of isoniazid (32; 49) and rifampicin (31) in *M. tuberculosis* clinical strains. Recently, Gupta *et al*. (23) demonstrated that efflux inhibition by verapamil can potentiate the activity of bedaquiline, a diarylquinoline recently approved for the treatment of MDRTB (66). It has also been shown that the addition of verapamil accelerates the bactericidal and sterilising activities of tuberculosis therapy in the mouse model (24). Beyond their antimycobacterial activity, these compounds also present immunomodulatory abilities on *M. tuberculosis*-infected macrophages. Previously, we had shown that inhibitors of calcium and potassium channels can enhance the killing of *M. tuberculosis* by macrophages (35; 41). Afterwards, it was demonstrated that the inhibition of macrophage calcium channels increases intracellular concentrations of calcium which, consequently, activates an immune response against intracellular *M. tuberculosis* (22). Further evidence in favour of the usefulness of the ion channel blockers is reported in the elegant studies by Adams *et al*. (2; 3), wherein the selective pressure exerted by the macrophage on internalized *M. tuberculosis* can induce the bacteria efflux pumps and thereby drug-tolerance. Additionally, the authors also show that this macrophage drug-induced tolerance can be reduced by verapamil or derived metabolites (2; 3).

Having established the significance of the use of ion channel blockers as adjuvants in tuberculosis chemotherapy, it is important to understand their antimycobacterial properties both *in vitro* and in the macrophage model. In this study, we demonstrate that these compounds are efflux inhibitors with direct antimycobacterial activity towards *M. tuberculosis*. We show that they can enhance the inhibitory activities of the antibiotics against *M. tuberculosis in vitro* and enhance the killing activity of the macrophage against intracellular *M. tuberculosis.* Furthermore, we demonstrate that these compounds are able to induce phagosome acidification, whose combined effect with other antimicrobial macrophage factors, promote the elimination

of the internalized mycobacteria. Our findings contribute to a better understanding of the mode of action of these ion channel blockers and provide insights to identify the mediators of their antimycobacterial and immunomodulatory activity.

4. Results

Ion channel blockers enhance the inhibitory activity of antibiotics against *M. tuberculosis*.

To determine whether the selected ion channel blockers known to act as efflux inhibitors show synergistic activity when combined with the antituberculosis drugs against the drug susceptible and resistant panel of *M. tuberculosis* strains used in this study, the susceptibility of isoniazid, rifampicin, amikacin and ofloxacin was investigated in the presence and absence of subinhibitory concentrations of verapamil, thioridazine, chlorpromazine, flupenthixol and haloperidol. The *in vitro* interaction of two agents in combination can be interpreted as synergistic, indifferent, or antagonistic depending on whether the activity of the combination is superior, comparable to, or smaller than the activities of the individual drugs, respectively. The efficiency of the drug combination was determined through the calculation of the fractional inhibitory concentrations (FIC) (29). The characterization of the nine *M. tuberculosis* strains is presented in Table IV.1. The minimum inhibitory concentrations (MICs) and FICs obtained with the combinations of the inhibitors and the antibiotics against the *M. tuberculosis* strains are listed in Table IV.2. As can be observed, the combination of isoniazid with the five compounds consistently demonstrated synergy, with FIC values ranging from 0.15 to 0.5, for the five clinical multi- and extensively drug resistant strains. For these, the MIC values of isoniazid were reduced from 2- to 20-fold depending on the strain and the inhibitor tested. No effect of the compounds was observed on the MIC of the isoniazid-monoresistant strains H37RvΔ*katG* and 294/09 (single *inhA* promoter C-15T mutation), demonstrating indifference. However, the combination between the phenotiazines with isoniazid demonstrated synergistic effect against the isoniazid-monoresistant strain 269/03 (single *katG* S315T mutation), being able to reduce 6-fold the MIC of isoniazid (FIC of 0.3). Contrary to the study by Warman *et al*. (62) we did not detect antagonistic activity between isoniazid and the

three phenotiazines in study. For rifampicin, significant synergistic activity was observed mainly with verapamil and chlorpromazine. The combination of verapamil and rifampicin reduced the MIC values of rifampicin 16-fold in four strains and 4-fold in one strain, all carrying the *rpoB* S531L mutation. The FIC for this combination was 0.06 and 0.25, respectively. Chlorpromazine reduced the MIC of rifampicin 16-fold in three strains and 4-fold in one strain, showing a FIC of 0.06 and 0.05, respectively. For strain 286/09, the MIC of rifampicin was reduced 20-fold by chlorpromazine and flupenthixol, and 4-fold by verapamil, thioridazine and haloperidol, originating a FIC of 0.05 and 0.25, respectively. For strains 149/09 and 29/12 rifampicin MIC values were reduced 8-fold. Only in one isolate (69/11), an heteroresistant strain for amikacin with simultaneous presence of drug susceptible and drug resistant genotype carrying the *rrs* A1401G mutation, was observed significant synergy when the combination of the compounds and amikacin was evaluated. For this strain, the MIC values were reduced 40-fold, to which corresponds a FIC of 0.03 for all the five inhibitors tested. For the remaining three amikacin-resistant strains carrying the *rrs* A1401G mutation, none of the inhibitors, except chlorpromazine, demonstrated any synergy. Chlorpromazine was found to have a FIC value of 0.13 for strains 149/09 and 29/12. The combination of the compounds with ofloxacin, for the three ofloxacin-resistant strains, was interpreted has being indifferent, with FIC of 1 for all the compounds tested. Collectively, these results support the hypothesis that using synergistic combinations of inhibitors with antituberculosis drugs can significantly enhance the activities of first and second line antituberculosis drugs. However, can this synergistic enhancement bring back the clinical and therapeutic effect of the antibiotics that became non-effective due to the presence of mutations associated with resistance? To address this question, we performed quantitative drug susceptibility testing for the four antibiotics in the presence of the inhibitors to assess the levels of resistance and correlate them with the mutations associated with clinical resistance.

Ion channel blockers reduce the resistance level of first and second line antituberculosis drugs.

To assess the clinical effect of these compounds in combination with antituberculosis drugs in respect to their levels of resistance, and evaluate its correlation with the

presence of mutations associated with resistance, the levels of resistance to isoniazid, rifampicin, amikacin, and ofloxacin were evaluated with the MGIT960 system and the Epicenter V5.80A software equipped with the TB eXIST module. The results are presented in Table IV.3. The addition of the compounds promotes a significant reduction of the resistance levels for all antibiotics except for ofloxacin.

In Figure IV.1 is shown the growth curves for strain 29/12, with and without verapamil, in the presence of isoniazid, as an example. This strain, which was initially resistant at 1 µg/ml of isoniazid, upon addition of ½ MIC of verapamil had its high-level of resistance decreased to low-level resistance (<1 µg/ml) (see full *vs*. dotted pink line in Figure IV.1). The resistance levels to isoniazid were reduced from high-level to low-level in 4/8 of the resistant strains, with verapamil, flupenthixol and chlorpromazine and 2/8 with thioridazine. The strain is considered resistant when the number of drug-resistant bacteria present in the drug-containing tube at critical concentration is greater than 1%, when compared with the drug-free growth control and correlates well with an effective clinical outcome (see Materials and Methods for critical concentrations) (10). None of the strains had reversal of resistance since the resistance levels did not drop below the isoniazid critical concentration of 0.1 µg/ml (Table IV.3). Correlating the resistance levels to the antibiotics with the presence of mutations associated with resistance: four strains for which the resistance level was reduced in the presence of the inhibitors harbored double mutations in *inhA* gene, 2 with C-15T/S94A and 2 with C-15T/I194A substitutions. The four remaining strains, where residual or no change of the resistance level was noticed in presence of the inhibitors, possessed single mutations: the C-15T mutation in *inhA* promoter in one strain, two strains with the S315T mutation in *katG*, and one with a complete deletion of *katG*. Strain H37Rv∆*katG* was included in the study as a negative control, as it harbors a complete deletion of the *katG* gene and demonstrates no efflux activity (32). Regarding the strains with the *katG* S315T mutation, only a slight enhancement of the antimicrobial effect of isoniazid was observed for both strains in the presence of the phenothiazines thioridazine, chlorpromazine and flupenthixol (Table IV.3). However, this potentiation was not enough to reach low-level isoniazid resistance. Regarding the strain 294/09 with the C-15T mutation in the promoter region of *inhA* gene, this strain display low-level resistance due to the overexpression of *inhA* and the effect of this overexpression on

isoniazid resistance cannot be overcome by the inhibitors tested. Concerning rifampicin, all strains presented high-level resistance due to the presence of the mutation S531L in *rpoB*. The resistance level was remarkably reduced from high- to low-level in presence of verapamil (5/5), chlorpromazine (4/5), and thioridazine, flupenthixol and haloperidol (1/5) (Tables IV.2 and IV.3). Four strains presented resistance to amikacin and for all was demonstrated the presence of a mutation in a gene associated with amikacin resistance. Three strains presented mutations in *rrs1400* region and one strain has a mutation in *eis* promoter region. The high-level resistance conferred by mutations in *rrs* could not be reduced in the presence of the inhibitors in 2/3 strains. Nevertheless, resistance to amikacin was reversed in strain 69/11. The amikacin resistance profile changed from high-level resistance to susceptible in presence of the five compounds. In this case, *rrs* hybridization pattern given by Genotype MTBDR*sl* assay indicated heteroresistance, *i.e*. the coexistence of a population of bacilli susceptible and resistant to amikacin. This is especially important since heteroresistance plays a role in development of resistance in *M. tuberculosis* (25). Our data demonstrates that these inhibitors are capable to prevent the development of resistance to antituberculosis drugs. The amikacin low-level resistance due to the mutation in *eis* promoter region could not be reduced. For ofloxacin, low-level resistance was detected in three strains and correlated with mutations in *gyrA* gene. With this panel of strains, ofloxacin resistance could not be reduced by any of the inhibitors tested.

With this methodology, we have been able to demonstrate that the addition of ion channel blockers enhances the therapeutic effect of the main antituberculosis drugs, isoniazid and rifampicin, in multi- and extensively drug resistant strains, despite the presence of a mutation conferring resistance. However, their effect on isoniazid resistance seems to be limited to strains with mutations in *inhA* gene. The fact that strains changed their resistance profile from high- to low-level upon the addition of the inhibitors brings clinical implications. The serum concentrations of isoniazid and rifampicin attained *in vivo*, 7 and 10 µg/ml respectively, are much higher than the respective MICs for the majority of strains (29). In the presence of a high-level resistance strain (see Material and Methods section for breakpoints), these drugs are predicted to be useless. However, in the presence of a low-level resistance strain it is possible to reach higher drug concentrations *in vivo*, since the effectiveness of the

synergistic combination of these inhibitors with conventional therapy, seen *in vitro*, is expected to be beneficial in reducing the resistance level of the strain.

The ion channel blockers display rapid and high killing activity.

One of the prerequisites for the development of new antituberculosis drugs includes the need for compounds that have bactericidal activity. This is important to prevent the development of drug resistance and act rapidly to reduce the duration of the treatment (19). To characterize the bactericidal activity of the ion channel blockers and evaluate if they can enhance the bactericidal efficacy of the antituberculosis drugs, we measured their antimycobacterial killing activity against the strains H37Rv (drug-susceptible) and 149/09 (drug-resistant) through time-kill studies. The time-kill kinetics of the compounds and antibiotics on *M. tuberculosis* H37Rv is shown in Figure IV.2. All the compounds exhibit time-dependent bactericidal activity. After seven days, flupenthixol, chlorpromazine, thioridazine, haloperidol, and verapamil were able to eliminate all *M. tuberculosis* cells. Flupenthixol reduced the cell number to zero at day one, chlorpromazine and thioridazine at day two, and verapamil and haloperidol at day seven. Moreover, at the end of 100 days all cultures remain negative. Isoniazid, rifampicin, amikacin, and ofloxacin were not able to reduce the viable number of *M. tuberculosis* cells to zero by the end of the seven days. On the seventh day of incubation, a $3\log_{10}$ CFU/ml decrease by rifampicin, amikacin, and ofloxacin was observed. For isoniazid was notice a $2\log_{10}$ reduction in the same time frame.

The most effective ion channel blocker previously tested, verapamil, was selected for the evaluation of the synergistic effect with the antibiotics through time-kill studies. The data presented in Figure IV.2B corresponds to the measurement of time-kill kinetics for verapamil with isoniazid, rifampicin, amikacin and ofloxacin for H37Rv. It can be observed that the activity of the antibiotics was strongly potentiated. By the end of day seven no bacteria remained viable for the five combinations tested. The combination of verapamil plus amikacin reduced the CFU/ml to zero at day two; verapamil and rifampicin at day three; verapamil and ofloxacin also at day three; and verapamil and isoniazid at day seven. Similar results were obtained for the multidrug resistant strain (data not shown). These results demonstrate that flupenthixol, chlorpromazine, thioridazine, haloperidol, and verapamil have direct bactericidal activity and when combined with the antibiotics can stimulate a rapid and high killing

effect against both drug susceptible and multidrug resistant *M. tuberculosis,* therefore fulfilling both criteria mentioned above.

ATP depletion occurs in response to the treatment with the ion channel blockers.

To determine whether the treatment with the ion channel blockers could have an effect on bacterial intracellular ATP levels, we monitored the intracellular ATP levels over seven days. ATP levels and killing activity was recorded after day one, two, three and seven of exposure to the compounds. The ATP levels in cultures of *M. tuberculosis* H37Rv, exposed to 5X the MIC for each compound, are shown in Figure IV.3A and viability, in Figure IV.3B. A rapid drop in intracellular ATP levels of *M. tuberculosis* H37Rv was notice after one day of exposure with all compounds, which progressively falls during the seven days of exposure. This decrease in the ATP levels was correlated with an increased killing activity. Isoniazid and rifampicin do not have a significant effect on the ATP levels. These results confirm that ATP depletion occurs in consequence of exposure to the compounds and indicates that the metabolic state of the cells is directly affected by them with anticipated effects on the bacterial energy available for active efflux.

Inhibition of ethidium bromide efflux demonstrates the role of the ion channel blockers as efflux inhibitors.

The ability of verapamil, thioridazine, chlorpromazine, flupenthixol and haloperidol to inhibit the efflux activity of the *M. tuberculosis* strains used in this study was carried out using an semi-automated ethidium bromide fluorometric method (60) with a modified protocol for *M. tuberculosis* (32). When data for the clinical strains were compared with those for the H37Rv strain, different levels of accumulation were observed for all isolates. The results showed that the clinical strains are able to handle higher ethidium bromide concentrations than H37Rv. This means that the clinical strains have higher efflux capacity than that of the reference strain as previously described (32). These assays were then performed in the presence of the same compounds at sub-inhibitory concentrations. In Figure IV.4A is shown the ethidium bromide accumulation assay for strain 29/12, in the presence of the inhibitors, as an example. The inhibitory indexes of activity of each compound against the *M. tuberculosis* strains are presented in Table IV.4. The relative final fluorescence index

corresponds to a measure of how efficient is the inhibitory effect of the compounds on efflux by comparison of the final fluorescence of the cells exposed to ethidium bromide plus the compound against cells exposed only to ethidium bromide (taken as 0). The accumulation of ethidium bromide increased in the presence of all inhibitors. Verapamil promoted the highest increase in accumulation in all strains, except H37RvΔ*katG* and 269/03 (6/8), followed by the phenotiazines. Among this class of compounds, flupenthixol demonstrated the highest inhibitory effect (3/8), followed by thioridazine and chlorpromazine, which demonstrated similar activity against 2/8 of the multidrug resistant strains. Haloperidol was the compound that demonstrated the lowest effect on the accumulation of ethidium bromide. The multidrug resistant strains showed significantly higher levels of ethidium bromide accumulation in presence of the inhibitors, whereas the clinical isoniazid monoresistant strains accumulated significantly lower levels of ethidium bromide when compared with those from the multidrug resistant strains. For the *katG* deleted strain, only a minor effect was observed with verapamil.

To directly assess efflux, we selected verapamil to measure the inhibition of ethidium bromide efflux on these strains (Figure IV.4B). The results showed that the ethidium bromide efflux is greatly inhibited in the presence of verapamil. Therefore, from the analysis of Figure IV.4B we correlate these results with the effect on the ethidium bromide accumulation observed in Figure IV.4A and conclude that the inhibition of efflux pumps retain ethidium bromide inside the cells. All together, the MICs results followed the same pattern as in the ethidium bromide accumulation promoted by the inhibitors, correlating the resistance levels of these strains with their ability to efflux noxious compounds.

Overexpression of efflux pumps in response to the antibiotic pressure leads to increased tolerance towards antibiotics.

In a previous work we have been able to correlate the increased ethidium bromide efflux in *M. tuberculosis* with efflux-mediated increased isoniazid resistance as the result of the activity of several overexpressed efflux pumps working in combination (32). Here, we assess the contribution of the *M. tuberculosis* membrane transporters for the increased levels of resistance towards isoniazid and rifampicin previously seen for the

multidrug resistant *M. tuberculosis* strains. For that, these strains were exposed to sub-inhibitory concentrations of isoniazid or rifampicin and the expression levels of genes that code for 10 efflux pumps and the global regulator *whiB7* were evaluated by RT-qPCR. Table IV.5 and IV.6 show the results obtained for the quantification of gene expression of the bacteria exposed to isoniazid and rifampicin, respectively. The relative expression of the efflux-pump genes was determined by comparison of the relative quantity of the respective mRNA in the presence of the antibiotic to the non-exposed condition. As can be observed, the majority of the efflux genes are overexpressed in response to isoniazid and rifampicin exposure. Although we cannot observe a specific pattern of expression, the genes *mmr*, *mmpl7*, *p55*, *Rv1217c* and *efpA* showed a significantly increase in their expression levels in the presence of isoniazid. Regarding the exposure to rifampicin, we can observe an increase in the expression of the majority of the genes albeit at lower absolute levels than those obtained for the isoniazid exposed strains. Of all pumps studied, P55 a member of the major facilitator superfamily efflux-pump, described in 2001 by Silva *et al*. (11), was found to be consistently expressed in all strains, presenting a 1.54 to 9.88-fold increase in the presence of rifampicin.

This systematic increase in efflux pumps gene expression after isoniazid and rifampicin exposure, correlated with increased efflux and antibiotic resistance, can occur due to two possible mechanisms. First, increase in gene expression due to a mutation in a regulatory or promoter region of the efflux pump gene leading to constitutive gene expression. Secondly, this increase can be due to the induction promoted by the compounds that are substrates of the efflux pumps. To clarify which inductive mechanism is promoting this overexpression, we searched for mutations on the transporters studied. Despite missense and silent mutations in the coding regions of these genes, no other mutations were found on putative promoter or regulatory regions in these strains (Table IV.S1). This indicates that the overexpression detected in these strains is induced by the antibiotic substrates and is not constitutive. Afterwards, we sought to see if the expression of some of these pumps can be induced upon macrophage residence. As proof of evidence, we selected one multidrug resistant strain, *M. tuberculosis* 82/09, to study the expression of five efflux genes, *mmr*, *mmpl7*, *Rv1258c*, *p55* and *efpA*, and determined their expression level upon exposure to isoniazid inside macrophages. The results show that *M. tuberculosis* efflux pumps are

also expressed inside the macrophages upon exposure to isoniazid (Figure IV.5) demonstrating that *M. tuberculosis* drug resistance in macrophages is mediated by the expression of pumps that help the bacteria to reduce the intracellular concentration of the antibiotic. Our results are in agreement with other previously reported data (2; 3). Collectively, these results reinforce the concept that induction of efflux pumps is one of the mechanisms involved in drug resistance in *M. tuberculosis*. Similar to our previous work, we conclude that efflux pumps are promiscuous in their activity, as we could not associate extrusion of isoniazid or rifampicin to a specific pump (32). Moreover, the results indicate that the compounds are not acting on a specific pump but on multiple pumps, and induction of efflux pumps seems to be a general stress response to the presence of noxious compounds, instead of a specific response of a specific pump. The differences observed in the gene expression profiles in each clinical strain most probably reflect their history of different antibiotic pressures in the clinical settings. The data obtained with the fluorometric method for the non-exposed strains and RT-qPCR for exposed strains indicates that these strains may be already primed to react in presence of toxic compounds.

The ion channel blockers enhance the killing activity of macrophages against intracellular *M. tuberculosis*.

Since *M. tuberculosis* infects mainly macrophages, the compounds to be applied as antimicrobials or enhancers of antimycobacterial agents should demonstrate activity against intracellular *M. tuberculosis*. For this purpose, we examined the intracellular activity of verapamil, flupenthixol, thioridazine, chlorpromazine, and haloperidol, and their ability to potentiate isoniazid activity, on human monocyte-derived macrophages. Isoniazid was chosen due to be one of the most effective antituberculosis agents known to date (37). We used one susceptible strain, H37Rv, and four isoniazid resistant strains with commonly described isoniazid-associated resistance mutations in *katG* and *inhA*. The enhancement of intracellular killing activity promoted by the compounds and their combined effect with isoniazid and the kinetics during 3 days post infection is presented in Figure IV.6. We found that all the compounds impaired growth of intracellular *M. tuberculosis* for both wild-type and resistant strains. When tested for the enhancement of the killing activity by the macrophage, thioridazine demonstrates to be the most active compound. Thioridazine enhanced the killing of mycobacteria by 58% to 88%,

whereas the remaining compounds demonstrated rates of killing of 12% to 64% with verapamil, 37% to 60% with chlorpromazine, 27% to 43% with flupenthixol, and 11% to 37% with haloperidol. The co-administration of isoniazid with any of the compounds studied in this work has led to a strong enhancement of the killing activity against the H37Rv strain. Conversely, no additional killing was observed in the isoniazid-resistant strains when isoniazid was used in combination with the compounds, as expected. This result is in agreement with the fact that all strains, except H37Rv, harbored a mutation that confers isoniazid resistance. Collectively, these results allow us to show that verapamil, flupenthixol, thioridazine, chlorpromazine, and haloperidol strengthen the killing activity of human macrophages against *M. tuberculosis.*

The ion channel blockers promote intracellular acidification.

