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INSTITUTO DE HIGIENE E MEDICINA TROPICAL

The antimycobacterial activity of thioridazine derivatives against drug resistant *Mycobacterium tuberculosis*:

***In vitro*, *ex vivo* and *in vivo* studies**

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Na presente dissertação incluem-se resultados que foram ou estão a ser alvo de publicação em co-autoria. Os artigos publicados ou submetidos para publicação serão integralmente apresentados em Anexo. Para efeitos do disposto no nº1 do Despacho nº2303/2000 do Regulamento de Programas de Doutoramento do Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa (Diário da República, 2ª série, nº 23, de 28 de Janeiro de 2000), o autor da dissertação declara que interveio na concepção e execução do trabalho experimental, na interpretação dos resultados e na redacção dos manuscritos publicados, submetidos ou que aguardam submissão.

Lisboa, 31 de Janeiro de 2008

Marta Sofia Lopes Martins

To my parents with all my love

"There is at bottom only one genuine treatment for all diseases,… to stimulate the phagocytes. Drugs are a delusion. … (when) the phagocytes are stimulated; they devour the disease…"

George Bernard Shaw

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From the bottom of my heart, I wish you all a wonderful life.

ABSTRACT

The main objective of this Thesis was to evaluate thioridazine (TZ) and chemically derived derivatives for anti-*Mycobacterium tuberculosis* activity in particular against multi-drug resistant (MDR) *M. tuberculosis*. Twenty-two TZ derivatives were obtained and screened for toxicity and mutagenicity by the trypan blue exclusion assay in human lymphocytes and the Ames test, respectively. The derivatives that were devoid of any toxicity and mutagenicity were then tested against Methicillin-resistant *Staphylococcus aureus* (MRSA) used as a model during the entire work, and against antibiotic resistant *M. tuberculosis* (MDR-TB) strains *in vitro* and subsequently in the macrophage that has phagocytosed the organism. Since tuberculosis is an infection of the alveolar macrophage it is important to evaluate the activity of these compounds inside the phagocytic cell where the mycobacteria are to be found. Thioridazine was also tested for its ability to cure the mouse of infection by *M. tuberculosis.* Therefore, animal studies using Balb/C mice infected with *M. tuberculosis* H37Rv ATCC27294 strain were initiated to parameterize the route of infection, dose of compound to be administered, among others. The results obtained showed that from the twenty-two TZ derivates none was toxic or mutagenic under the conditions tested. Therefore, all the twenty-two derivatives were selected for *in vitro* evaluation against *S. aureus* and *M. tuberculosis* strains. From the *in vitro