Restriction of mycobacterial growth requires acidification of phagosomes (64). Since the inhibitors enhance the antimycobacterial activity of human macrophages, we hypothesized that these compounds can induce phagosome acidification. To test this hypothesis, human monocyte-derived macrophages infected with *M. bovis* BCG-GFP were treated with the compounds and stained with LysoSensor, an acidotropic probe that emits fluorescence when pH reaches 4.2. Figure VI.7 depicts representative images of vesicles positive and negative with LysoSensor. As can be observed, LysoSensor staining is weak in non-treated human monocyte-derived macrophages. In the presence of the ion channel blockers the fluorescence intensity increases indicating a decreased pH. We also observed colocalization of *M. bovis* and LysoSensor inside the vesicles which demonstrates that the compounds mediate acidification of *M. bovis* BCG-containing vesicles. The quantification of vesicle acidification in human monocyte-derived macrophages was measured by the accumulation of Lysotracker Red, a lysosomotropic probe, using flow cytometry. First, we analysed the increase in the fluorescence of Lysotracker RED positive cells. The results show that all the compounds increase significantly the percentage of cells stained positive for Lysotracker RED (Figure VI.8). Next, we measured the increase on the overall number of Lysotracker RED stained vesicles per cell (average fluorescence intensity). We observed that all the compounds increase the overall number of stained cells (Figure IV.S1). In both cases, verapamil, thioridazine and haloperidol induced the strongest effects. These results clearly show that the compounds are able to induce the phagosome

acidification. However, it is unlikely that pH alone is responsible for the death of intracellular bacilli. It is described that *M. tuberculosis* is able to survive in the acidic environment of the phagolysosome of activated macrophages and its survival or elimination depends on the ability of the macrophage to activate its pH depended lysosomal hydrolases (58). Following this rational, we assessed the impact of pH on the survival of *M. tuberculosis in vitro* and we observed that *M. tuberculosis* can cope in an acidic environment (Figure IV.S2). However, *M. tuberculosis* becomes sensitive to low pH in combination with other antimicrobial factors such as low pH activated lysosomal hydrolases, so we measured the activity of cathepsin B, a lysosomal cysteine protease, which enters in the phagosome early in their maturation (54). Increased activity of cathepsin B was detected in cultures treated with the compounds (Figure IV.9). When compared with the untreated control cultures, cathepsin B activity was induced by 21% by verapamil, 42% by thioridazine, 33% by chlorpromazine and flupenthixol, and 32% by haloperidol, in average, regardless the macrophage is infected or not infected. These observations indicate that the inhibitors prompted the acidification of the phagosomes containing *M. tuberculosis* and enhance the expression of cathepsin B promoting the hydrolytic features of the phagosome. These results show that phagosome acidification is important for the function of degradative hydrolases as well as other phagosomal functions, as previously suggested (58). Furthermore, it is known that phagosomal acidification is established by the V-ATPases (55) and that the accumulation of V-ATPases is associated with an enhancement of the expression of the aspartic protease cathepsin D (54). We are currently evaluating the expression of cathepsin D and V-ATPases and determining if the compounds-mediated acidification could be inhibited by a V-ATPase inhibitor and, if this inhibition can lead to enhanced mycobacterial replication inside the macrophages.

Multidrug resistant *M. tuberculosis* strains demonstrate enhanced fitness.

We have noticed that the activity of the compounds was influenced by the genotype and fitness of the strains. In this sense, we characterized the genotype of these strains and evaluated the growth patterns *in vitro* and *ex vivo* as indicators of fitness. Five strains were found to belong to LAM genotype: two belonging to cluster Q1, two from cluster Lisboa3, and one non-clustered strain; one Beijing strain, and one strain with a non-defined genotype (Table IV.1). This strain was further analyzed at SITVITWEB

database and the corresponding MIRU12-VNTR pattern was designated as VNTR international type VIT21. Next, we determined the growth rates for all strains grown in liquid media. First, we examined the time necessary for the strains to adapt to a new environment in liquid culture starting with a well-defined inoculum. All grew slightly faster when compared with the control strain, H37Rv (Figure IV.S3A), except strains 286/09 and 69/11. Second, we evaluated the growth rate, *i.e*., the time necessary to reach 400 GU (the acceleration phase). All strains demonstrated similar growth rates, except strains belonging to cluster Q1 (149/09 and 29/12) and the isoniazid resistant strain 294/09. We observed that strains belonging to cluster Q1 take more time to reach the same growth units than strains from cluster Lisboa3 and Beijing, and less than H37Rv (Figure IV.S3B). Strain 294/09 showed a dramatically slower replication rate when compared with the other strains with the same mutation. Whilst 294/09 is resistant just to isoniazid, the other strains are multidrug resistant. Collectively, the results show that multidrug resistant strains included in this study have a similar ability to adapt to a new environment but differ in their growth rates *in vitro*. Next, we evaluate the ability of some of these strains to grow inside macrophages as a surrogate of intracellular fitness. For that, human monocyte-derived macrophages were infected with multidrug resistant strains belonging to cluster Lisboa3 and Q1, the isoniazid monoresistant strains 294/09, 269/03, and the H37Rv *katG* deletion mutant. As a control, we used the H37Rv reference strain. We found that the *M. tuberculosis* multidrug resistant strains have enhanced fitness when compared with susceptible or monoresistant strains. As can be observed in Figure IV.10, both strains grew more quickly in macrophages than all other strains. At the end of five days, the number of viable bacteria was significantly higher than the one reached by the susceptible strain H37Rv. Moreover, H37Rv was able to replicate much faster than the isoniazid monoresistant strains. Regarding H37RvΔ*katG*, this strain is highly resistant to isoniazid *in vitro* and does not have catalase activity (data not shown). We observed that this strain has reduced ability to survive in human macrophages. However, *katG* deletion mutants can cause disease in humans. One possible explanation is that the bacteria can acquire compensatory mutations to resist the oxidative burst *in vivo* (51). We also observed a reduction of the fitness of the strain with the mutation S315T on *katG*. Strain 269/03 retains catalase activity and demonstrates a physiological cost inside macrophages. *katG*315 is the most common

isoniazid resistance-conferring mutation found in clinical settings. Although, it is assumed that strains with *katG*315 can maintain *katG* activity and virulence in mice without significant loss of fitness (44); others show that *M. tuberculosis* strains with *katG*315 mutation are less fit on murine macrophages (47). It has been shown that the genetic background of a strain influences the fitness of drug-resistant *M. tuberculosis* (18). Altogether, it seems that intracellular fitness of the *M. tuberculosis* strains varies according to their resistance pattern and the mutations associated. The multidrug resistant strains studied showed an increased replication rate inside macrophages indicating a higher fitness for strains with this genotype, clusters Lisboa3 and Q1. Furthermore, overexpression of efflux pumps was more evident on multidrug resistant strains than in isoniazid monoresistant strains and the compounds were shown to be more effective on the multidrug resistant strains. From these results we can establish association between drug resistance, the genetic background and overexpression of efflux pumps.

5. Discussion

The global emergence of multi- and extensively drug resistant tuberculosis has hampered the perspectives of tuberculosis control and elimination. Multidrug resistant tuberculosis requires a prolonged treatment with second-line drugs that are less effective and more toxic. In addition, for extensively drug resistant tuberculosis the chances to achieve a successful treatment are further reduced. The therapeutic options for these patients can be limited to second-line oral bacteriostatic agents and to drugs with questionable reliability and reproducibility known as WHO Group V (65). Therefore, there is an urgent need for the identification of compounds with different mechanisms of action that can be used in combination with the existing antituberculosis drugs in order to shorten the treatment. Some new drugs have been developed in the latest years which have demonstrated potential to achieve this objective (48). However, they are still limited in number which emphasizes the need for new approaches.

In this study, we provide evidence that verapamil, flupenthixol, thioridazine, chlorpromazine, and haloperidol act synergistically with the main first line antibiotics to restrict *M. tuberculosis* growth in either drug susceptible or resistant strains. However, they were not able to restore full drug susceptibility. Nevertheless, they substantially

assist in the reduction of the resistance level to the antibiotics and, by this manner, potentiate their activity. This evidence was confirmed by time-kill studies through which we were able to demonstrate that these compounds are capable to prevent the emergence of resistance and preserve the activity of the co-administered antibiotics. Besides, the low mutation frequencies determined for each ion channel blocker tested (10^{-10}-10^{-11}) indicate that they are less subject to acquire spontaneous mutations for resistance. Assuming that the *M. tuberculosis* burden in the lungs is around 10^{8}-10^{9} and the mutations rates for isoniazid and rifampicin are approximately 10^{-8} and 10^{-10} (12), respectively, the ion channel blockers when combined with antituberculosis drugs currently in use, will reduce the probability of development of spontaneous mutations during tuberculosis drug therapy.

At this moment we do not know the exact mechanism or the assembly of mechanisms by which these inhibitors act as antimicrobial agents against *M. tuberculosis*. We observed that all compounds lead to a significant decrease in the intracellular mycobacterial load as result of phagosome acidification inducement and enhanced expression of lysosomal hydrolases. Our previous research has leaded us to propose that the enhancement of the killing activity of macrophages by these type of compounds was dependent upon the availability of potassium and calcium ions inside the cells. (6; 35; 61). In support of these, Gupta *et al.* (22) elegantly demonstrate that the inhibition of L-type channels results in significant increase of calcium from intracellular stores within macrophages leading to a reduction in bacterial burden. This rise of protons was assumed to be responsible for the phagosome acidification and activation of lysosomal hydrolases that reduce the bacterial load (6; 35; 61). Consequently, it is expected that the use of inhibitors of potassium and calcium transport, such as verapamil, haloperidol and the phenothiazines, will enhance the killing of internalized *M. tuberculosis*, as we have observed in this study. Additionally, in support of this it was recently proposed that phagosomal acidification is controlled by the Abelson (Abl) tyrosine kinase by increasing the transcription and expression of V-ATPases and that the inhibition of tyrosine kinase triggers the phagosome acidification (9). The authors also show that imatinib, an inhibitor of the tyrosine kinase used for the treatment of cancer and pulmonary arterial hypertension, induce phagosomal acidification and growth restriction of *M. tuberculosis* in human macrophages (9; 40). Since tyrosine

kinase expression is dependent on calcium influx, partially through L-type channels (46), the impact of calcium on tyrosine inhibition can be associated with a decrease in the intracellular ATP availability. The increase of the expression of V-ATPase activity can induce phagosome acidification and consequently, increase the concentration of calcium on the cytosol resulting in enhanced bacterial killing. Both models act synergistically and strongly suggest the involvement of calcium and potassium ions on the mechanism of action of these inhibitors.

Our *in vitro* results show that the ion channel blockers are good antimycobacterial agents, especially when used in combination with antituberculosis drugs, indicating that they interfere directly with the bacteria. In eukaryotic cells it is known that these drugs act by blocking receptors for neurotransmitters such as dopamine and their neuroleptic activity is attributed to their role on the mitochondrial electron transport chain; haloperidol and chlorpromazine inhibit the mitochondrial NADH: ubiquinone oxidoreductase (complex I) (7). In *M. tuberculosis* it is believed that phenotiazines also inhibit one of the key enzymes of the mycobacterial respiratory chain, the type II NADH: menaquinone oxidoreductase (NDH-2) (62; 63). *M. tuberculosis* possesses two NADH dehydrogenases: the NDH-1, encoded by the *nuo* operon, which is non-essential in *M. tuberculosis* (45) and, the non-proton-translocating NDH-2, encoded by the *ndh* gene, which is essential for *M. tuberculosis* survival (36). NDH-1 is an equivalent of mitochondrial complex I, while NDH-2 is a single subunit enzyme with NADH/quinol oxidoreductase activity (68). In *M. tuberculosis*, energy generation is mainly performed by NDH-2 (45) whereas NDH-1 is mostly implicated on anti-apoptotic activity of host cells and, not on its original function of generation of energy (59). Rao *et al*. (45) established a link between NDH-2 and the proton motive force (PMF) through the demonstration that the inhibition of NDH-2 by thioridazine dissipates the membrane potential. Since thioridazine and chlorpromazine act as partial uncouplers of oxidative phosphorylation in mitochondria (38) and besides their inhibitory effect on NDH-2 they also act as uncoplers of oxidative phosphorylation in *Staphylococcus aureus* (50), it is expected that they indirectly promote the collapse of PMF. Uncouplers of oxidative phosphorylation are able to transport protons across biological membranes (17). By transporting protons down the electrochemical potential gradient, these agents collapse the PMF. The collapse of the PMF has detrimental effects on the bacteria such as

increased production of reactive oxygen species (ROS), inhibition of ATP synthesis (without affecting the respiratory chain and ATP synthase), and disruption of calcium homeostasis.

All the compounds tested were able to promote intracellular accumulation of ethidium bromide, a broad substrate for PMF dependent efflux pumps, in both drug susceptible and resistant strains. Hence, we can conclude that ethidium bromide accumulation promoted by the ion channel blockers is a result of efflux pump inhibition caused by dissipation of PMF. This was previously shown in our studies with susceptible and antibiotic-adapted *M. tuberculosis* strains (32). In this study it is now demonstrated, for the first time, on *M. tuberculosis* multidrug resistant clinical strains. Moreover, we showed that several efflux pumps are induced upon exposure to isoniazid and rifampicin in these multi- and extensively drug resistant clinical strains. We have detected a general and marked expression of almost all efflux genes tested, which is consistent with a stress response to a hostile environment. Afterwards, we sought to see if some of these pumps are also expressed within the macrophage. We found that some of the efflux genes were expressed inside macrophage upon drug exposure mimicking the inducible adaptative response seen *in vitro*. Using the zebrafish larval model, Adams *et al*. (2) demonstrated that drug-tolerant bacteria originate in macrophages dependent on the inducible overexpression and activity of bacterial efflux pumps. In their model, a bacterial subpopulation tolerant to drugs arise after few days post-infection, in response to host defenses, by the inducement of bacterial efflux pumps, that rendered the bacteria tolerant to several antituberculosis drugs (2; 3). This result further strengthens the importance of the design of rational strategies to prevent the emergence of drug resistance in *M. tuberculosis*. Furthermore, during infection, pathogens like *M. tuberculosis* have developed strategies to neutralize NADPH oxidase function and to inhibit phagosomal generation of ROS. Velmurugan *et al*. (59) identified *nuoG* gene of *M. tuberculosis*, which encodes a subunit of the NADH dehydrogenase type I, as an anti-apoptotic gene in *M. tuberculosis*. Therefore, a complete functional NDH-1 complex mediates the anti-apoptotic properties of the bacteria (59). We hypothesize that the disruption of the membrane potential triggered by the compounds, will lead to the impairment of the respiratory chain, promoting apoptosis during infection. It is not clear whether the inhibition promoted by the compounds is due to their interaction with

specific targets or is just a consequence of the dissipation of the membrane potential. We have tried to obtain mutants for these compounds in an attempt to identify their target. So far, we were unable to isolate such mutants. The reason for this can be related with the reduced mutation rates for these compounds and also with the essentiality of their putative targets as is the case of *ndh* and the phenothiazines. Further analyses are under way to explore this subject.

These compounds have been used for years as antipsychotics and antihypertensives, are affordable and, can be taken as oral formulation. Several other drugs whose mechanism of action is known to affect the mycobacterial metabolic energy and respiration are now in the pipeline. These include bedaquiline, an ATP synthase inhibitor (14; 26), and PA-824 and demalanid, which target the cytochrome oxidase (34). Although haloperidol, thioridazine and chlorpromazine are widely used for the treatment of psychosis, these compounds have significant toxic effects on mitochondrial bioenergetics function, which may result in serious tardive dyskinesia and Parkinson-like disease (7; 38) and cardiotoxicity (20). As such, caution should be taken when designing new compounds that target the respiratory chain, since that due to the impairment of the oxidative phosphorylation, mitochondria became unable to meet the energetic needs of cell. On the other hand, verapamil is shown to be extremely well tolerated with few side-effects documented (52). The significant differences observed in the effective concentrations of the ion channel blockers required to inhibit *M. tuberculosis in vitro* versus that needed to produce similar effects on macrophages (Table IV.S2), indicates an interaction that involves (i) the biochemical mechanism underlying the inhibitory effect of the drugs within the bacteria, (ii) the ability of the macrophages to concentrate the compounds (41) so they can reach therapeutic concentrations, and (iii) the consequences of the stimulation of macrophage bactericidal mechanisms. Altogether they result in the elimination of the bacteria. The activity of verapamil, thioridazine, chlorpromazine, flupenthixol, and haloperidol against drug resistant *M. tuberculosis* at concentrations usually reached in the human serum when they are employed clinically was demonstrated. The results presented in this study enable us to propose the following mechanism of action for these compounds: a) in the bacteria: after entering the cell the compounds will generate a cascade of events that starts with the inhibition of the respiratory chain complexes. If the respiratory chain is a

direct target, we cannot say at the present moment. The inhibition of the respiratory chain will lead to: i) dissipation of membrane potential; ii) reduction of ATP levels; iii) ROS generation; iv) increase in intracellular calcium levels; and v) cell death; b) on the host cell, the treatment with the inhibitors will lead to an increased transcription of the V-ATPases which results in the phagosome acidification. The phagosomal acidification will synergize with several components of the host immune response, such as lysosomal hydrolases and reactive oxygen and nitrogen species, and lead to bacterial growth restriction.

Ongoing work aims to test these hypotheses and determine the exact mechanism by which these compounds can eliminate *M. tuberculosis*. Furthermore, it will be interesting to evaluate the impact of the genotype of the strains studied on their capacity to trigger the mechanisms described above. *M. tuberculosis* strains of the Latino-American-Mediterranean (LAM) genotype, specifically the Lisboa3 and Q1 clusters, is the main genotype circulating in Lisbon (42). Our study provides evidence that strains from both clusters interact differently with the host, when compared with the other strains. Strains from Lisboa3 and Q1 clusters demonstrated enhanced fitness inside the macrophages. In conclusion, our study should provide valuable information for the improvement of known drugs and for the design and development of new ones. Such increased knowledge will allow for better epidemiological predictions and assist in the development of new therapeutic strategies to fight drug resistant tuberculosis.

6. Materials and Methods

Mycobacterial strains and growth conditions. A panel of nine *M. tuberculosis* strains were selected for this study: *M. tuberculosis* H37Rv ATCC27294^{T}, *M. tuberculosis* H37RvΔ*katG* (32) and seven drug-resistant clinical strains obtained from the culture collection of the Mycobacteriology laboratory of the Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (Table IV.1). *M. bovis* BCG Pasteur ATCC35734 expressing green fluorescent protein (BCG-GFP) was kindly provided by Prof. M. Niederweis (University of Alabama). Cultures of *M. tuberculosis* were routinely grown in MGIT media (Becton Dickinson, Diagnostic Systems, Sparks, MD, USA) supplemented with 10% OADC (oleic acid/albumin/dextrose/catalase) (Becton-Dickinson), except when indicated otherwise. Susceptibility testing, quantitative drug

susceptibility testing and minimum inhibitory concentration determinations (MIC) were conducted using the BACTEC™ MGIT™ 960 system (MGIT960).

Antibiotics and chemicals. Isoniazid, rifampicin, ofloxacin, amikacin, capreomycin, verapamil, flupenthixol, thioridazine, chlorpromazine, haloperidol, and the efflux substrate ethidium bromide (EtBr), were purchased from Sigma-Aldrich (St. Louis, MO, USA). All drugs were prepared in sterile deionized water except rifampicin and haloperidol which were prepared in dimethyl sulfoxide (DMSO). The lyophilized drugs (BACTEC™ MGIT™ 960 SIRE and PZA kits; SIRE: streptomycin, isoniazid, rifampicin and ethambutol; PZA: pyrazinamide) used in standard susceptibility testing were purchased from Becton Dickinson and the stock solutions prepared as per manufacturer's instructions.

Susceptibility testing. **(i) First and second line drug susceptibility testing.** The MGIT960 was used for first and second line drug susceptibility testing according to the manufacturer's instructions. MGIT tubes were inoculated with 0.8 ml SIRE supplement (Becton Dickinson), 0.1 ml of antibiotic at the critical concentrations (0.1 µg/ml for isoniazid, 1 µg/ml for rifampicin, 1 µg/ml for streptomycin, 5 µg/ml for ethambutol, 100 µg/ml for pyrazinamide, 1 µg/ml for amikacin, 2.5 µg/ml for capreomycin, and 1 µg/ml for ofloxacin) and 0.5 ml of the suspension of the strain. For preparation of the drug-free proportional control strain suspension was diluted 1:100 (1:10 for PZA) and 0.5 ml inoculated in the tube. The results were interpreted as follows: at the time of positivity of the proportional control (Growth units [GU] =400), the comparison between this tube and the tubes containing the drugs(s) was performed. If the GU of the tubes containing the drug were >100, they were considered to be resistant to that concentration. If the GU of the tube containing the drug was <100 they were considered susceptible (53). Growth of the cultures was monitored with the Epicenter V5.80A software equipped with the TB eXIST module.

(ii) MIC determination of antibiotics and inhibitors. MIC determination was done within MGIT960 and the growth monitored with the Epicenter V5.80A software. Verapamil and haloperidol were tested at concentrations ranging from 16 to 512 µg/ml; thioridazine, chlorpromazine and flupenthixol were tested at concentrations from 3.75 to 60 µg/ml. Isoniazid was tested a concentration ranging from 0.1 to 120 µg/ml,

rifampicin and amikacin were tested from 1 to 640 µg/ml and ofloxacin was tested from 1 to 10 µg/ml. At the time of testing, two-fold serial dilutions were prepared to achieve the desired concentrations. The MIC was considered as the lowest concentration with GU <100 when the drug-free control tube reached a GU value of 400.

(iii) Quantitative drug susceptibility testing of antibiotics in presence and absence of inhibitors.

Quantitative drug susceptibility testing of rifampicin, isoniazid, ofloxacin and amikacin was conducted using the MGIT960 and the Epicenter V5.80A/TB eXIST. Isoniazid was tested at 0.1, 0.4, 1, 3 and 10 µg/ml, rifampicin and amikacin at 1, 4 and 20 µg/ml, and ofloxacin at 1, 2 and 10 µg/ml. The interpretation of the results was performed as per Springer *et al*. (53). For the quantification of the resistance levels of each strain, the following criteria was used: isoniazid low-level resistance when resistant (R) at 0.1 and susceptible (S) at 1 µg/ml; isoniazid high-level resistance when R ≥ 1; rifampicin and amikacin low-level resistance when R at 4 and S at 20 µg/ml; rifampicin and amikacin high-level resistance when R ≥ 20 µg/ml; ofloxacin low-level resistance when R at 1 and S at 2 µg/ml; ofloxacin high-level resistance when R ≥ 2 µg/ml. The inhibitors were used at ½ of the respective MIC.

(iv) Determination of the synergistic effect of the inhibitors. The analysis of the effect of drug combination was achieved by calculating the fractional inhibitory concentration (FIC) index as follows: FIC= $MIC_{ATB\ in\ combination}/MIC_{ATB\ alone}$. The FIC was interpreted as: FIC≤0.5, synergism; >0.5-1, indifference; and >1, antagonism (29).

Time-kill kinetics. To assess the bactericidal activity of the inhibitors, time-kill studies were performed with two strains: the reference strain H37Rv (drug-susceptible) and one clinical strain, 149/09 (drug-resistant with XDR pattern – see Table IV.1). Mid-log phase cultures were diluted to 1×10^5 CFU/ml and challenged with the compounds alone or in combination with antibiotics. Strains were inoculated in 100 ml of MB7H9 (DIFCO, Madrid, Spain) containing 10% OADC enrichment, 0.05% Tween 80 and the compounds at five times the MIC. A drug-free control was included in the assay to monitor the normal growth of the strains. Cultures were sampled for CFU determination after 1, 2, 3 and 7 days of incubation at 37ºC. For CFU determination, the samples were pelleted once by centrifugation at 16060 x *g* for 10 minutes to reduce drug carry-over and resuspended in drug-free MB7H9. Colony forming units' were determined with the

MGIT960 and the Epicenter software. Briefly, to generate calibration curves, the number of viable cells was first determined by standard plate counting method. Cell suspensions were grown to an optical density at 600 nm (OD_{600}) of 0.8 and 1 ml was added to 9 ml of MB7H9. Tenfold serial dilutions (10^0 to 10^{-7}) were made in MB7H9 and 100 µl from each dilution plated onto MB7H11 plates (DIFCO) supplemented with 10% OADC. Plates were sealed and the CFU´s determined after incubation at 37ºC during 21 days. Simultaneously, MGIT tubes supplemented with 10% OADC were inoculated in triplicate with 100 µl of the same serial dilutions, incubated in the MGIT960 system and the time to detection (TTD, the time to reach the threshold of 75 GU) recorded. Calibration growth curves for each dilution were generated with the Epicenter software and the TTD for each dilution plotted against $\log_{10}$ CFU. To obtain the cell concentration for each drug exposed culture, CFU´s were predicted by the theoretical $\log_{10}$ CFU based TTD multiplied by the corresponding dilution factor. The killing effect of the compounds on *in vitro M. tuberculosis* growth was defined as the lack of growth in MGIT tubes after 100 days of incubation (16).

Quantification of intracellular ATP

Intracellular ATP was quantified by using the ATP Determination Kit (Invitrogen, Life Technologies, Paisley, UK) according to the manufacturer's instructions. Briefly, strains were exposed to the compounds and at various time points, aliquots of 1 ml of bacteria were collected, heat inactivated and immediately deep frozen. Cell lysates were transferred into white flat bottom 96-well plates and the ATP content measured using a Tecan's Infinite® M200 plate reader (Tecan Trading AG, Switzerland) and expressed as relative luminescence units. ATP standards were used in all experiments as internal controls. Sample ATP levels were calculated from the calibration curve, with correction for background luminescence. Isoniazid and rifampicin were used as controls. Cultures were sampled for CFU determination as described above.

Growth rate measurements

Cell suspensions were adjusted to 150 GU with sterile saline solution and 0.5 ml transferred into a MGIT tube containing 10% OADC. Growth rates were measured at 37°C with the MGIT960 and the Epicenter software. First, we determined the TTD as a measure of the capacity of the strain to adapt to a new environment. The relative growth

rates were determined according to Gullberg *et al*. (21) with modifications. The calculations were based on TTD values between 75 and 400 GU which corresponds to OD_{600} values between ≈0.02 and 0.1 (data not shown). The relative growth rates were determined as the ratio between the growth rate of each strain and the growth rate of the control strain. Data was normalized against the control strain *M. tuberculosis* H37Rv.

Mutation frequency determinations

M. tuberculosis H37Rv was grown in 200 ml Erlenmeyer flasks supplemented with 10% OADC and 0.05% Tween 80 and incubated at 37ºC until they reach an OD_{600} of 0.8-0.9. Cultures were diluted to 1×10^4 CFU/ml in MB7H9. Spontaneous mutants resistant to the inhibitors were detected by plating 0.1 ml of this dilution onto MB7H11 agar plates containing the compounds at concentrations five times the MIC. Plates were sealed in plastic bags and the colonies counted after three weeks of incubation at 37ºC. The mutation frequency corresponds to the ratio of the average number of mutants per milliliter to the total number of cells per milliliter (12).

Detection of catalase activity

Catalase activity was assessed according to the protocol described by David (13), at room temperature. The *M. tuberculosis* H37Rv strain served as a positive control.

Acid resistance evaluation

M. tuberculosis H37Rv was grown in 25 ml of MB7H9 containing 10% OADC and 0.05% Tween 80 until they reach an OD_{600} of 0.8. Cells were washed by centrifugation at 2940 x *g* during 3 minutes, to remove Tween 80. Washed cells were diluted to 1×10^5 CFU/ml in 100 ml of MB7H9 at different pH's ranging from 6.8 to 3.5 plus 10% OADC without Tween 80 (58). Cultures were sampled for CFU determination after 1, 2, 3 and 7 days of incubation at 37ºC. For CFU determination, samples were washed once by centrifugation at 16060 x *g* for 10 minutes and resuspended in MB7H9. Colony forming units were determined with the MGIT960 and the Epicenter software as described above.

Antimycobacterial activity on infected macrophages

i) Mycobacterial cultures. *M. tuberculosis* strains and *M. bovis* BCG-GFP were cultured in MB7H9 broth supplemented with 10% OADC and 0.05% tyloxapol (Sigma-Aldrich) at 37ºC, with occasional shaking. For BCG-GFP, 50 µg/ml hygromycin (Sigma-Aldrich) were added to the media. Prior to any experiment, bacteria were prepared in order to achieve a single cell suspension. Bacterial cultures on exponential growth phase were centrifuged at 3000 x *g* for 10 minutes, washed in phosphate buffered saline (PBS) solution and resuspended in cell culture medium without antibiotics. In order to disrupt bacterial clumps, the cell suspension was passed through a 21G needle. Residual clumps were removed by 1 minute centrifugation at 500 x *g*.

ii) Isolation and culture of human monocyte-derived macrophages. Human monocyte-derived macrophages were obtained from buffy coat preparations kindly donated by Instituto Português do Sangue. The buffy coat was diluted (1:1) in PBS containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA and then gently overlaid on Ficoll-Paque Plus (GE Healthcare, Freiburg, Germany) at a ratio of 2:1 followed by 800 x *g* centrifugation for 20 minutes at room temperature. The interface was then recovered and washed two times. Selection of CD14 monocytes was performed using MACS LS cell separation system (MiltenyiBiotec, Cologne, Germany) according to the manufacturer instructions. Briefly, cells were incubated during 30 minutes at 4°C with anti-CD14 MicroBeads (MiltenyiBiotec) and then passed through a magnetic separator. CD14 monocytes were recovered and differentiated into macrophages during 7 days in macrophage medium containing RPMI-1640 medium with 10% fetal calf serum (FCS), 1% GlutaMAX™, 1 mM sodium pyruvate, 10 mM HEPES at pH 7.4, 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco, Life Technologies), and 20 ng/ml M-CSF (Immunotools, Friesoythe, Germany) and incubated at 37ºC in 5% CO_2 atmosphere. Fresh medium was added at day 4 post isolation.

iii) Determination of macrophage viability after treatment with compounds. Human monocyte-derived macrophages were seeded $3x10^5$ cells per well in 24-well plates and treated with the compounds. After 3 days of treatment, cell viability was determined using AlamarBlue (Molecular Probes, Life Technologies) following the manufacturer's indications. Briefly, 10% AlamarBlue reagent was added to each well

and incubated for 4 hours at 37ºC and 5% CO_2. Then, fluorescence was measured at an excitation of 570 nm and emission of 595 nm in a Tecan's Infinite M200 plate spectrophotometer. For the subsequent intracellular assays, the compounds were used at concentrations that were shown to be non-toxic to the macrophages. Isoniazid was used at critical concentration of 0.1 µg/ml.

iv) Quantification of intracellular bacterial survival. Human monocyte-derived macrophages were infected with *M. tuberculosis* strains at a multiplicity of infection (MOI) 1:1 and were allowed to uptake the bacteria for 3 hours. Next, they were washed three times with PBS and maintained in macrophage medium without antibiotics. At 3 hours, and 1, 3, 5 and 7 days post-infection, cells were lysed with 0.05% Igepal (Sigma-Aldrich) solution in water. Serial dilutions of the lysate were plated on MB7H11 medium supplemented with 10% OADC. Colony forming units were counted upon 3 weeks of incubation at 37ºC. The capacity of intracellular growth of each strain was calculated as the ratio between the CFU during the 5 day protocol and the CFU on day 0 (47).

v) Quantification of compound-induced macrophage acidification. Human monocyte-derived macrophages were infected with BCG-GFP at MOI 1:10 as described above. At 24 hours post-infection, cells to be analysed by fluorescence microscopy were incubated with LysoSensor™ Yellow/Blue DND-160 (Molecular Probes) at 1 µM for 10 minutes and then washed and observed under a fluorescence microscope (Axioskop®, ZEISS, Germany). Quantification of acidified cells by flow cytometry was performed by detaching the cells with 0.05% Trypsin-EDTA (Sigma-Aldrich). After cells were washed with PBS and further incubated with 100 nM LysoTracker Red DND-99 (Molecular Probes) for 2 hours at 37ºC and 5% CO_2. Cells were washed and analyzed on an easyCyte™ 5HT flow cytometer (Millipore Corporation, Billerica, MA, USA).

vi) Measurement of cathepsin B activity. Human monocyte-derived macrophages were infected with *M. tuberculosis* H37Rv at MOI 1:1 as described above. After 24 hours post-infection cells were washed with PBS and transferred into black flat bottom 96-well plates. The cells were incubated with the compounds during 24 hours at 37ºC with 5% CO_2. Cathepsin B activity was measured using a fluorometric cathepsin B activity assay kit (BioVision, California, USA) in accordance with the manufacturer's

instructions. Briefly, cells were lysed in chilled cathepsin B cell lysis buffer and incubated on ice for 10 minutes. Cell lysates were recovered and cathepsin B substrate (Ac-RR-AFC) added to a final concentration of 200 µM. Cells were further incubated during 2 hours at 37°C. Cathepsin B activity was measured at 400 nm excitation and 505 nm emission using a Tecan's Infinite® M200 plate reader and expressed as relative fluorescence units (RFU).

Assessment of efflux activity. The detection of ethidium bromide efflux was performed by a semi-automated fluorometric method using a Rotor-Gene 3000™ thermocycler (Corbett Research, Sidney, Australia) (60) with a modified protocol for *M. tuberculosis* (32). The semi-automated fluorometric method was applied to all strains. Briefly, the strains were grown in 10 ml of MB7H9 medium containing 10% OADC enrichment (Becton Dickinson) and 0.05% Tween 80. Cultures were incubated at 37ºC, without stirring, until they reached an OD_{600} of 0.8. After the cultures reached the desired OD_{600}, cultures were centrifuged at 2940 x *g*, during 3 minutes at 25ºC. After this, the supernatant were discarded, the pellet washed, resuspended in PBS and centrifuged as before.

(i) Accumulation of ethidium bromide. For the ethidium bromide accumulation assays, the washed cells were resuspended in PBS and the OD_{600} adjusted to 0.8. In order to determine the lowest concentration of ethidium bromide that causes accumulation, 50 µl of the bacterial suspension was added to 0.2 ml PCR tubes containing different concentrations of ethidium bromide that ranged from 0.125 to 5 µg/ml and glucose at a final concentration of 0.4%. The final OD_{600} of the bacterial suspension in the assay was 0.4. The assays were conducted at 37ºC in a Rotor-Gene 3000™, and the fluorescence of ethidium bromide was measured (530/585 nm) at the end of each cycle of 60 seconds, for 60 minutes. After determining the higher concentration of ethidium bromide that do not causes accumulation, the effect of the inhibitors on the accumulation of ethidium bromide was evaluated. These assays were performed as described above with each efflux inhibitor at ½ of the MIC (a subinhibitory concentration that do not affect bacterial viability but inhibits efflux), ethidium bromide at the higher concentration that do not cause accumulation (determined for each strain), 37ºC and with and without glucose. To better evaluate the effect of the inhibitors in the accumulation of ethidium bromide, for each assay we

determined the relative final fluorescence (RFF) at the last time point (minute 60) of the assay in comparison with the control condition using the formula RFF = ($RF_{treated}$-$RF_{non\text{-}treated}$)/($RF_{non\text{-}treated}$) (33). Here, $RF_{treated}$ corresponds to the relative fluorescence for each strain treated with the compound at the last time point of the accumulation assay and the $RF_{non\text{-}treated}$ corresponds to the relative fluorescence of the cells only in the presence of ethidium bromide at the same time point. High RFF values indicated that cells accumulate more ethidium bromide under the condition used than those of the control (non-treated cells). Negative RFF values indicated that treated cells accumulated less ethidium bromide than those of the control condition. Each assay was performed in triplicate and the results presented correspond to the average ±SD of three independent assays.

ii) Efflux of ethidium bromide. For the efflux assays, the washed cells were resuspended in PBS and the OD_{600} adjusted to 0.4. The ethidium bromide loaded cells were prepared by incubating the bacterial suspension, in glass test tubes, with the higher concentration of ethidium bromide that do not cause accumulation (determined above), under conditions that promote accumulation: the most effective efflux inhibitor at ½ of the MIC, absence of glucose at 25ºC during 60 minutes. For all strains tested in this work, the most effective inhibitor was verapamil. After ethidium bromide accumulation, the cells were collected by centrifugation at 4860 x *g* during 5 minutes at 25ºC and resuspended in PBS to an OD_{600} of 0.8. After adjusting de OD_{600}, 50 µl of the bacterial suspension was added to 0.2 ml PCR tubes containing (1) PBS only; (2) PBS plus glucose, to a final concentration of 0.4%; (3) verapamil at ½ of MIC only; and (4) glucose to a final concentration of 0.4% plus verapamil at ½ of MIC. The final OD_{600} of the bacterial suspension in the assay was 0.4. The fluorescence of ethidium bromide was measured, as described above. The fluorescence was acquired first, at the end of 15 seconds and at the end of every 30 seconds during the following 30 minutes. The efflux activity was quantified by comparing the fluorescence data obtained for the cells under conditions that allow maximum efflux (incubation at 37ºC in the presence of glucose and absence of a compound) against the data from the control tube that contains the ethidium bromide-load cells under conditions that inhibit the efflux (with a compound and absence of glucose).

Efflux pump gene expression. **(i) RNA extraction.** Total RNA was isolated from the cells using a GTC/Trizol based method. Briefly, cultures were centrifuged at 2940 x *g* during 16 minutes. Then, supernatant was removed leaving about 500 µl above the pellet. Five volumes of a 5M GTC solution were added to each culture and incubated 5 minutes at room temperature. The mixture was then centrifuged at 2940 x *g* for 30 minutes and the supernatant was discarded. The pellet was resuspended in 1 ml of TRI reagent (Sigma). The suspension was transferred to lysis tubes containing glass beads (QIAGEN, GmbH, Hilden, Germany) and sonicated at 35 kHz (Gen-Probe, California, USA) during 3 x 5 minutes with 1 minute of cooling on ice between intervals. The disrupted cells were incubated at room temperature during 10 minutes and centrifuged at 16060 x *g* for 45 seconds. Supernatant above the beads and cellular debris were transferred to a tube containing 300 µl of chloroform: isoamyl alcohol (24:1). Afterwards, the mixture was incubated during 5 minutes at room temperature followed by centrifugation at 16060 x *g* for 10 minutes. The top aqueous layer was then transferred to a clean tube containing an equal volume of isopropanol and the mixture incubated overnight at -20°C. Precipitated nucleic acids were recovered by centrifugation at 18000 x *g* for 30 minutes at 4ºC and RNA pellet washed twice with 1 ml ice cold 70% ethanol. RNA was dissolved in RNase-free water and contaminating chromosomal DNA was digested with RNase-free DNase I (QIAGEN) at room temperature during 30 minutes. RNA was purified with phenol: chloroform (4:1) during 10 minutes of incubation on ice and the mixture centrifuged at 18000 x *g* for 10 minutes at room temperature. The top aqueous layer was transferred to a new tube, to which 0.1 volumes of RNase-free sodium acetate pH 5.2 and 2.5 volumes of 100% RNase-free ethanol were added. Samples were incubated overnight at -20°C. After this incubation, RNA was collected by centrifugation at 18000 x *g* for 30 minutes at 4ºC, and washed twice with 1 ml ice cold 70% ethanol. The purified RNA was air-dried and then dissolved in 70 µl RNase-free water. Quantity and quality of the purified RNA was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, USA).

(ii) RT-qPCR analysis. The relative expression level of 10 efflux genes that code for membrane efflux transporters in *M. tuberculosis* and the transcriptional regulator *whiB7* (Table IV.6 and IV.7) were analysed by RT-qPCR in the strains exposed to sub-

inhibitory concentrations of isoniazid or rifampicin. The primers employed are available upon request. The RT-qPCR procedure was performed in a Rotor-Gene™ 3000 thermocycler and followed the protocol recommended for use with the QuantiTect SYBR Green RT-PCR Kit (QIAGEN). The following amplification program was used: reverse transcription for 30 minutes at 50ºC; initial activation step for 15 minutes at 95ºC; 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 52ºC for 30 seconds and extension at 72ºC for 30 seconds; a final extension step at 72ºC for 5 minutes; and an additional step at 50ºC for 15 seconds followed by melt analysis (50-99ºC). The determination of the relative mRNA expression level was performed using the comparative quantification cycle (Cq) method (28). The relative expression of the 11 genes was determined by comparison of the relative quantity of the respective mRNA in the presence of the antibiotic to the non-exposed condition. Each strain was assayed in triplicate using total RNA obtained from three independent cultures. Data was normalized to the *M. tuberculosis* 16S rDNA reference gene and presented as the mean fold change (±SD) compared with the control.

iii) RNA extraction and RT-qPCR from infected macrophages. Total RNA was isolated from control and isoniazid-treated *M. tuberculosis*-infected macrophages at day 1, 3, 5 and day 7 post-infection as described above. The RT-qPCR procedure was performed in a Rotor-Gene™ 3000 thermocycler and followed the protocol recommended for use with the QuantiTect SYBR Green RT-PCR Kit. The amplification program was as follows: reverse transcription for 30 minutes at 50ºC; initial activation step for 15 minutes at 95ºC; 45 cycles of denaturation at 94ºC for 1 minute, annealing at 52ºC for 1 minute and extension at 72ºC for 1 minute; a final extension step at 72ºC for 5 minutes; and an additional step at 50ºC for 15 seconds followed by melt analysis. Data was normalized to the *M. tuberculosis* 16S rDNA reference gene and presented as the mean fold change (±SD) compared with the control.

Genetic characterization. **(i) Detection of mutations associated with resistance.** Genomic DNA was extracted using the QIAamp DNA mini kit (QIAGEN) according to the manufacturer's instructions. The most common mutations in *katG* gene and *inhA* regulatory region were investigated using Genotype MTBDR*plus* (HainLifescience GmbH, Nehren, Germany). For the detection of the most frequent mutations in *gyrA* and *rrs* genes was used Genotype MTBDR*sl* (HainLifescience) according to the

manufacturer's instructions. Genomic analysis of the complete *inhA* gene and *eis* promoter region was performed by PCR amplification and DNA sequencing using the primers described previously (27; 39; 43). The reaction mixtures were prepared for a total reaction volume of 50 µl consisting of 1x Taq buffer (Fermentas, Ontario, Canada), 1.5 mM $MgCl_2$, 200 mM of each dNTP, 20 pmol of each primer (10 pmol for *eis*), 1.5U Taq DNA Polymerase (Fermentas), and 5 µl of chromosomal DNA. The PCR reactions were performed with the following amplification profile: initial denaturation at 94ºC for 5 minutes, followed by denaturation at 94ºC for 1 minute, annealing at 60ºC (56 ºC for *eis*) for 1 minute and extension at 72ºC for 1 minute during 40 cycles. The final extension occurred at 72ºC for 10 minutes.

(ii) Detection of mutations in drug transporters. The genes were analysed by PCR amplification and DNA sequencing to search for mutations. The primer sequences are available upon request from the authors. The reaction mixtures were prepared for a total reaction volume of 50 µl consisting of 1x Taq buffer (Fermentas), 1.5 mM $MgCl_2$, 200 mM of each dNTP, 20 pmol of each primer, 1.5U Taq DNA Polymerase (Fermentas), and 5 µl of chromosomal DNA. The PCR reactions were performed with the following amplification profile: initial denaturation at 94ºC for 5 minutes, followed by denaturation at 94ºC for 1 minute, primer dependent annealing temperature for 1 minute and extension at 72ºC for 1 minute during 40 cycles. The final extension occurred at 72ºC for 10 minutes.

(iii) Strain typing. Mycobacterial interspersed repetitive unit-variable number of tandem repeats (MIRU-VNTR) genotyping was performed for each strain by multiplex PCR amplification 24 MIRU-VNTR loci, as described by Supply *et al*. (5). The genotype of these strains was analysed using the MIRU-VNTR*plus* web application (4) and the SITVITWEB database (15) when relevant.

7. Figures

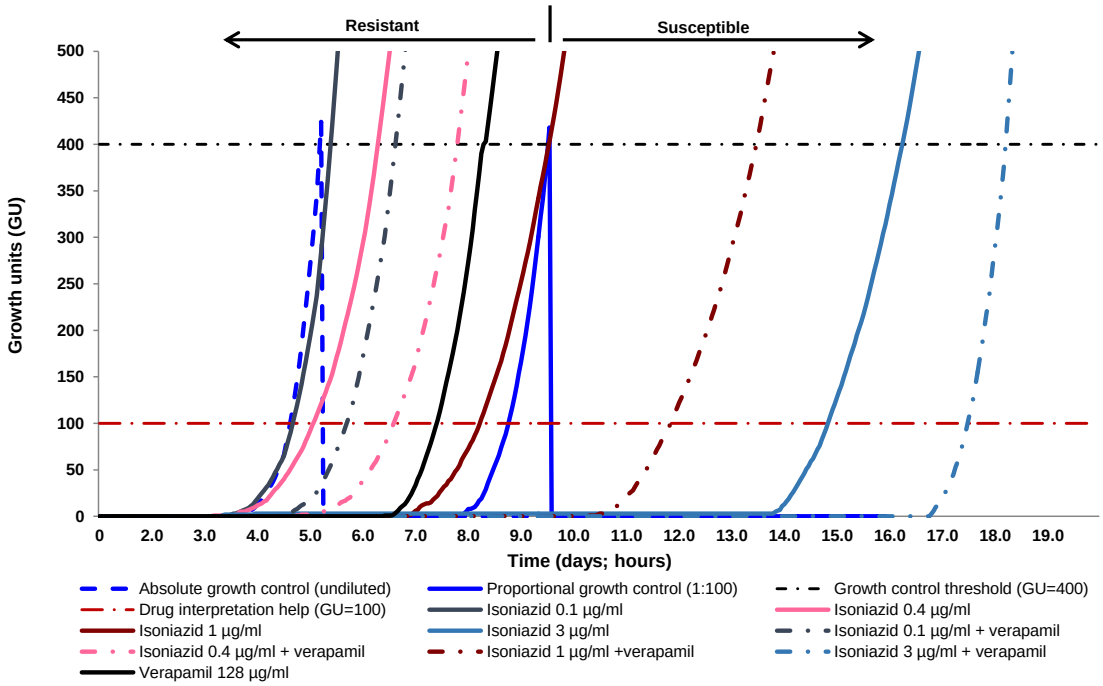

Figure IV.1. Quantitative drug susceptibility testing of isoniazid for the *M. tuberculosis* 29/12 strain, in the presence or absence of verapamil. Quantitative drug susceptibility testing of isoniazid was conducted using the BACTEC 960 system and the Epicenter V5.80A software equipped with the TB eXIST module. Full and dotted dark-blue line indicates the drug-free proportional and absolute growth control, respectively. Coloured full lines correspond to the growth curves of the strain in the presence of isoniazid; the dotted lines correspond to the growth curves with isoniazid in combination with verapamil. At the time of growth of the proportional growth control (GU=400), the comparison between this tube and the tubes containing the drugs(s) was performed. If the GU of the tubes containing the drug were >100, they were considered to be resistant to that concentration. If the GU of the tube containing the drug was <100 they were considered susceptible. Isoniazid was tested at 0.1, 0.4, 1, 3 and 10 µg/ml with and without ½ MIC verapamil.

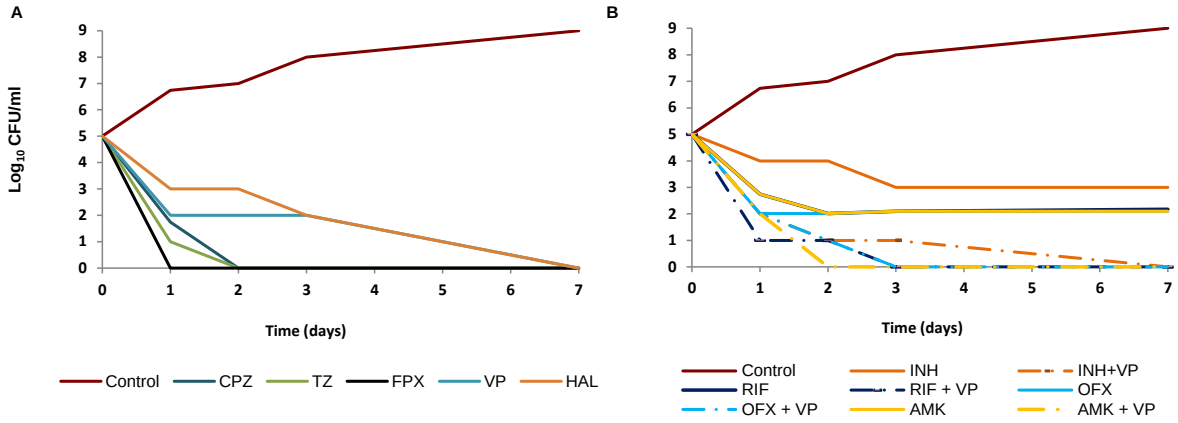

Figure IV.2. Time-kill curves for flupenthixol, chlorpromazine, thioridazine, haloperidol, verapamil and the antituberculosis drugs isoniazid, rifampicin, amikacin and ofloxacin. Compounds were used at 5X MIC and CFU/ml was determined at days 1, 2, 3 and 7. A) Killing activity of the ion channel blockers; B) Killing activity of the antibiotics in the presence or absence of verapamil. In the Figure, is represented the assay for the H37Rv strain. Data corresponds to the average of two experiments. VP, verapamil; TZ, thioridazine; CPZ, chlorpromazine; HAL, haloperidol; FPX, flupenthixol; INH, isoniazid; RIF, rifampicin; AMK, amikacin; OFX, ofloxacin.

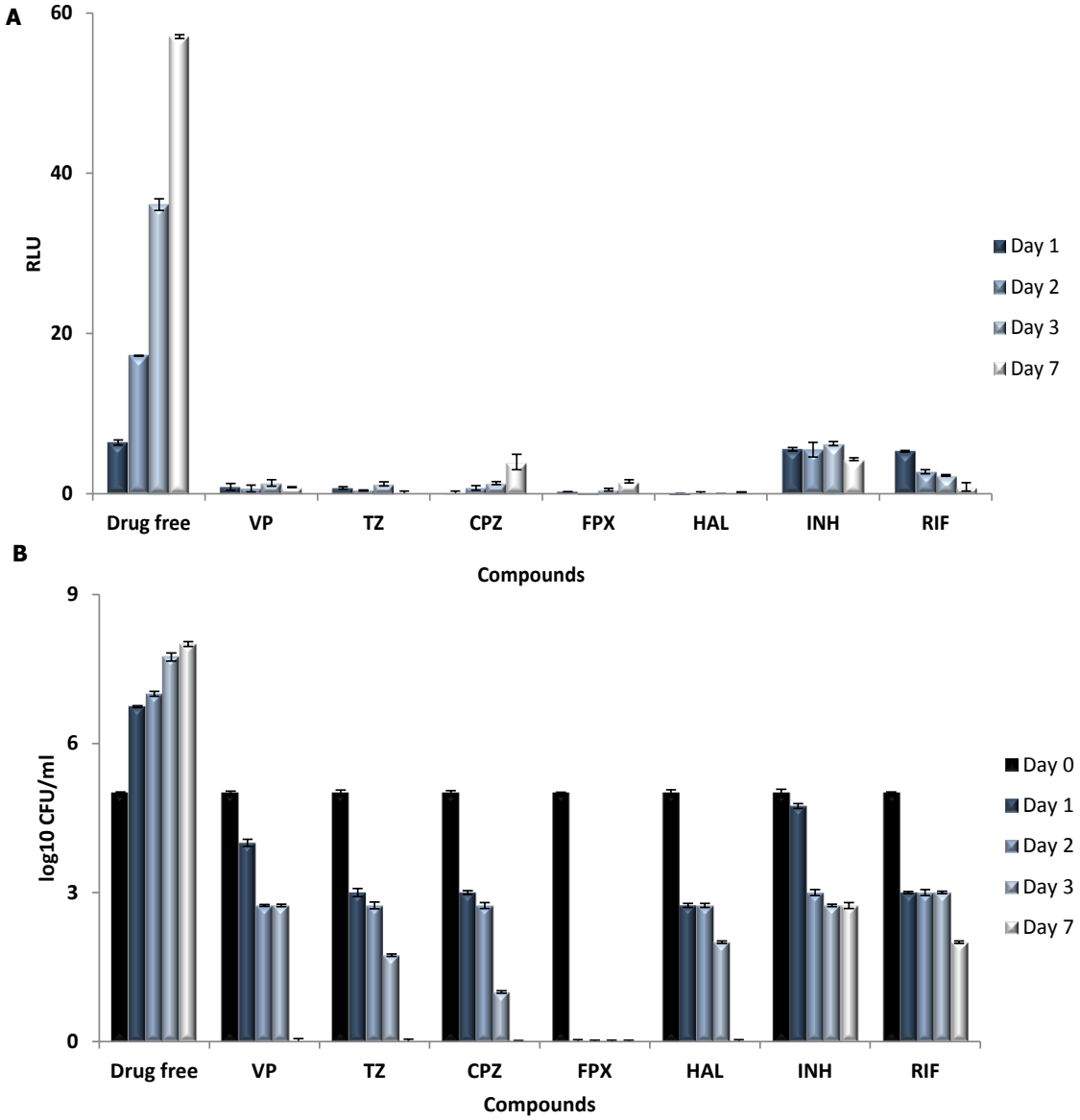

Figure IV.3. Mycobacterial intracellular ATP levels and viability. Cultures of *M. tuberculosis* H37Rv were exposed to the inhibitors during 7 days. ATP was determined by using a luciferin-luciferase bioluminescence detection system at day 1, 2, 3 and 7. Cell viability was measured in parallel with MGIT960 system as described in Material and Methods. Isoniazid (INH) and rifampicin (RIF) were used as controls. VP, verapamil; TZ, thioridazine; CPZ, chlorpromazine; FPX, flupenthixol; HAL, haloperidol. RLU, relative fluorescence units.

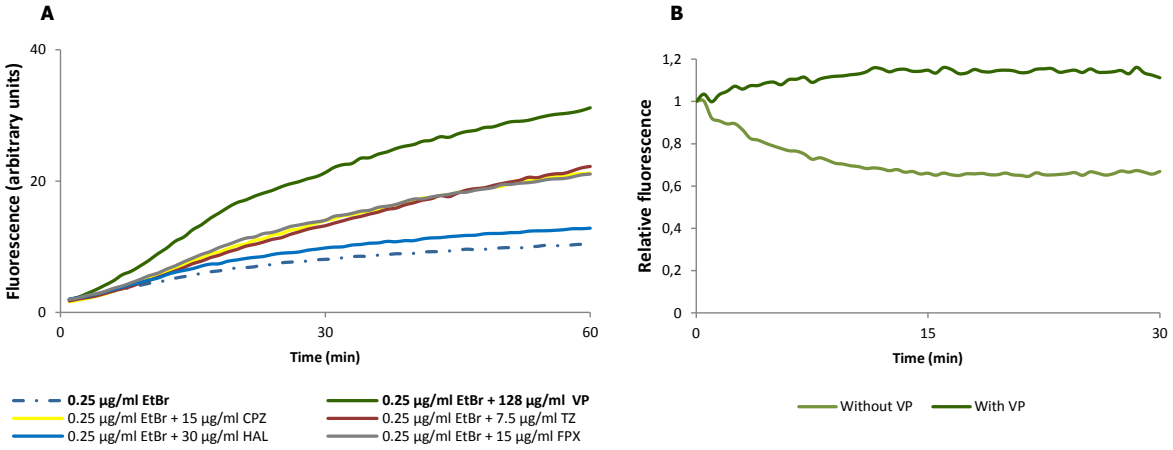

Figure IV.4. Effect of the inhibitors on the accumulation and efflux of ethidium bromide by the *M. tuberculosis* strains. In the Figure is presented an assay for the strain 29/12 as an example. A) Accumulation and B) efflux of ethidium bromide. Ethidium bromide was tested at 0.25 µg/ml. Efflux inhibitors were tested at ½ the MIC. EtBr, ethidium bromide; VP, verapamil; TZ, thioridazine; CPZ, chlorpromazine; HAL, haloperidol; FPX, flupenthixol.

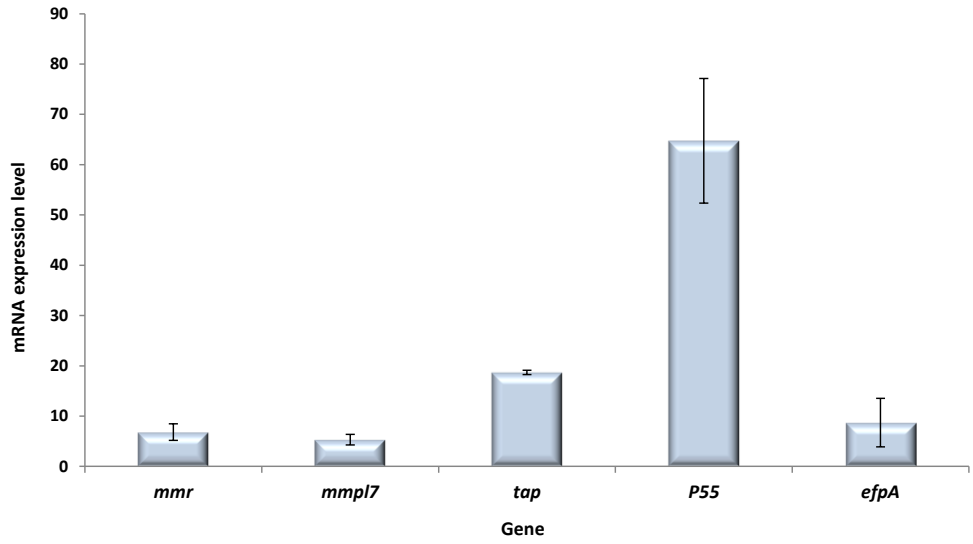

Figure IV.5. Relative expression of genes coding for efflux pumps in *M. tuberculosis* 82/09 strain during intracellular growth when exposed to isoniazid. *M. tuberculosis* infected macrophages were exposed to 0.1 µg/ml isoniazid during 3 days. Relative expression of isoniazid-treated cells was compared with untreated control cells.

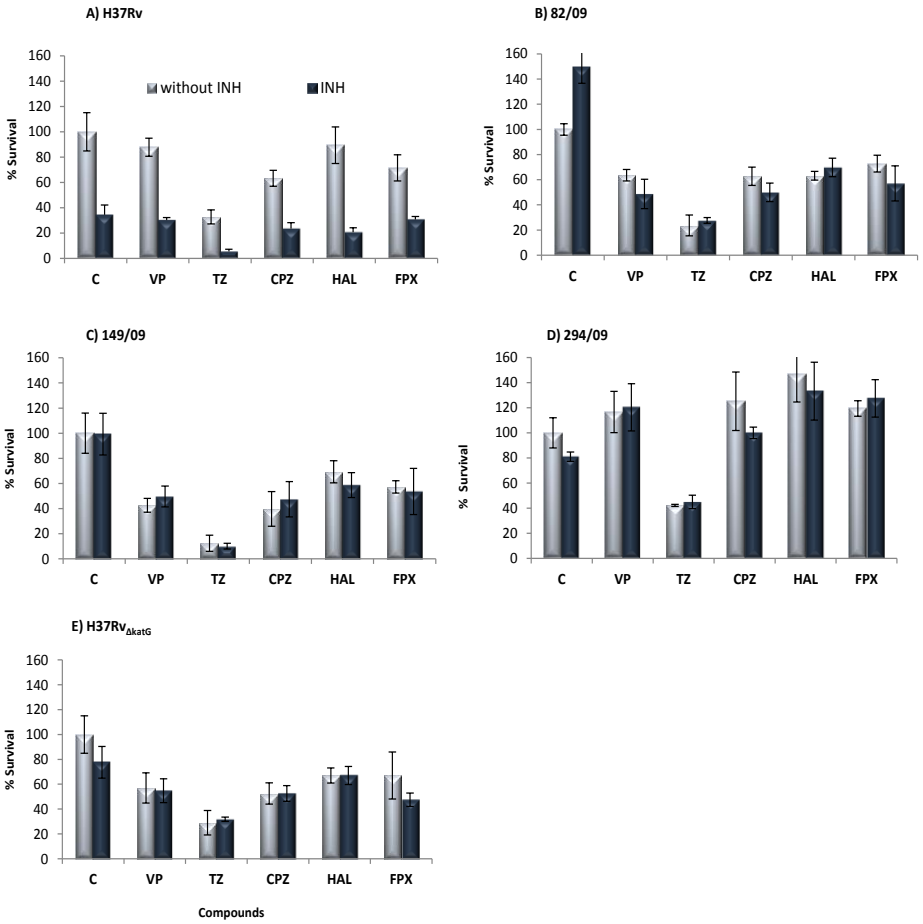

Figure IV.6. Antimycobacterial activity of the ion channel blockers on *M. tuberculosis* infected macrophages. Effect of the inhibitors on the intracellular survival of *M. tuberculosis* within human monocyte-derived macrophages, 3 days post infection. INH was tested at 0.1 µg/ml, VP, 10 µg/ml; TZ, 2.5 µg/ml; CPZ, 1.25 µg/ml; HAL, 1.25 µg/ml; FPX, 1.25 µg/ml. INH: isoniazid; VP: verapamil; TZ: thioridazine; CPZ: chlorpromazine; HAL: haloperidol; FPX: flupenthixol. The results are presented as a mean of the percentage of the survival ± SD.

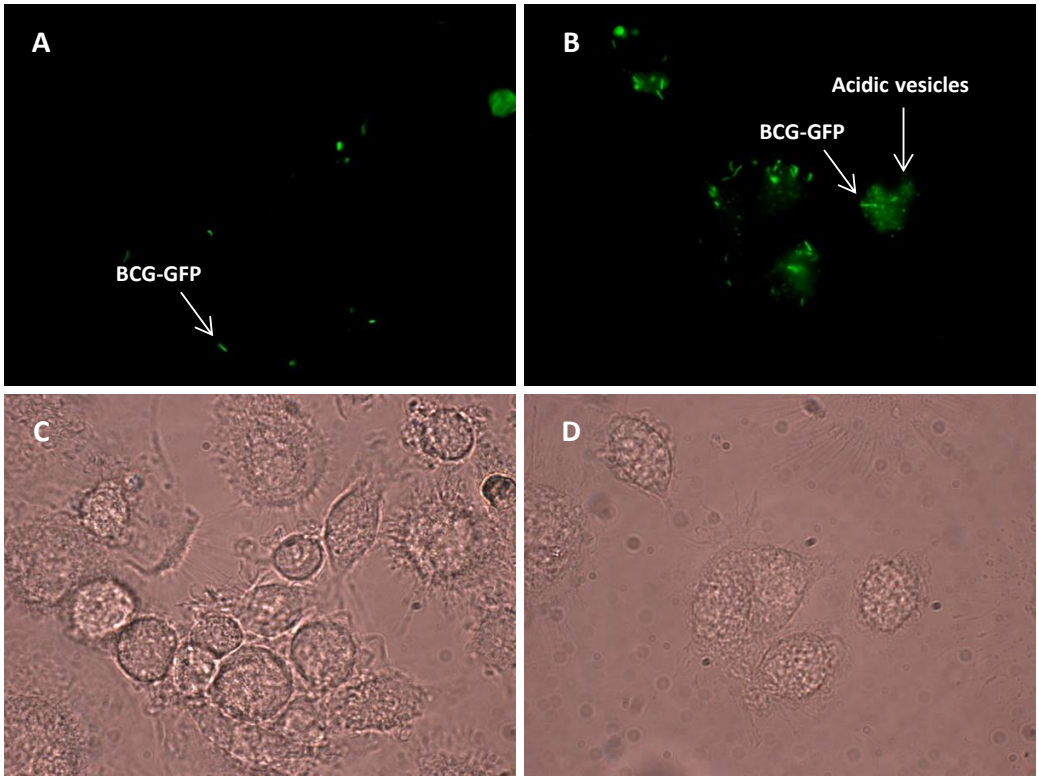

Figure IV.7. Fluorescence microscopy images of *M. bovis* BCG-GFP-infected human monocyte-derived macrophages stained with LysoSensor. A) Non-treated cells, B) cells treated with 2.5 µg/ml thioridazine; C) bright-field for non-treated cells, and D) bright-field for cells treated with 2.5 µg/ml thioridazine.

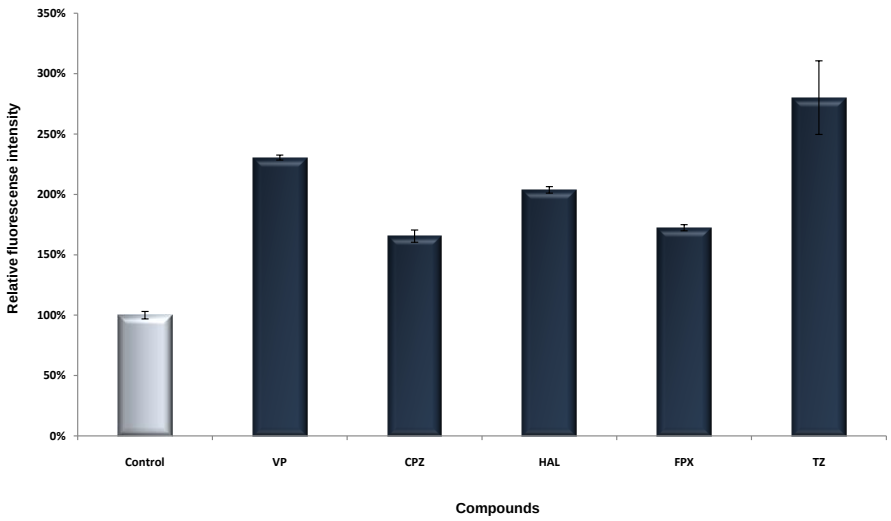

Figure IV.8. Quantification of phagolysosome acidification. Human monocyte-derived macrophages were infected with *M. bovis* BCG-GFP and treated with verapamil, thioridazine, chlorpromazine, flupenthixol, and haloperidol, stained with Lysotracker Red, and analyzed by flow cytometry. VP was tested at 10 µg/ml; TZ at 2.5 µg/ml; CPZ at 1.25 µg/ml; HAL at 1.25 µg/ml; FPX at 1.25 µg/ml. VP, verapamil; TZ, thioridazine; CPZ, chlorpromazine; HAL, haloperidol; FPX, flupenthixol.

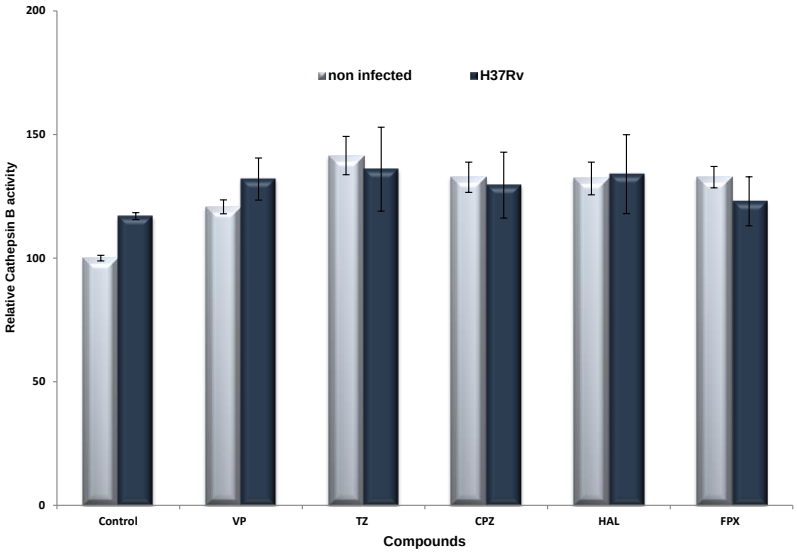

Figure IV.9. Determination of cathepsin B activity. Human monocyte-derived macrophages were treated with verapamil, thioridazine, chlorpromazine, flupenthixol, and haloperidol. Cathepsin activity was evaluated also with cultures infected with *M. tuberculosis* H37Rv and treated with the compounds. VP was tested at 10 µg/ml; TZ at 2.5 µg/ml; CPZ at 1.25 µg/ml; HAL at 1.25 µg/ml; FPX at 1.25 µg/ml. INH: isoniazid; VP: verapamil; TZ: thioridazine; CPZ: chlorpromazine; HAL: haloperidol; FPX: flupenthixol.

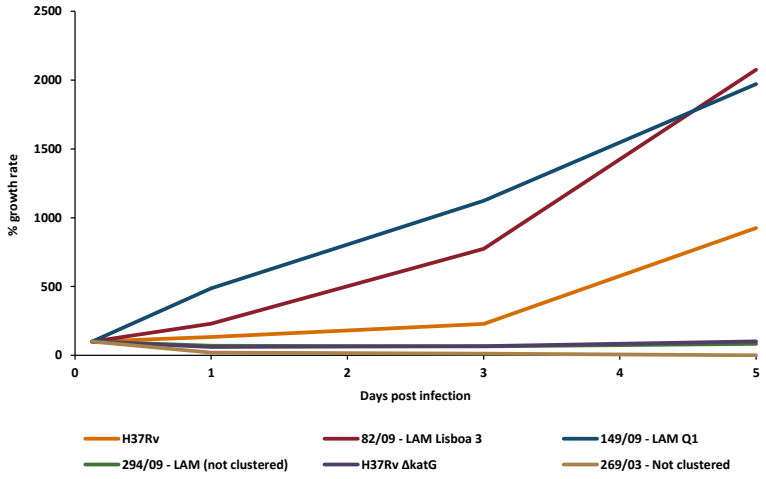

Figure IV.10. Growth rate of *M. tuberculosis* strains in human monocyte-derived macrophages during a 5 days period. CFU were determined after 3h and 1, 3 and 5 days post infection.

8. Tables

Table IV.1. Molecular characterization, resistance pattern, and MIC values of the antibiotics and inhibitors for the nine *M. tuberculosis* strains.

Strain	Genotype	Resistance pattern	Gene mutations	Antibiotics (µg/ml)				Inhibitors (µg/ml)				
				INH	RIF	AMK	OFX	VP	TZ	CPZ	FPX	HAL
H37Rv	H37Rv	Susceptible	none	0.1	1	1	1	256	15	30	30	64
H37RvΔ*katG*	H37Rv	INH	*katG* deletion	128	1	1	1	128	15	30	30	32
82/09	LAM –Lisboa3	INH; RIF - MDR	*inhA* C-15T/S94A; *rpoB* S531L	3	320	1	1	256	15	30	30	64
149/09	LAM – Q1	INH; RIF; AMK; CAP; OFX - XDR	*inhA* C-15T/I194T; *rpoB* 531L; *gyrA* D94A; *rrs* A1401G	3	320	640	10	256	15	30	30	64
286/09	LAM –Lisboa3	INH; RIF; AMK; OFX – XDR	*inhA* C-15T/S94A; *rpoB* S531L; *gyrA* S91P; *eis* G-10A	20	80	4	10	128	15	30	30	32
69/11	Beijing	INH; RIF; AMK; CAP; OFX - XDR	*katG* S315T; *rpoB* S531L; *rrs* A1401G/wt*	20	320	40	1	256	15	30	30	32
29/12	LAM – Q1	INH; RIF; AMK; CAP; OFX - XDR	*inhA* C-15T/I194T; *rpoB* S531L; *gyrA* D94A; *rrs* A1401G	3	320	640	10	256	15	30	30	64
269/03	Unclustered*	INH	*katG* S315T	10	1	1	1	256	15	30	30	64
294/09	LAM – unclustered	INH	*inhA* C-15T	0.4	1	1	1	256	15	30	30	64

Δ, deletion; MDR, multidrug resistant; XDR, extensively drug resistant; INH, isoniazid; RIF, rifampicin; AMK, amikacin; CAP, capreomycin; OFX, ofloxacin; VP, verapamil; TZ, thioridazine; CPZ, chlorpromazine; FPX, flupenthixol; HAL, haloperidol. *mixed pattern: simultaneous presence of drug susceptible and drug resistant genotype. *VNTR international type 21 (see text for details). LAM, Latino-American-Mediterranean.

Table IV.2. Fractional inhibitory concentration (FIC) values of the inhibitors when combined with isoniazid, rifampicin, amikacin or ofloxacin, against the nine *M. tuberculosis* strains.

Drug combinations	*M. tuberculosis* strains [MIC in combination(µg/ml)/FIC]								
	H37Rv	**H37Rv Δ*katG***	**82/09**	**149/09**	**286/09**	**69/11**	**29/12**	**269/03**	**294/09**
INH	0.1	128	3	3	20	20	3	10	0.4
INH + VP	0.1/1	128/1	**1/0.33**	**1/0.33**	**1/0.05**	**10/0.50**	**1/0.33**	10/1	0.4/1
INH + TZ	0.1/1	128/1	**1/0.33**	**1/0.33**	20/1	**3/0.15**	3/1	**3/0.30**	0.4/1
INH + CPZ	0.1/1	128/1	**1/0.33**	**1/0.33**	**1/0.05**	**3/0.15**	**1/0.33**	**3/0.30**	0.4/1
INH + FPX	0.1/1	128/1	**1/0.33**	**1/0.33**	**1/0.05**	**3/0.15**	**1/0.33**	**3/0.30**	0.4/1
INH + HAL	0.1/1	128/1	**1/0.33**	**1/0.33**	**1/0.05**	**10/0.50**	**1/0.33**	10/1	0.4/1
RIF	1	-	320	320	80	320	320	-	-
RIF + VP	1/1	-	**20/0.06**	**20/0.06**	**20/0.25**	**20/0.06**	**20/0.06**	-	-
RIF + TZ	1/1	-	320/1	320/1	**20/0.25**	320/1	320/1	-	-
RIF + CPZ	1/1	-	320/1	**20/0.06**	**4/0.05**	**20/0.06**	**20/0.06**	-	-
RIF + FPX	1/1	-	320/1	320/1	**4/0.05**	320/1	320/1	-	-
RIF + HAL	1/1	-	320/1	320/1	**20/0.25**	320/1	320/1	-	-
AMK	1	-	-	640	4	40	640	-	-
AMK + VP	1/1	-	-	640/1	4/1	**1/0.03**	640/1	-	-
AMK+ TZ	1/1	-	-	640/1	4/1	**1/0.03**	640/1	-	-
AMK + CPZ	1/1	-	-	**40/0.13**	4/1	**1/0.03**	**40/0.13**	-	-
AMK + FPX	1/1	-	-	640/1	4/1	**1/0.03**	640/1	-	-
AMK + HAL	1/1	-	-	640/1	4/1	**1/0.03**	640/1	-	-
OFX	1	-	-	10	10	-	10	-	-
OFX + VP	1/1	-	-	10/1	10/1	-	10/1	-	-
OFX+ TZ	1/1	-	-	10/1	10/1	-	10/1	-	-
OFX + CPZ	1/1	-	-	10/1	10/1	-	10/1	-	-
OFX + FPX	1/1	-	-	10/1	10/1	-	10/1	-	-
OFX + HAL	1/1	-	-	10/1	10/1	-	10/1	-	-

Δ, deletion; INH, isoniazid; RIF, rifampicin; AMK, amikacin, OFX, ofloxacin; VP, verapamil; TZ, thioridazine; CPZ, chlorpromazine; FPX, flupenthixol; HAL, haloperidol. FIC, fractional inhibitory concentration; MIC, minimum inhibitory concentration; The lowest concentration tested corresponds to the critical concentration for each antibiotic (See Material and Methods section for details). The FIC was interpreted as: FIC≤0.5, synergism; >0.5-1, indifference; and >1, antagonism (29).

Table IV.3. Quantitative drug susceptibility testing for isoniazid, rifampicin, amikacin and ofloxacin in the presence of inhibitors for the *M. tuberculosis* strains.

Strain	Isoniazid							Rifampicin							Amikacin							Ofloxacin						
	Conc. (µg/ml)	- ICB	+VP	+TZ	+CPZ	+FPX	+HAL	Conc. (µg/ml)	-- ICB	+VP	+TZ	+CPZ	+FPX	+HAL	Conc. (µg/ml)	- ICB	+VP	+TZ	+CPZ	+FPX	+HAL	Conc. (µg/ml)	- ICB	+VP	+TZ	+CPZ	+FPX	+HAL
H37Rv	0.1	S	S	S	S	S	S	1	S	S	S	S	S	S	1	S	S	S	S	S	S	1	S	S	S	S	S	S
	0.4	S	S	S	S	S	S	4	S	S	S	S	S	S	4	S	S	S	S	S	S	2	S	S	S	S	S	S
	1	S	S	S	S	S	S	20	S	S	S	S	S	S	20	S	S	S	S	S	S	10	S	S	S	S	S	S
	3	S	S	S	S	S	S																					
	10	S	S	S	S	S	S																					
H37Rv ΔkatG	0.1	R	R	R	R	R	R	ND							ND							ND						
	0.4	R	R	R	R	R	R																					
	1	R	R	R	R	R	R																					
	3	R	R	R	R	R	R																					
	10	R	R	R	R	R	R																					
82/09	0.1	R	R	R	R	R	R	1	R	R	R	R	R	R	ND							ND						
	0.4	R	R	R	R	R	R	4	R	R	R	R	R	R														
	1	R	S	S	S	S	S	20	R	S	R	R	R	R														
	3	S	S	S	S	S	S																					
	10	S	S	S	S	S	S																					
149/09	0.1	R	R	R	R	R	R	1	R	R	R	R	R	R	1	R	R	R	R	R	R	1	R	R	R	R	R	R
	0.4	R	R	R	R	R	R	4	R	R	R	R	R	R	4	R	R	R	R	R	R	2	R	R	R	R	R	R
	1	R	S	S	S	S	S	20	R	S	R	S	R	R	20	R	R	R	R	R	R	10	S	S	S	S	S	S
	3	S	S	S	S	S	S																					
	10	S	S	S	S	S	S																					
286/09	0.1	R	R	R	R	R	R	1	R	R	R	R	R	R	1	R	R	R	R	R	R	1	R	R	R	R	R	R
	0.4	R	R	R	R	R	R	4	R	R	R	S	S	R	4	S	S	S	S	S	S	2	R	R	R	R	R	R
	1	R	S	R	S	S	S	20	R	S	S	S	S	S	20	S	S	S	S	S	S	10	S	S	S	S	S	S
	3	R	S	R	S	S	S																					
	10	R	S	R	S	S	S																					
69/11	0.1	R	R	R	R	R	R	1	R	R	R	R	R	R	1	R	S	S	S	S	S							
	0.4	R	R	R	R	R	R	4	R	R	R	R	R	R	4	R	S	S	S	S	S							
	1	R	R	R	R	R	R	20	R	S	R	S	R	R	20	R	S	S	S	S	S	ND						
	3	R	R	S	S	S	R																					
	10	R	S	S	S	S	S																					
29/12	0.1	R	R	R	R	R	R	1	R	R	R	R	R	R	1	R	R	R	R	R	R	1	R	R	R	R	R	R
	0.4	R	R	R	R	R	R	4	R	R	R	R	R	R	4	R	R	R	R	R	R	2	R	R	R	R	R	R
	1	R	S	R	S	S	S	20	R	S	R	S	R	R	20	R	R	R	R	R	R	10	S	S	S	S	S	S
	3	S	S	S	S	S	S																					
	10	S	S	S	S	S	S																					
294/09	0.1	R	R	R	R	R	R	ND							ND							ND						
	0.4	S	S	S	S	S	S																					
	1	S	S	S	S	S	S																					
	3	S	S	S	S	S	S																					
	10	S	S	S	S	S	S																					
269/03	0.1	R	R	R	R	R	R	ND							ND							ND						
	0.4	R	R	R	R	R	R																					
	1	R	R	R	R	R	R																					
	3	R	R	S	S	S	R																					
	10	S	S	S	S	S	S																					

Δ, deletion; INH, isoniazid; RIF, rifampicin; AMK, amikacin, OFX, ofloxacin; VP, verapamil; TZ, thioridazine; CPZ, chlorpromazine; FPX, flupenthixol; HAL, haloperidol. R, resistant; S, susceptible; ICB, ion channel blocker; ND, not determined.

Table IV.4. Relative final fluorescence (RFF) based on the accumulation of ethidium bromide for the *M. tuberculosis* strains in the presence of the inhibitors.

Strain	RFF of the inhibitors				
	VP	**TZ**	**CPZ**	**FPX**	**HAL**
H37Rv	**1.58**	0.97	0.84	0.90	0.32
H37Rv$_{\Delta katG}$	0.37	0.09	0.03	0.15	-0.06
82/09	**2.17**	0.96	0.63	**1.24**	0.44
149/09	**2.31**	**1.13**	**1.16**	**1.45**	0.58
69/11	**1.19**	0.23	0.47	0.95	0.21
29/12	**2.00**	**1.14**	**1.04**	**1.03**	0.24
294/09	**1.21**	0.52	0.44	0.98	-0.18
269/03	0.73	0.17	0.07	0.35	-0.02

VP, verapamil; TZ, thioridazine; CPZ, chlorpromazine; FPX, flupenthixol; HAL, haloperidol.
Strain 286/09 was not evaluated due to poor growth under the conditions required for the accumulation assays.
Results in boldface (>1) indicate significant inhibition.

Table IV.5. Average quantification of the relative expression level, by RT-qPCR, of the genes that code for efflux pumps in the *M. tuberculosis* exposed to isoniazid.

Gene	Relative expression level ± SD						
	82/09	**149/09**	**286/09**	**29/12**	**69/11**	**294/09**	**269/03**
mmr	**6.29 ± 3.23**	**2.22 ±0.11**	0.78 ± 0.22	**1.63 ± 0.16**	0.76 ± 0.07	0.64 ± 0.03	0.74 ± 0.04
mmpl7	0.49 ± 0.38	**2.49 ± 0.49**	**2.23 ± 0.33**	**4.03** ± 2.87	0.76 ± 0.00	0.83 ± 0.24	0.72 ± 0.21
Rv1258c	0.79 ± 0.40	**1.34 ± 0.26**	0.48 ± 0.40	**2.22 ± 0.11**	0.79 ± 0.30	0.85 ± 0.12	0.64 ± 0.03
p55	**26.06 ± 2.55**	**1.65 ± 0.92**	0.67 ± 0.13	**4.04** ± 0.78	0.86 ± 0.41	**1.38 ± 0.20**	0.69 ± 0.17
whiB7	**3.38 ± 1.29**	0.19 ± 0.01	0.71 ± 0.07	0.87 ± 0.00	0.61 ± 0.15	0.67 ± 0.13	**2.55** ± 1.32
1217c	**5.94 ± 1.44**	0.08 ± 0.01	0.60 ± 0.30	**5.12** ± 1.96	0.69 ± 0.17	**1.33 ± 0.26**	0.62 ± 0.06
1218c	**1.36 ± 0.91**	0.04 ± 0.00	0.50 ± 0.00	**2.17 ± 0.42**	0.44 ± 0.08	**1.28 ± 0.06**	0.72 ± 0.13
efpA	**10.11 ± 3.82**	**1.38 ± 0.21**	0.80 ± 0.18	**1.76 ± 0.34**	**1.39** ± 0.33	0.81 ± 0.00	**1.52** ± 0.15
Rv2459	0.44 ± 0.15	**1.62 ± 0.00**	0.44 ± 0.26	**1.86 ± 0.63**	0.68 ± 0.19	0.73 ± 0.04	0.94 ± 0.09
pstB	**1.54 ±0.66**	**1.12 ± 0.16**	0.49 ± 0.24	**1.94 ± 0.28**	0.74 ± 0.11	**1.08 ± 0.21**	0.55 ± 0.11
iniA	0.26 ± 0.05	0.05 ± 0.00	**1.41 ± 0.23**	**1.53 ± 0.30**	0.46 ± 0.02	**1.00 ± 0.00**	0.38 ± 0.18

The relative expression of the 11 genes was determined by comparison of the relative quantity of the respective mRNA in the presence of isoniazid to the non-exposed condition. Each strain was assayed in triplicate using total RNA obtained from three independent cultures. Data was normalized to the *M. tuberculosis* 16S rDNA reference gene and presented as the mean fold change (±SD) compared with the control. Results in bold type were considered to be overexpression. Relative expression levels above 2 were considered significantly overexpressed.

Table IV.6. Average quantification of the relative expression level, by RT-qPCR, of the genes that code for efflux pumps in the *M. tuberculosis* exposed to rifampicin.

Gene	Relative expression level ± SD				
	82/09	**149/09**	**286/09**	**69/11**	**29/12**
mmr	**1.31 ± 0.62**	0.73 ±0.49	**2.49 ± 0.49**	**1.00 ± 0.33**	0.71 ± 0.00
mmpl7	**1.85 ± 0.87**	0.46 ±0.23	**3.23 ± 1.09**	0.39 ± 0.08	**1.02 ± 0.30**
Rv1258c	**1.40 ± 0.66**	**1.31 ± 0.44**	0.94 ± 0.18	0.20 ± 0.03	0.93 ± 0.31
p55	**4.27 ± 1.44**	**2.30 ± 0.23**	**9.88 ± 0.97**	**1.54 ± 0.66**	**2.59 ± 0.63**
whiB7	0.87 ± 0.29	**1.61 ± 0.76**	**1.04 ± 0.16**	**2.47 ± 0.24**	**1.83 ± 0.44**
1217c	0.71 ± 0.07	0.52 ± 0.07	0.98 ± 0.76	0.14 ± 0.08	**1.12 ± 0.57**
1218c	0.72 ± 0.40	0.36 ± 0.12	0.69 ± 0.10	0.51 ± 0.05	**1.44 ± 0.62**
efpA	**1.94 ± 0.09**	**1.83 ± 0.44**	**1.41 ± 0.47**	**1.81 ± 0.27**	**3.47 ± 1.17**
Rv2459	0.69 ± 0.35	0.41 ± 0.04	0.74 ± 0.04	0.28 ± 0.08	**1.08 ± 0.21**
pstB	0.41 ± 0.30	0.46 ± 0.11	0.47 ± 0.04	0.22 ± 0.01	**1.41 ± 0.47**
iniA	**1.94 ± 0.28**	0.89 ± 0.25	**54.50 ± 21.35**	**1.47 ± 0.21**	**3.11 ± 1.20**

The relative expression of the 11 genes was determined by comparison of the relative quantity of the respective mRNA in the presence of rifampicin to the non-exposed condition. Each strain was assayed in triplicate using total RNA obtained from three independent cultures. Data was normalized to the *M. tuberculosis* 16S rDNA reference gene and presented as the mean fold change (±SD) compared with the control. Results in bold type were considered to be overexpression. Relative expression levels above 2 were considered significantly overexpressed.

9. References

1. Abbate E, Vescovo M, Natiello M, Cufré M, García A, Montaner P, Ambroggi M, Ritacco V, van Soolingen D. Successful alternative treatment of extensively drug-resistant tuberculosis in Argentina with a combination of linezolid, moxifloxacin and thioridazine. J Antimicrob Chemother 2012; 67: 473-7.

2. Adams K, Takaki K, Connolly L, Wiedenhoft H, Winglee K, Humbert O, Edelstein P, Cosma C, Ramakrishnan L. Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. Cell; 2011: 145: 39-53.

3. Adams K, Szumowski J, Ramakrishnan L. Verapamil, and its metabolite norverapamil, inhibit macrophage-induced, bacterial efflux pump-mediated to multiple anti-tubercular drugs. J Infect Dis. 2014; doi: 10.1093/infdis/jiu095.

4. Allix-Béguec C, Harmsen D, Weniger T, Supply P, Niemann S. Evaluation and strategy for use of MIRU-VNTR*plus*, a multifunctional database for online analysis of genotyping data and phylogenetic identification of *Mycobacterium tuberculosis* complex isolates. J Clin Microbiol. 2008; 46: 2692-99.

5. Amaral L, Kristiansen J, Abebe L, Millet W. Inhibition of the respiration of multi-drug resistant clinical isolates of *Mycobacterium tuberculosis* by thioridazine: potential use for initial therapy of freshly diagnosed tuberculosis. J Antimicrob Chemother. 1996; 38: 1049-53.

6. Amaral L, Martins M, Viveiros M. Enhanced killing of intracellular multidrug-resistant *Mycobacterium tuberculosis* by compounds that affect the activity of efflux pumps. J Antimicrob Chemother. 2007; 59: 1237-46.

7. Balijepalli S, Boyd M, Ravindranath V. Inhibition of mitochondrial complex I by haloperidol: the role of thiol oxidation. Neuropharmacology. 1999; 38: 567-77.

8. Böttger E. The ins and outs of *Mycobacterium tuberculosis* drug susceptibility testing. Clin Microbiol Infect. 2011; 17: 1128-34.

9. Burns H, Stegelmann F, Fabri M, Döhner K, van Zandbergen G, Wagner M, Skinner M, Modlin R, Stenger S. Abelson tyrosine kinase controls phagosomal acidification required for killing of *Mycobacterium tuberculosis* in human macrophages. J Immunol. 2012; 189: 4069-78.

10. Canetti G, Froman S, Grosset J, Hauduroy P, Langerova M, Mahler H, Meissener G, Mitchison DA, Šula L. Mycobacteria: laboratory methods for testing drug sensitivity and resistance. Bull World Health Organ. 1963; 29: 565-78.

11. Da Silva P, Bigi F, Santangelo M, Romano M, Martín C, Cataldi A, Aínsa A. Characterization of P55, a multidrug efflux pump in *Mycobacterium bovis* and *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2001; 45: 800-4.

12. David H. Probability distribution of drug-resistant mutants in unselected populations of *Mycobacterium tuberculosis*. Appl Microbiol. 1970; 20: 810-4.

13. David H. 1989. Méthodes de laboratoire pour mycobacteriologie clinique. Institute Pasteur, Paris.

14. de Jonge M, Koymans L, Guillemont J, Koul A, Andries K. A computational model of the inhibition of *Mycobacterium tuberculosis* ATPase by a new drug candidate R207910. Proteins. 2007; 67: 971-80.

15. Demay C, Liens B, Burguière T, Hill V, Couvin D, Millet J, Mokrousov I, Sola C, Zozio T, Rastogi N. SITVITWEB – A publicly available international multimarker database for studying

Mycobacterium tuberculosis genetic diversity and molecular epidemiology. Infect Genet Evol. 2012; 12: 755-66.

16. Filippini P, Iona E, Piccaro G, Peyron P, Neyrolles O, Fattorini L. Activity of drug combinations against dormant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2010; 56: 2712-5.

17. Gage D, Neidhardt F. Adaptation of *Escherichia coli* to the uncoupler of oxidative phosphorylation 2,4-dinitrophenol. J Bacteriol. 1993; 175: 7105-8.

18. Gagneux S, Burgos M, DeRiemer K, Enciso A, Muñoz S, Hopewell P, Small P, Pym A. Impact of bacterial genetics on the transmission of isoniazid-resistant *Mycobacterium tuberculosis*. PloS Pathog. 2006; 2: e61.

19. Ginsberg A. Drugs in development for tuberculosis. Drugs; 2010: 70: 201-2214.

20. Glassman A, Bigger Jr T. Antipsychotic drugs: prolonged QTc interval, torsade de pointes, and sudden death. Am J Psychiatry. 2001; 158: 1774-82.

21. Gullberg E, Cao S, Berg O, Ilbäck C, Sandegren L, Hughes D, Andersson D. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog. 2011; 7: e1002158.

22. Gupta S, Salam N, Srivastava V, Singla R., Behera D, Khayyam K, Korde R, Malhotra P, Saxena R, Natarajan K. Voltage gated calcium channels negatively regulate protective immunity to *Mycobacterium tuberculosis*. PLoS One. 2009; 4: e5305.

23. Gupta S, Cohen K, Winglee K, Maiga M, Diarra B, Bishai W. Efflux inhibition with verapamil potentiates bedaquiline in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2013; 58: 574-6.

24. Gupta S, Tyagi S, Almeida D, Maiga M, Ammerman N, Bishai W. Acceleration of tuberculosis treatment by adjunctive therapy with verapamil as an efflux inhibitor. Am J Respir Crit Care Med. 2013; 188: 600-7.

25. Hofmann-Thiel S, van Ingen J, Feldmann K, Turaev L, Uzakova G, Murmusaeva G, van Soolingen D, Hoffmann H. Mechanisms of heteroresistance to isoniazid and rifampin of *Mycobacterium tuberculosis* in Tashkent, Uzbekistan. Eur Respir J. 2009; 33: 368-74.

26. Koul A, Dendouga N, Vergauwen K, Molenberghs B, Vranckx L, Willebrords R, Ristic Z, Lill H, Dorange I, Guillemont J, Bald D, Andries K. Diarylquinolines target subunit c of mycobacterial ATP synthase. Nat Chem Biol. 2007; 3: 323-4.

27. Leung E, Ho P, Yuen K, Woo W, Lam T, Kao R, Seto W, Yam W. Molecular characterization of isoniazid resistance in *Mycobacterium tuberculosis*: identification of a novel mutation in *inhA*. Antimicrob Agents Chemother 2006; 50: 1075-8.

28. Livak K, Schmittgen T. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt method. Methods. 2001; 25: 402-8.

29. Lorian V. Antibiotics in laboratory medicine, 4th ed. Williams and Wilkins, Baltimore, USA. pp 330-396. 1996.

30. Louw GE, Warren R, van Pittius N, McEvoy C, van Helden P, Victor T. A balancing act: efflux/influx in mycobacterial drug resistance. Antimicrob Agents Chemother. 2009; 53: 3181-9.

31. Louw G, Warren R, van Pittius N, Leon R, Jimenez A, Hernandez-Pando R, McEvoy C, Grobbelaar M, Murray M, van Helden P, Victor T. Rifampicin reduces susceptibility to ofloxacin in rifampicin-resistant *Mycobacterium tuberculosis* through efflux. Am J Respir Crit Care Med. 2011; 184: 269-76.

32. Machado D, Couto I, Perdigão J, Rodrigues L, Portugal I, Baptista P, Veigas B, Amaral L, Viveiros M. Contribution of efflux to the emergence of isoniazid and multidrug resistance in *Mycobacterium tuberculosis*. PLoS One. 2012; 7: e34538.

33. Machado L, Spengler G, Evaristo M, Handzlik J, Molnár J, Viveiros M, Kiec-Kononowicz K, Amaral L. Biological activity of twenty-three hydantoin derivatives on intrinsic efflux pump system of Salmonella enterica serovar Enteritidis NCTC 13349. In Vivo. 2011; 25: 769-72.

34. Manjunatha U, Boshoff H, Barry, III C. 2009. The mechanism of action of PA-824. Commun Integr Biol. 2009; 2: 215-8.

35. Martins M, Viveiros M, Amaral L. Inhibitors of Ca^{2+} and K^{+} transport enhance intracellular killing of *M. tuberculosis* by non-killing macrophages. In Vivo. 2008; 22: 69-76.

36. Miesel L, Weisbrod T, Marcinkeviciene J, Bittman R, Jacobs Jr W. NADH dehydrogenase defects confer isoniazid resistance and conditional lethality in *Mycobacterium smegmatis*. J Bacteriol. 1998; 180: 2459-67.

37. Mitchison D, Davies G. The chemotherapy of tuberculosis: past, present and future. Int J Tuberc Lung Dis. 2012; 16: 724-32.

38. Modica-Napolitano J, Lagace C, Brennan W, Aprille J. Differential effects of typical and atypical neuroleptics on mitochondrial function *in vitro*. Arch Pharm Res. 2003; 26: 951-9.

39. Morlock G, Metchock B, Sikes D. *ethA*, *inhA*, and *katG* loci of ethionamide-resistant clinical *Mycobacterium tuberculosis* isolates. Antimicrob Agents Chemother. 2003; 47: 3799-805.

40. Napier R, Rafi W, Cheruvu M, Powell K, Zaunbrecher M, Bornmann W, Salgame P, Shinnick T, Kalman D. Imatinib-sensitive tyrosine kinases regulate mycobacterial pathogenesis and represent therapeutic targets against tuberculosis. Cell. 2011; 10: 475-85.

41. Ordway D, Viveiros M, Leandro C, Bettencourt R, Almeida J, Martins M, Kristiansen J, Molnar J, Amaral L. Clinical concentrations of thioridazine kill intracellular multidrug-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2003; 47: 917-22.

42. Perdigão J, Macedo R, João I, Fernandes E, Brum L, Portugal I. Multidrug-resistant tuberculosis in Lisbon, Portugal: a molecular epidemiological perspective. Microbial Drug Resistance. 2008; 14: 133-43.

43. Perdigão J, Macedo R, Silva C, Machado D, Couto I, Viveiros M, Jordão L, Portugal I. From multidrug-resistant to extensively drug-resistant tuberculosis in Lisbon, Portugal: the stepwise mode of resistance acquisition. J Antimicrob Chemother. 2013; 68: 23-33.

44. Pym A, Saint-Joanis B, Cole S. Effect of *katG* mutations on the virulence of *Mycobacterium tuberculosis* and the implication for transmission in humans. Antimicrob Agents Chemother. 2002; 70: 4955-60.

45. Rao S, Alonso S, Rand L, Dick T, Pethe K. The protonmotive force is required for maintaining ATP homeostasis and viability of hypoxia, nonreplicating *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA. 2008; 105: 11945-50.

46. Raymond R, Millhorn D. Regulation of tyrosine hydroxylase gene expression during hypoxia: Role of Ca^{2+} and PKC. Kidney Int. 1997; 51: 536-41.

47. 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: 2277-80.

48. Rivers E, Mancera R. New anti-tuberculosis drugs in clinical trials with novel mechanisms of action. Drug Discov Today. 2008; 13: 1090-98.

49. Rodrigues L, Machado D, Couto I, Amaral L, Viveiros M. Contribution of efflux activity to isoniazid resistance in the *Mycobacterium tuberculosis* complex. Infect Genet Evol. 2011; 12: 695-700.

50. Schurig-Briccio L, Yano T, Rubin H, Gennis R. Characterization of the type 2 NADH:menaquinone oxidoreductases from *Staphylococcus aureus* and the bactericidal action of phenothiazines. Biochim Biophys Acta. 2014; doi.org/10.1016/j.bbabio.2014.03.017.

51. Sherman D, Mduli K, Hickey M, Arain T, Morris S, Barry III C, Stover C. Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. Science. 1996; 272: 1641-3.

52. Singh B, Ellrodt G, Peter C. Verapamil: a review of its pharmacological properties and therapeutic use. Drugs. 1978; 15: 169-97.

53. Springer B, Lucke K, Calligaris-Maibach R, Ritter C, Böttger E. Quantitative drug susceptibility testing of *Mycobacterium tuberculosis* using MGIT960 and the EpiCenter instrumentation. J Clin Microbiol. 2009; 47: 1773-80.

54. Sturgill-Koszycki S, Schaible U, Russel D. Mycobacterium-containing phagosomes are accessible to early endosomes and reflect a transitional state in normal phagosome biogenesis. EMBO J. 1996; 15: 6960-8.

55. Sun-Wada G, Tabata H, Kawamura N, Aoyama M, Wada Y. Direct recruitment of H^+-ATPase from lysosomes for phagosomal acidification. J Cell Sci. 2009; 122: 2504-13.

56. Supply P, Allix-Béguec C, Lesjean S, Cardoso-Oelemann M, Rüsch-Gerdes S, Willery E, Savine E, de Haas P, van Deutekom H, Roring S, Bifani P, Kurepina N, Kreiswirth B, Sola C, Rastogi N, Vatin V, Gutierrez M, Fauville M, Niemann S, Skuce R, Kremer K, Locht C, van Soolingen D. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol. 2006; 44: 4498-510.

57. van Soolingen D, Hernandez-Pando R, Orozco H, Aguilar D, Magis-Escurra C, Amaral L, van Ingen J, Boeree M. The antipsychotic thioridazine shows promising therapeutic activity in a mouse model of multidrug-resistant tuberculosis. PLoS One. 2010; 5: e12640.

58. Vandal O, Pierini L, Schnappinger D, Nathan C, Ehrt S. A membrane protein preserves intrabacterial pH in intraphagossomal *Mycobacterium tuberculosis*. Nat Med. 2008; 14: 849-54.

59. Velmurugan K, Chen B, Miller J, Azogue S, Gurses S, Hsu T, Glickman M, Jacobs Jr W, Porcelli S, Briken V. *Mycobacterium tuberculosis nuoG* is a virulence gene that inhibits apoptosis of infected host cells. PLoS Pathog. 2007; 3: e110.

60. Viveiros M, Rodrigues L, Martins M, Couto I, Spengler G, Martins A, Amaral L. Evaluation of efflux activity of bacteria by a semi-automated fluorometric system. Methods Mol Biol. 2010; 642: 159-72.

61. Viveiros M, Martins M, Rodrigues L, Machado D, Couto I, Ainsa J, Amaral L. Inhibitors of mycobacterial efflux pumps as potential boosters for anti-tubercular drugs. Expert Rev Anti Infect Ther. 2012; 10: 983-98.

62. Warman A, Rito T, Fisher N, Moss D, Berry N, O´Neill P, Ward S, Giagini G. Antitubercular pharmacodynamics of phenothiazines. J Antimicrob Chemother. 2013; 68: 869-80.

63. Weinstein E, Yano T, Li L, Avarbock D, Avarbock A, Helm D, McColm A, Duncan K, Lonsdale J, Rubin H. Inhibitors of type II NADH: menaquinone oxidoreductase represent a class of antitubercular drugs. Proc Natl Acad Sci USA. 2005; 122: 4548-53.

64. Welin A, Raffetseder J, Eklund D, Stendahl O, Lerm M. Importance of phagosomal functionality for growth restriction of *Mycobacterium tuberculosis* in primary human macrophages. J Innate Immun. 2011; 3: 508-18.

65. World Health Organization. Anti-tuberculosis drug resistance in the world. Fourth Global Report. The WHO/IUATLD Global Project on Anti-tuberculosis Drug Resistance Surveillance. 2002–2007. WHO/HTM/TB/2008.394. Geneva, Switzerland, World Health Organization. 2008.

66. World Health Organization. The use of bedaquiline in the treatment of multidrug-resistant tuberculosis: interim policy guidance. WHO/HTM/TB/2013.6. Geneva, Switzerland, World Health Organization. 2013.

67. World Health Organization. Global tuberculosis report 2013. WHO/HTM/TB/2013.11. Geneva, Switzerland, World Health Organization. 2013.

68. Yano T, Li L, Weinstein E, Teh J, Rubin H. Steady-state kinetics and inhibitory action of antitubercular phenothiazines on *Mycobacterium tuberculosis* type-II NADH-menaquinone oxidoreductase (NDH-2). J Biol Chem. 2006; 281: 11456-63.

10. Supplementary Figures

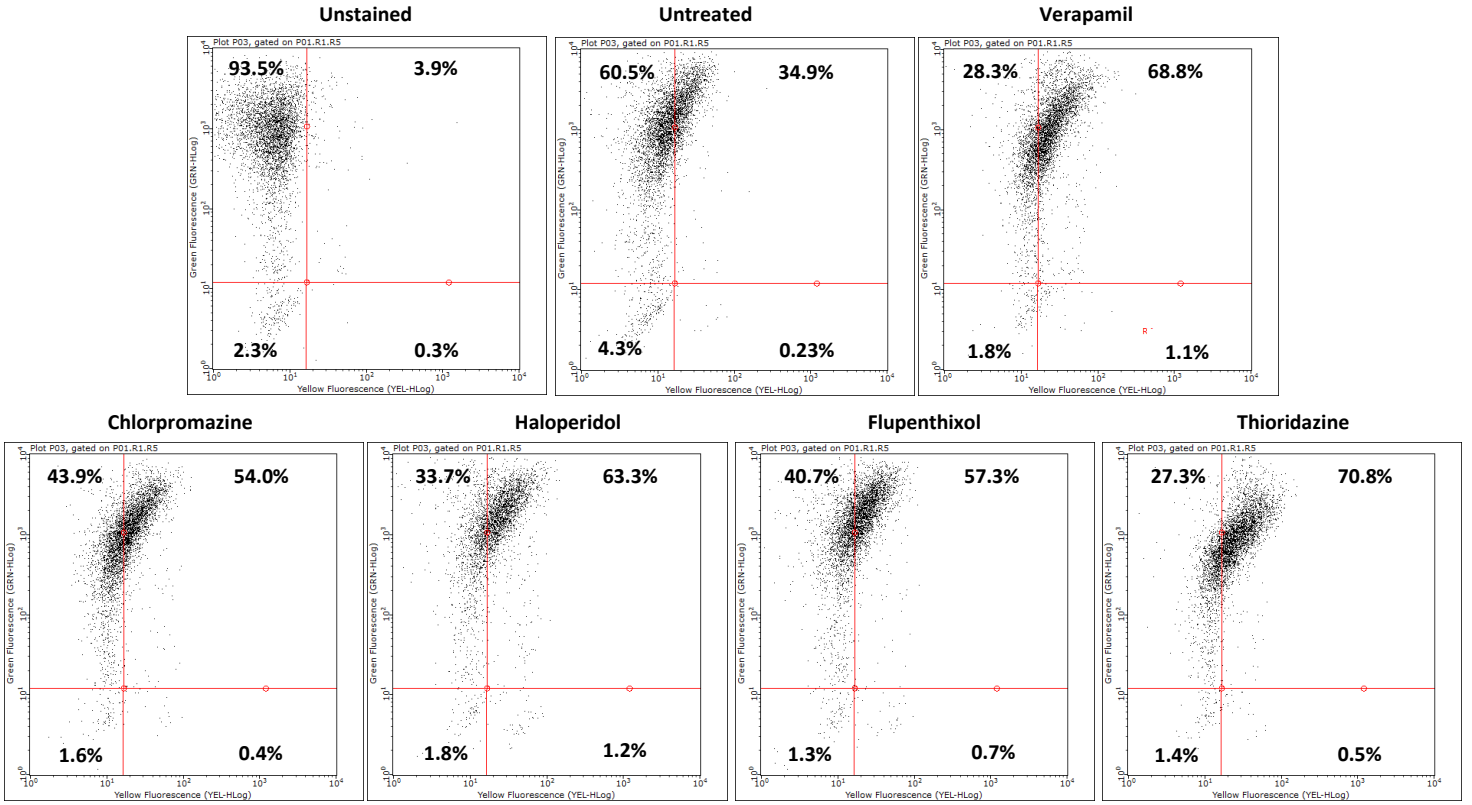

Figure IV.S1. Quantification of acidification in treated human macrophages by flow cytometry. Human monocyte-derived macrophages were infected *M. bovis* BCG-GFP, and treated with verapamil, thioridazine, chlorpromazine, flupenthixol, haloperidol, stained with Lysotracker Red, and analyzed by flow cytometry. VP was tested at 10 µg/ml; TZ at 2.5 µg/ml; CPZ at 1.25 µg/ml; HAL at 1.25 µg/ml; FPX at 1.25 µg/ml. INH: isoniazid; VP: verapamil; TZ: thioridazine; CPZ: chlorpromazine; HAL: haloperidol; FPX: flupenthixol.

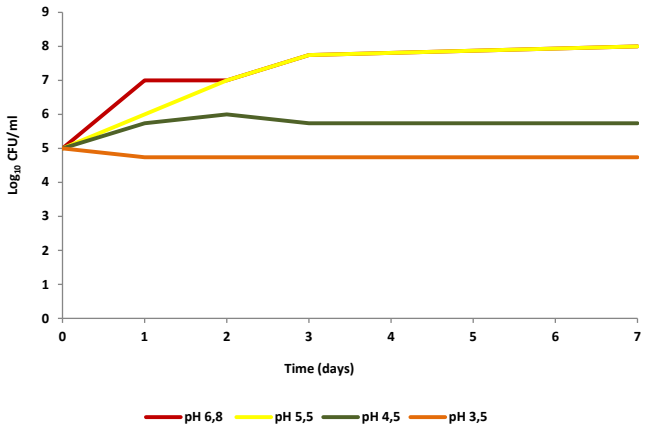

Figure IV.S2. Effect of pH on survival of *M. tuberculosis* H37Rv. Cells, at a concentration of 10^5, were incubated in MB7H9, without Tween 80, at pHs from 6.8 to 3.5 during 7 days. CFU was determined with BACTEC MGIT 960 as described in Material and Methods section.

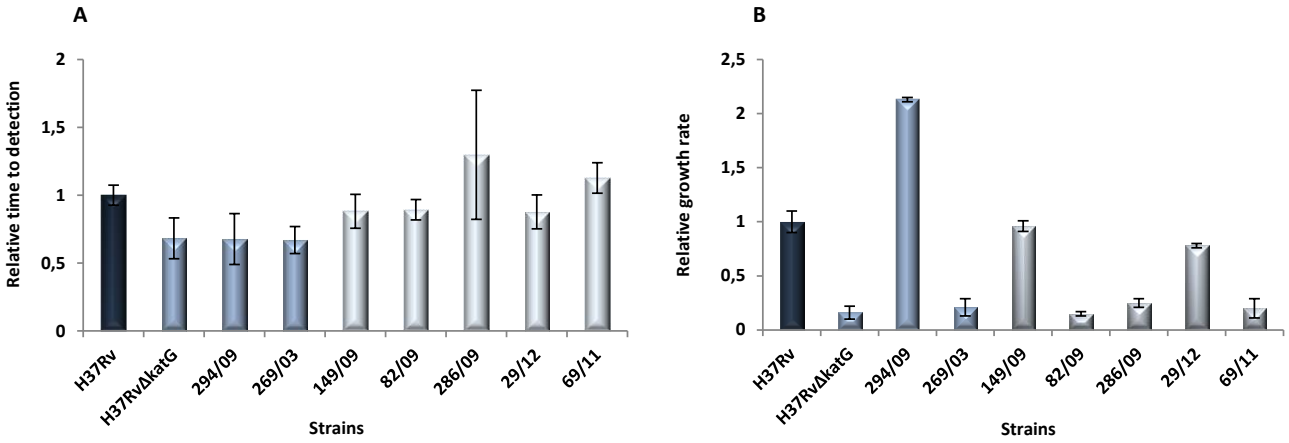

Figure IV.S3. *In vitro* determination of the fitness of the *M. tuberculosis* strains. A) Comparison between the capacities of the strains to adapt to a new environment measured as the time to reach the positivity threshold of 75 GU; B) Relative growth rates determined as the ratio between the growth rate of each strain and the growth rate of the control strain. The growth rates which were based on the time to detection between 75 and 400 GU.

11. Supplementary Tables

Table IV.S1. Genetic characterization of the efflux transporters studied.

Gene	Strain						
	82/09	**149/09**	**286/09**	**69/11**	**29/12**	**269/03**	**294/09**
iniA	ΔGCG after nt 285	wt	ΔGCG after nt 285	wt	wt	wt	wt
Rv1258c	wt	wt	wt	C194del	wt	wt	wt
p55	wt	Q506	Q506	Q506	Q506	wt	Q506
Rv1217c	wt	A531	A531	A173T/S204/A531	A531	wt	wt
Rv1218c	wt	wt	wt	Q243R	wt	wt	wt
pstB	T61M	T61M	T61M	wt	T61M	wt	T61M

Wt, wild type; Δ, deletion

Table VI.S2. Concentrations required to inhibit *M. tuberculosis in vitro* versus that needed to produce similar effects on macrophages.

Compound	Concentration (μM)	
	in vitro	***ex vivo***
Verapamil	260	20.4
Thioridazine	18.4	6.1
Chlorpromazine	42.2	3.5
Flupenthixol	29.6	2.5
Haloperidol	79.8	3.3

CHAPTER V

Chapter V. General discussion and future perspectives

"Every wild strain of tubercle bacilli contains some mutants resistant to antibacterial drugs. The difference between a resistant strain and a susceptible strain is that the proportion of resistant bacteria among the total number of bacteria making up the strain is much higher in a resistant strain than in a susceptible one."
Canetti *et al*. (7)

Despite several efforts to control the disease, tuberculosis remains the world's leading cause of death due to a single bacterial infectious agent. The emergence of multi- and extensively drug-resistant tuberculosis suggests that *M. tuberculosis* has been adapting and evolved towards drug resistance. Drug resistance in *M. tuberculosis* is essentially a manmade phenomenon and occurs when resistant mutants that are naturally present in the mycobacterial population are selected as a consequence of irregular drug supply, inadequate drug prescription, and/or non-adherence to the treatment. At the site of the infection, *M. tuberculosis* bacterial burden can be up to 10^8-10^9 cells and the mutations rates for isoniazid and rifampicin are approximately 10^{-8} and 10^{-10}, respectively (8). Since the chromosomal loci responsible for the resistance to the various antibiotics are not linked, the probability of the development of double mutations is extremely reduced. Over the last two decades, numerous studies have shown that *M. tuberculosis* drug resistance emerges through the sequential acquisition of chromosomal mutations in the genes that encode for the drug targets (11; 20; 29). In this work (**Chapter II**) we have identified a panel of drug resistance markers among the multidrug resistant *M. tuberculosis* strains circulating in Lisbon. The results show that there is a strong correlation between the development of gene mutations in the drug targets and the resistance phenotype of a strain, and that the chromosomal alterations associated with resistance are highly conserved (**Chapter IIA**). Moreover, the application of quantitative drug susceptibility testing (6; 24) allowed us to determine the levels of resistance for each strain and its correlation with the mutations associated with drug resistance. It was known that, in *M. tuberculosis* clinical strains, different levels of phenotypic drug resistance can be found, from low, intermediate to high-level drug resistance (11). We know now that these different levels of resistance are associated with distinct genetic profiles with a high degree of association (6). Therefore, mutations

in *rpoB* (rifampicin and rifabutin), *inhA* (isoniazid and ethionamide), *ethA* (ethionamide), *rpsL* (streptomycin), and *rrs* (amikacin) were associated with high-level resistance, and mutations in *embB* (ethambutol), *gidB* (streptomycin), *tlyA* and *rrs* (capreomycin), *eis* (amikacin), and *gyrA* (quinolones) confer only low-level resistance. The standard drug susceptibility testing fails to detect the clinically relevant levels of drug resistance. From the clinical point of view, the report of resistance to a given antibiotic will have the same consequence whether it is low- or high-level resistance- the antibiotic is excluded from the therapeutic regimen. However, low-level drug resistance does not always correspond to clinical resistance, whereas for a high-level resistance strain, the antibiotic will be useless. Therefore, in the presence of a low-level resistant strain it might be possible to attain a good clinical outcome if a high dose of the antibiotic is implemented. Susceptibility testing based on the critical concentration of a drug is unquestionably appropriate for screening of drug resistance, however, when in the presence of a drug resistant strain this information should be complemented by quantitative drug susceptibility testing (3). In support of this rational we demonstrated that the use of high dose isoniazid for the therapy of multidrug drug resistant strains circulating in Lisbon will not improve the clinical outcome of the patient due to the presence of double mutations in *inhA* that confer high-level resistance (**Chapter IIB**). The prompt identification of patients infected with a drug resistant *M. tuberculosis* strain is thus of major importance in order to prevent the transmission of drug resistant tuberculosis. We applied two molecular methods for the detection of mutations associated with resistance to first- and second-line antituberculosis drugs. These assays proved to be reliable methods for predicting multidrug resistance and high-level resistance. Nevertheless, the results showed that low-level resistance will remain undetected by these methods. Therefore, these findings indicate that molecular methods for the diagnosis of drug resistance cannot replace the phenotypic drug susceptibility testing. Finally, by MIRU-VNTR typing we detected two related genetic clusters in circulation, Lisboa3 and Q1, as previously demonstrated by Perdigão and colleagues (15; 16; 17; 18). The analysis of the distribution of the mutational pattern by genetic cluster showed that cluster Lisboa3 can be characterized by five gene mutations, *inhA* S94A, *rpsL* K43R, *tlyA* GT 755/766, *eis* G-10A, and *gyrA* S91P; and cluster Q1 by six gene mutations *inhA* I194T, *embB* M306V, *pncA* V125G, *gidB* A80P, *rrs* A1401G, and

gyrA D94A. GidB A80P was previously described as a surrogate marker for Q1 isolates with strong association with multi- and extensively drug resistance (19). The identification of the mutational pattern of the *M. tuberculosis* strains circulating in Lisbon will permit the application of more suitable methods for the detection and quantification of drug resistance, clinical management and therapeutic selection of these patients. From the above described laboratory and basic scientific findings it's easy to perceive how difficult is, for the clinicians, to manage these patients, not only in Portugal and in Europe but worldwide (12; 14).

In order to prevent the emergence of drug resistance it is necessary to understand the chain of events that explains how *M. tuberculosis* develops drug resistance. In **Chapter III**, we focused our attention on the understanding of the chain of events leading to the development of resistance in *M. tuberculosis*, with particular emphasis on the differential activity of genes that code for the main *M. tuberculosis* efflux pumps prior to, during and, after the establishment of the resistance phenotype and their interaction with the genetically based resistance. From the results obtained in this study we propose the following hypothesis for the emergence of drug resistance in a susceptible strain: the addition of isoniazid at therapeutic effective concentrations will activate KatG leading to the ineffectiveness of InhA with consequent inhibition of biosynthesis of mycolic acids and cell death (25) (Figure V.1.I). However, the addition of isoniazid at subtherapeutic concentrations will select the drug resistant subpopulation, the so-called persisters (28). After the exposure to the antibiotic, the activity of efflux pumps will increase in response to antibiotic stress as previously demonstrated by our group (26). This increased activity results in the reduction of intracellular levels of the antibiotic, which may enable the survival of the bacterial subpopulation under constant stress promoted by a sub-lethal level of the antibiotic. During this period, mutants with alterations in the genes that favour resistance can be selected, therefore insuring the establishment of an antibiotic resistant population presenting high-level resistance that is clinically significant (Figure V.1.II). This induced resistance against the antibiotic can be reduced by the use of efflux inhibitors (Figure V.1.III). In this chapter, we further described a panel of five genes that encode for efflux pumps associated with isoniazid resistance in *M. tuberculosis*: *mmr*, *p55*, *mmpL7*, *Rv1258c*, *Rv2459* and *efpA*. These results points toward efflux pump gene expression as an underlying mechanism of

isoniazid resistance. Although we detected a general and marked increase of efflux pumps genes, we also noted the absence of a clearly defined pattern of specific gene expression in response to isoniazid exposure. Altogether these results indicated that efflux pumps are promiscuous in their activity as we cannot associate extrusion of isoniazid to a specific gene as reported by others (13).

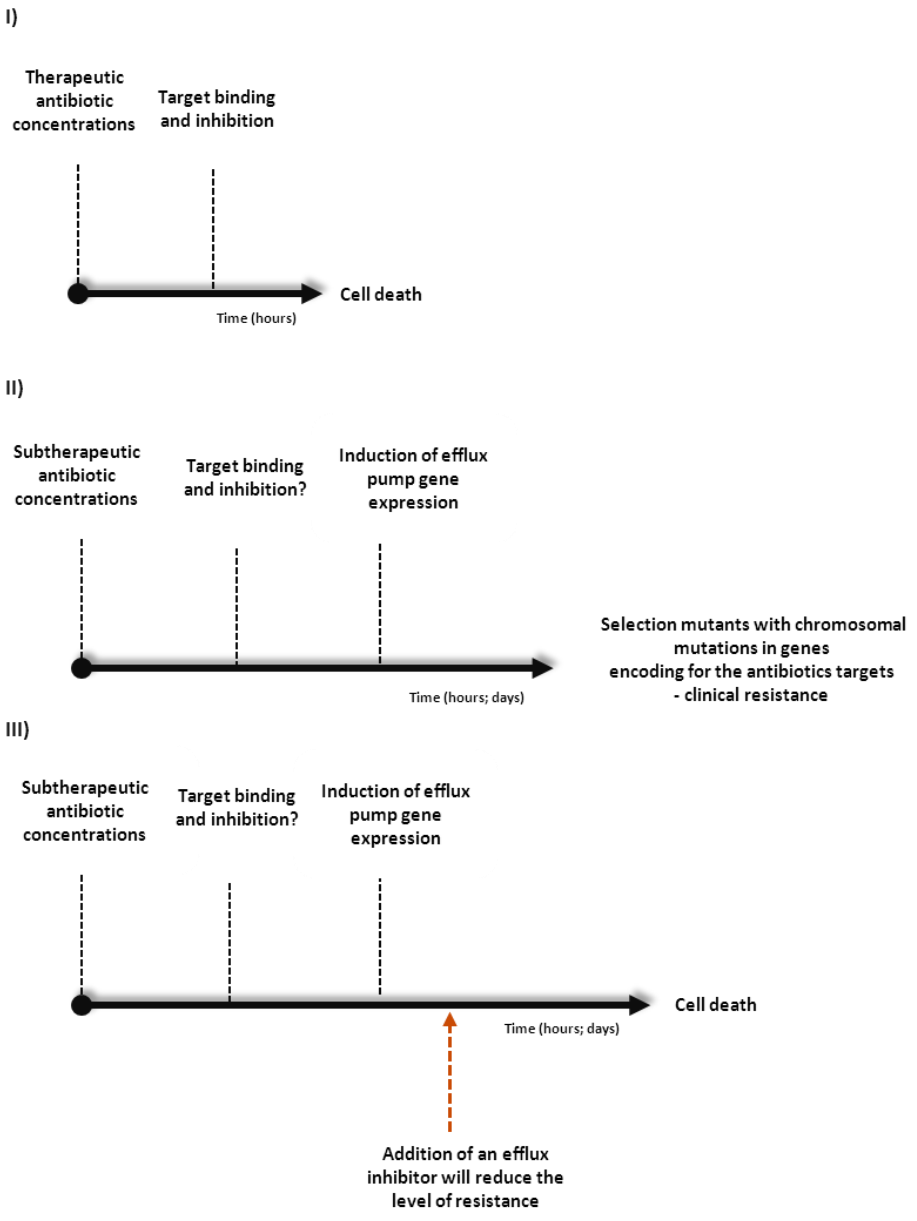

Figure V.1. Evolution towards high level resistance in *M. tuberculosis* strains susceptible to drugs.

The major conclusions of Chapter III are that the increased activity of efflux pumps, as a result of the exposure to subinhibitory concentrations of isoniazid, allows a sustainable resistant population to be maintained in the patient under therapy, from which genetically resistant mutants will emerge with increased frequency and the addition of an efflux inhibitor is able to reduce this level of resistance.

From the work developed in Chapter III several questions arised such as: what is the role played by the efflux systems in *M. tuberculosis* strains that carry mutations in the drug target genes? Do they respond in the same way to the same antituberculosis antibiotic pressure? What is the mechanism by which the efflux inhibitors can reduce the level of resistance that leads to cell death? To address these questions a panel of drug resistant *M. tuberculosis* strains were selected to investigate the *in vitro* activity of the putative efflux inhibitors, verapamil, thioridazine, chlorpromazine, haloperidol and flupenthixol, on the resistance levels of isoniazid, rifampicin, amikacin and ofloxacin. From here we also wanted to evaluate changes in the expression of efflux pump genes upon exposure to isoniazid and rifampicin and evaluate the antimycobacterial properties of these inhibitors, both *in vitro* and in the macrophage model, against these strains (**Chapter IV**).

Concerning the first question: what is the role played by the efflux systems *in M. tuberculosis* strains that carry mutations in the drug target genes? The results obtained in **Chapter IV** revealed that the addition of inhibitors enhances the clinical effect of the main antituberculosis drugs, isoniazid and rifampicin, against multidrug resistant strains, despite the presence of a mutation conferring resistance. In the presence of a isoniazid and rifampicin high-level resistance strain, these drugs are obviously predicted to be useless. However, we have demonstrated the effectiveness of a synergistic combination of drugs with conventional therapy that showed to be beneficial since it reduced the resistance level of the strain and these results strongly support the relevance of these inhibitors as adjuvants in tuberculosis chemotherapy. Importantly, we found that the activity of these compounds is limited to strains with *inhA* mutations which points to a genotype-specificity of these compounds. However, how drug resistant *M. tuberculosis* strains responds to the antibiotic pressure imposed by the antibiotics? At the light of that described above for *M. tuberculosis* susceptible strains we suggest the

following chain of events (Figure V.2.I): with the addition of isoniazid to an isoniazid resistant strain, the presence of a mutation in the target of isoniazid- *inhA* - will reduce the affinity of the *katG*-activated isoniazid for NAD (21; 22). The inhibition of the INH-NAD adduct will lead to isoniazid resistance. The presence of a mutation in *inhA* will prevent the binding of isoniazid to its target resulting in the accumulation of isoniazid inside the cells. The creation of such toxic effect will make the cell respond by changing the expression of several genes, such as stress genes, which includes the genes that code for efflux pumps in order, to reduce the intracellular concentration of the drug. The addition of an efflux inhibitor will reduce the efflux activity leading to an increase in intracellular concentration of the drug resulting in cell death (Figure V.2II).

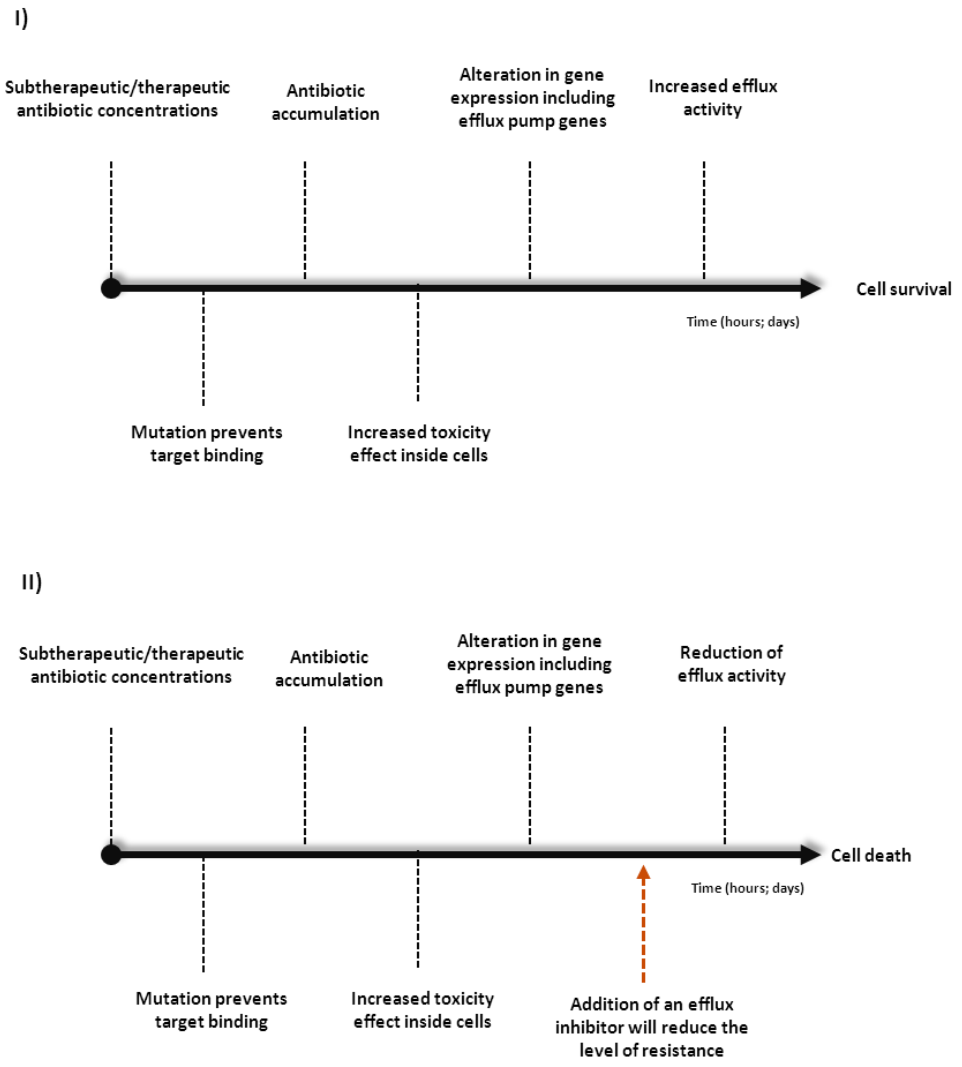

Figure V.2. The effect of isoniazid exposure on a drug resistant *M. tuberculosis* strain.

We also demonstrated that the compounds were able to promote intracellular accumulation of ethidium bromide, a broad range substrate for efflux pumps, on *M. tuberculosis* multidrug resistant strains, clearly putting in evidence that active efflux is inhibited by these compounds. We increased the panel of efflux pumps related factors to be studied and included the regulator *whib7* that was recently described to be involved in the regulation of intrinsic antibiotic resistance in *M. tuberculosis* (4). Similar to the results obtained in **Chapter III**, we have noticed a general inducible overexpression of almost all efflux genes studied upon exposure to isoniazid and rifampicin in multi- and extensively drug resistant strains. These results allow us to conclude that the level of drug resistance in *M. tuberculosis* is a combination between the presence of a mutation

in drug target genes and a general stress response to the presence of noxious compounds that regulates the intracellular level of a drug whose primary line of defensive response involves the overexpression of efflux pumps. Therefore, the results obtained in **Chapter IV,** combined with the all sum of results from **Chapter II** and **III,** demonstrated that the main mechanisms associated with drug resistance in *M. tuberculosis* correlates mutations in target genes with increased efflux and compounds that inhibit efflux can significantly reduce the phenotypic level of such resistance.

Next we focused on the understanding of the mechanism of action of these compounds both on bacterial and host cell. Specifically we were interested to unravel the chain of events occurring between the addition of an inhibitor and the death of the bacilli (Figure V.2). For that purpose, we evaluated the antimycobacterial activity of these compounds against a panel of drug resistant *M. tuberculosis* strains, *in vitro*, and assessed their effect on the enhancement of the killing activity of human monocyte-derived macrophages. The results obtained showed that verapamil, thioridazine, chlorpromazine, flupenthixol and haloperidol are efflux inhibitors with antimycobacterial activity towards *M. tuberculosis in vitro* and *ex vivo*. Their bactericidal activity was investigated through time-kill studies and the results demonstrated that these compounds display rapid and high killing activity. This increased bactericidal activity is correlated with intracellular ATP depletion. For *ex vivo* studies we evaluated the activity of the compounds against intracellular *M. tuberculosis*, their potential to induce phagosome acidification, the expression of lysosomal hydrolases and the efflux pump gene expression of internalized *M. tuberculosis*. Gene expression analysis showed that, mimicking what was seen previously *in vitro*, efflux genes were also overexpressed in response to isoniazid *ex vivo*, demonstrating that *M. tuberculosis* drug resistance inside macrophages is also mediated by expression of efflux pumps that can be inhibited by such efflux inhibitors. All compounds lead to a significant decrease in the intracellular mycobacterial load through a dual effect: enhanced synergistic killing activity with antibiotics plus the acidification of phagosomes and expression of lysosomal hydrolases.

Coming to the completion of this thesis we can affirm that the studies developed and the results obtained herein provided a preliminary, yet detailed, description of the molecular mechanisms by which these agents act in *M. tuberculosis*. The results obtained so far

allow us to rename these compounds from efflux inhibitors to ion channel blockers, as a more appropriate designation for their plethora of direct and indirect inhibitory effects, and to propose the following model for their mechanism of action: a) on the host cell, their inclusion on the antituberculosis treatment will lead to an increased transcription of the V-ATPases which results in the phagosome acidification. The phagosomal acid will synergize with several components of the host immune response, such as lysosomal hydrolases, promoting the bacterial growth restriction, therefore being strong cell-immune boosters, the most important component of the host defenses against tuberculosis (Figure V.3); in the bacteria: and after entering the cell, the compounds will generate a cascade of events that starts with the inhibition of the respiratory chain complexes due to the collapse of PMF by transporting protons down the electrochemical potential gradient. The collapse of PMF has detrimental effects on the bacterial cell such as increased production of reactive oxygen species (ROS), inhibition of ATP synthesis, and disruption of calcium homeostasis. We hypothesize that the disruption of PMF results in the inhibition of *M. tuberculosis* efflux systems that depends on PMF. As such, the inhibition of the respiratory chain will lead to i) dissipation of membrane potential; ii) reduction of ATP levels; iii) ROS generation; iv) increase in intracellular calcium levels; v) intracellular retention of any efflux substrate (*e.g*. ethidium bromide or antituberculosis drugs); and ultimately vi) cell death (Figure V.4). As a corollary of the above detailed demonstration of this hypothesis one can easily correlate these effects with the increased retention of the antituberculosis drugs, such as isoniazid and rifampicin (whose *in vitro* synergistic activity was demonstrated), if these ion channel blockers are to be included in the multidrug long-term antituberculosis therapy and the anticipated benefits that they will bring to the treatment outcome and shortening of the therapeutic regimen.

In conclusion, due to their dual role as antimicrobial agents and enhancers of macrophage killing activity, this work highlights the value of these compounds as adjuvants of tuberculosis therapeutics, with particular relevance for the treatment of the multidrug resistant forms. The objectives of this thesis were fully achieved and the several experimental procedures applied during this work revealed to be appropriate for the intended objectives and can be used to characterize and evaluate the bactericidal activity and efficacy of any combination of compounds and antibiotics with the

potential to be used as antituberculosis agents. The study was divided into three main chapters each focusing on the major aspects of *M. tuberculosis* drug resistance, namely: (i) the description of the molecular genetic basis of drug resistance in *M. tuberculosis* clinical strains (**Chapter II**); (ii) the study on the contribution of efflux to the emergence of drug resistance in *M. tuberculosis* (**Chapter III**), and (iii) the description of the mechanism of action of putative efflux inhibitors that can be used as adjuvants on tuberculosis therapy (**Chapter IV**).

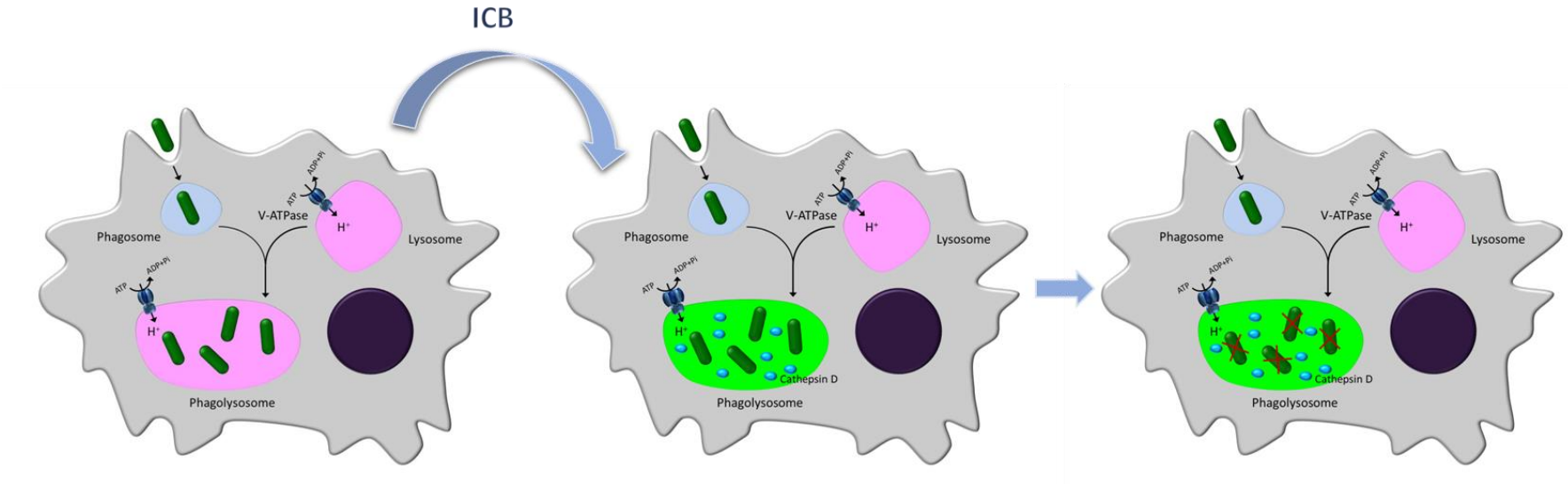

Figure V.3. Hypothetical model for the mechanism of action of the ion channel blockers on human macrophages. The treatment with the inhibitors will lead to an increased transcription of the V-ATPases which results in the phagosome acidification. The phagosomal acid will synergize with several components of the host immune response, such as lysosomal hydrolases (*e.g.* cathepsin D) leading to bacterial growth restriction. ICB, ion channel blocker.

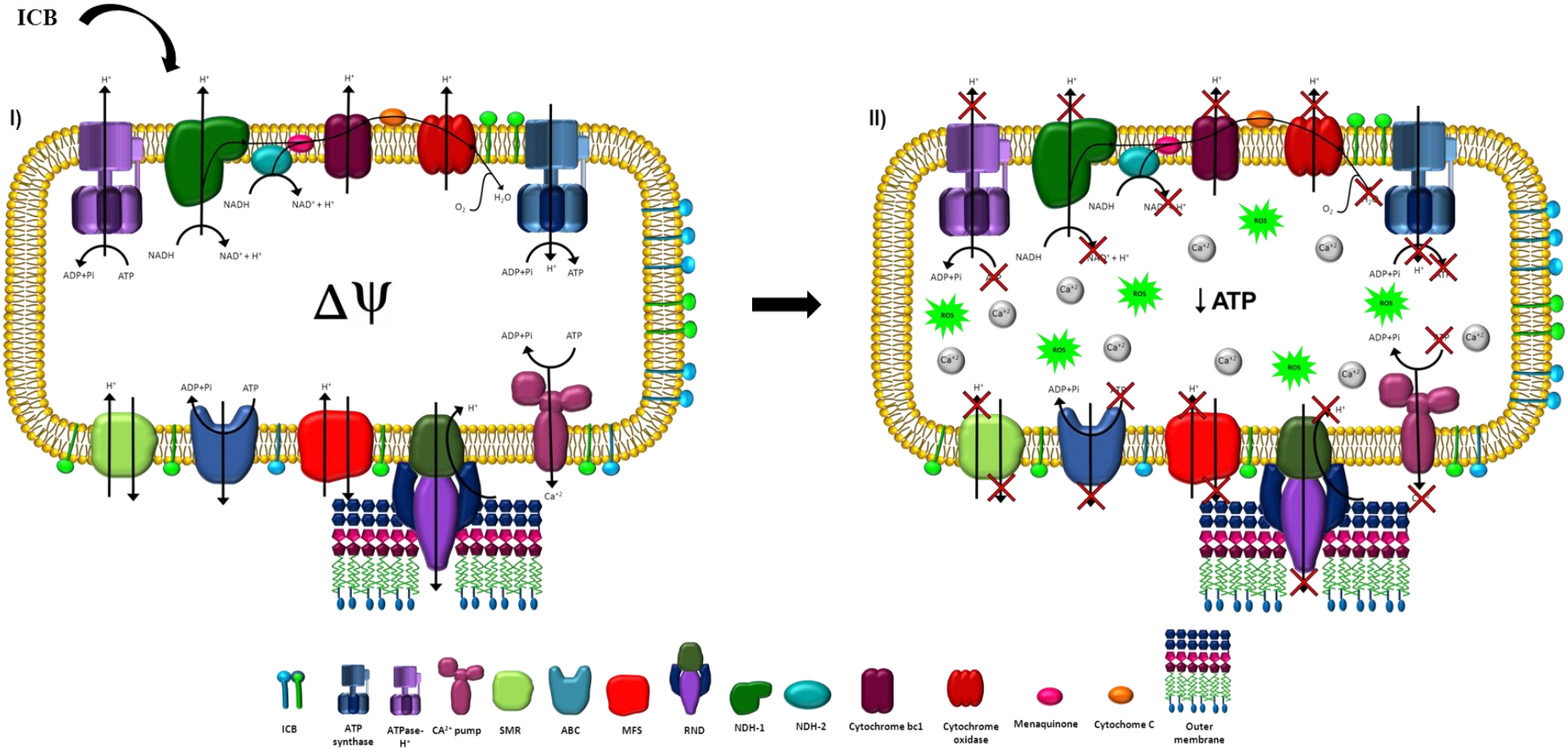

Figure V.4. Proposed mechanism of action of the ion channel blockers in *M. tuberculosis*. I) Ion channel blockers cross the membrane and inhibit calcium and potassium channels. The loss of these ions resulted in immediate dissipation of the membrane potential (Δψ) and II) hydrolysis of internal ATP, leading to an eventual collapse of the pH gradient at the membrane, ROS generation; increase in intracellular calcium levels; and cell death. The disruption of PMF results in the inhibition of *M. tuberculosis* efflux systems that depends on PMF. ICB, ion channel blockers; ROS, reactive oxygen species; PMF, proton motive force; SMR, small multidrug resistance; RND, resistance-nodulation division; MFS, major facilitator superfamily ; ABC, ATP-binding cassette.

The above enumerated conclusions of this work provides insights to the biological foundations of the mechanisms of acquired resistance in tuberculosis that will certainly contribute to design new therapeutic agents and strategies to circumvent its development, but it's necessary go further and deeper with the characterization of these mechanisms and compounds. Important issues remain to be elucidated and they open a new range of future research perspectives. The following aspects can be listed and should be the focus of our efforts and attention in the near future – It's important to:

- Determine the membrane potential, ROS production, and the effect of ROS scavengers when exposed to the compounds. This will allow the precise determination of the relation between the mechanism of action of the compounds and the integrity of the mycobacterial membrane;
- Study the interplay between calcium, ROS and NO. Calcium and ROS are known to be involved in the response to various cell stresses and NO production has been shown to be calcium dependent;
- Quantify the intracellular concentration of calcium upon exposure to the compounds and its correlation with the reduction of ATP levels. The increase on intracellular calcium levels is one of the major consequences of the inhibition of the respiratory chain. The study of the involvement of calcium homeostasis in *M. tuberculosis* will definitely help to elucidate the mechanism of action of the ion channel blockers studied in this thesis;
- Identify the precise molecular target of these compounds. The biological targets of the compounds are a matter of debate (23; 27). It is important to know if the inhibition promoted by the compounds is due to their interaction with specific target or is just a consequence of the dissipation of membrane potential;
- Identify the genes required for *M. tuberculosis* survival in an intracellular acidic pH environment. The identification of pathways utilized by *M. tuberculosis* to cope within an acidic environment can and should be looked as potential targets for tuberculosis chemotherapy;
- Determine the intracellular concentration of calcium inside macrophages and its correlation with the ATP-induced killing activity upon exposure to the compounds.

- Investigate the effect of the compounds on the activity of the Abelson (Abl) tyrosine kinase. Abl tyrosine kinase is described to control phagosome acidification (5). Given the effect of these compounds on phagosome acidification is likely that they could also have an effect on Abl tyrosine kinase;
- Evaluate the expression of cathepsin D and V-ATPases. Phagosomal acidification is established by the V-ATPases and the accumulation of V-ATPases is associated with an enhancement of the expression of the aspartic protease cathepsin D. It will be interesting to determine the expression of cathepsin D and V-ATPases and understand if the compounds-mediated acidification could be inhibited by a V-ATPase inhibitor and, if this inhibition can lead to enhanced mycobacterial replication inside the macrophages;
- Evaluate the mitochondrial membrane potential and intracellular calcium concentrations of *M. tuberculosis* infected macrophages. We postulate that the ion channel blockers have an effect on the bacterial membrane potential but do not affect macrophage membrane potential. It is of major importance to investigate their effect on the host cell since increased intracellular calcium concentrations is required for the manteinance of mitocodrial functions and drugs that increase calcium concentration and protect mitocondrial integrity can be suitable tools to boost microbiocidal mechanisms of the macrophage against *M. tuberculosis* as is expected from the preliminary evidences on the effect of these compounds on the phagosomal V-ATPases and the mechanism of acidification;
- Characterize the patterns of cytokine production from drug-treated human macrophages and its correlation with the genetic background of the strains. In Chapter III we have noticed that the *in vitro* effect of the ion channel blockers is dependent on the genetic background of the strains. It would be of interest to determine whether these strains demonstrate a strain-specific pattern of cytokine production that may explain the success of these strains, principally of Lisboa Family, in the infection, disease and drug resistance development among the patients of the Lisbon metropolitan area;
- Explore the role of NDH-1 during macrophage infection upon exposure to the inhibitors. A complete functional NDH-1 complex mediates the anti-apoptotic

properties of the bacteria. We hypothesize that the disruption the membrane potential triggered by the tested compounds, will lead to the impairment of the respiratory chain and initiate apoptosis.

The complete characterization of the *in vitro* and *ex vivo* antimycobacterial activity of these compounds, initiated with this thesis, will serve as the basis for the future characterization of their mechanism of action *in vivo* using, *e.g*. a zebrafish larval model, as previously described (1). Other groups have already started to demonstrate that the foundations of our initial hypothesis is true – the acceleration of tuberculosis treatment by adjunctive therapy with an efflux inhibitor (1; 2; 10).

The understanding of the mechanism of action of these compounds will definitely contribute for the design of new ones and can provide the basis for the development of new therapeutic strategies to fight drug resistant tuberculosis. In Europe and in Portugal these new therapeutic strategies will be of utmost importance since the management of multidrug and extensively drug resistant tuberculosis patients is a challenge for the clinicians whose support and therapeutic weapons to fight the disease is scarce due to the evident failures of the World Health Organization standardized second and third line therapeutic regimens and the reduced drugs available with known efficacy against drug resistant tuberculosis (12; 14). Furthermore, these new therapeutic strategies will be of tremendous assistance for the management of the national tuberculosis programs in order to cut and stop the chain of events that promotes acquired resistance during treatment and the transmission of drug resistant strains (10).

It's our wish and belief that, with a concerted action between biological, medical and public health sciences, the problems posed by drug resistant tuberculosis will be severely restrained in the near future by the development of new combined therapeutic strategies and new mycobacteria laboratory diagnostic procedures to assist the clinicians and the health system in its treatment and control – we hope to have modestly contributed with this work.

References

1. Adams K, Takaki K, Connolly L, Wiedenhoft H, Winglee K, Humbert O, Edelstein P, Cosma C, Ramakrishnan L. Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. Cell; 2011: 145: 39-53.

2. Adams K, Szumowski J, Ramakrishnan L. Verapamil, and its metabolite norverapamil, inhibit macrophage-induced, bacterial efflux pump-mediated to multiple anti-tubercular drugs. J Infect Dis. 2014; doi: 10.1093/infdis/jiu095.

3. Böttger E. The ins and outs of *Mycobacterium tuberculosis* drug susceptibility testing. Clin Microbiol Infect. 2011; 17: 1128-34.

4. Burian J, Ramón-García S, Howes C, Thompson C. WhiB7, a transcriptional activator that coordinates physiology with intrinsic drug resistance in *Mycobacterium tuberculosis*. Expert Rev Anti Infect Ther. 2012; 10: 1037-47.

5. Burns H, Stegelmann F, Fabri M, Döhner K, van Zandbergen G, Wagner M, Skinner M, Modlin R, Stenger S. Abelson tyrosine kinase controls phagosomal acidification required for killing of *Mycobacterium tuberculosis* in human macrophages. J Immunol. 2012; 189: 4069-78.

6. Cambau E, Viveiros M, Machado D, Raskine L, Ritter C, Tortoli E, Fauville-Dufaux M, Hoffner S, Richter E, Perez del Molino M, Cirillo D, van Soolingen D, Boettger E. Quantitative antibiotic susceptibility testing of multidrug resistant *Mycobacterium tuberculosis* and molecular resistance mechanisms; a European multicenter evaluation. 2014. Submitted.

7. Canetti G, Froman S, Grosset J, Hauduroy P, Langerova M, Mahler H, Meissener G, Mitchison DA, Šula L. Mycobacteria: laboratory methods for testing drug sensitivity and resistance. Bull World Health Organ. 1963; 29: 565-78.

8. David H. Probability distribution of drug-resistant mutants in unselected populations of *Mycobacterium tuberculosis*. Appl Microbiol. 1970; 20: 810-4.

9. Dheda K, Gumbo T, Gandhi N, Murray M, Theron G, Udwadia Z, Migliori G, Warren R. Global control of tuberculosis: from extensively drug-resistant to untreatable tuberculosis. Lancet Respir Med. 2014; 2: 321-38.

10. Gupta S, Tyagi S, Almeida D, Maiga M, Ammerman N, Bishai W. Acceleration of tuberculosis treatment by adjunctive therapy with verapamil as an efflux inhibitor. Am J Respir Crit Care Med. 2013; 188: 600-7.

11. Johnson R, Streicher E, Louw GE, Warren R, van Helden P, Victor T. Drug resistance in *Mycobacterium tuberculosis*. Curr Issues Mol Biol. 2006; 8: 97-112.

12. Lange C, Abubakar I, Alffenaar J, Bothamley G, Caminero J, Carvalho A, Chang K, Codecasa L, Correia A, Crudu V, Davies P, Dedicoat M, Drobniewski F, Duarte R, Ehlers C, Erkens C, Goletti D, Günther G, Ibraim E, Kampmann B, Kuksa L, de Lange W, van Leth F, van Lunzen J, Matteelli A, Menzies D, Monedero I, Richter E, Rüsch-Gerdes S, Sandgren A, Scardigli A, Skrahina A, Tortoli E, Volchenkov G, Wagner D, van der Werf M, Williams B, Yew W, Zellweger J, Cirillo D; for the TBNET. Management of patients with multidrug-resistant/extensively drug-resistant tuberculosis in Europe: a TBNET consensus statement. Eur Respir J. 2014; 10.1183/09031936.00188313.

13. Louw G, Warren R, van Pittius N, Leon R, Jimenez A, Hernandez-Pando R, McEvoy C, Grobbelaar M, Murray M, van Helden P, Victor T. Rifampicin reduces susceptibility to ofloxacin in rifampicin-resistant *Mycobacterium tuberculosis* through efflux. Am J Respir Crit Care Med. 2011; 184: 269-76.

14. Oliveira O, Gaio R, Villar M, Duarte R. Predictors of treatment outcome in multidrug-resistant tuberculosis in Portugal. Eur Respir J. 2013; 42:1747-9.

15. Perdigão J, Macedo R, João I, Fernandes E, Brum L, Portugal I. Multidrug-resistant tuberculosis in Lisbon, Portugal. A molecular epidemiological perspective. Microb Drug Resist. 2008; 14: 133-43.

16. Perdigão J, Macedo R, Malaquias A, Ferreira A, Brum L, Portugal I. Genetic analysis of extensively drug-resistant *Mycobacterium tuberculosis* strains in Lisbon, Portugal. J Antimicrob Chemother. 2010; 65: 224-7.

17. Perdigão J, Macedo R, Silva C, Pinto C, Furtado C, Brum L, Portugal I. Tuberculosis drug resistance in Lisbon, Portugal: a 6-year overview. Clin Microbial Infect. 2011; 17: 1397-402.

18. Perdigão J, Macedo R, Silva C, Machado D, Couto I, Viveiros M, Jordão L, Portugal I. From multidrug-resistant to extensively drug-resistant tuberculosis in Lisbon, Portugal: the stepwise mode of resistance acquisition. J Antimicrob Chemother. 2013; 68: 23-33.

19. Perdigão J, Macedo R, Machado D, Silva C, Jordão L, Couto I, Viveiros M, Portugal I. GidB mutation as a phylogenetic marker for Q1 cluster *Mycobacterium tuberculosis* isolates and intermediate-level streptomycin resistance determinant in Lisbon, Portugal. Clin Microbiol Infect. 2013. 10.1111/1469-0691.12392.

20. Ramaswamy S, Musser J. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: update. Tuber Lung Dis. 1998; 79: 3-29.

21. Rawat R, Whitty A, Tonge P. The isoniazid-NAD adduct is a slow, tight-binding inhibitor of InhA, the *Mycobacterium tuberculosis* enoyl reductase: adduct affinity and drug resistance. Proc Natl Acad Sci USA. 2003; 100: 13881-6.

22. Rozwarski D, Grant G, Barton D, Jacobs Jr. W, Sacchettini J. Modification of the NADH of the isoniazid target (InhA) from *Mycobacterium tuberculosis*. Science. 1998; 279: 98-102.

23. Schurig-Briccio L, Yano T, Rubin H, Gennis R. Characterization of the type 2 NADH:menaquinone oxidoreductases from *Staphylococcus aureus* and the bactericidal action of phenothiazines. Biochim Biophys Acta. 2014; doi.org/10.1016/j.bbabio.2014.03.017.

24. Springer B, Lucke K, Calligaris-Maibach R, Ritter C, Böttger E. Quantitative drug susceptibility testing of *Mycobacterium tuberculosis* using MGIT960 and the EpiCenter instrumentation. J Clin Microbiol. 2009; 47: 1773-80.

25. Vilchèze C, Jacobs Jr W. The mechanism of isoniazid killing: clarity through the scope of genetics. Annu Rev Microbiol. 2007; 61: 35-50.

26. Viveiros M, Portugal I, Bettencourt R, Victor T, Jordaan A, Leandro C, Ordway D, Amaral L. Isoniazid-induced transient high-level resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2002; 46: 2804-10.

27. Warman A, Rito T, Fisher N, Moss D, Berry N, O´Neill P, Ward S, Giagini G. Antitubercular pharmacodynamics of phenothiazines. J Antimicrob Chemother. 2013; 68: 869-80.

28. Zhang Y, Yew W, Barer M. Targeting persisters for tuberculosis control. Antimicrob Agents Chemother. 2012; 56: 2223-30.

29. Zhang Y, Yew W. Mechanisms of drug resistance in *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis. 2009; 13: 1320-30.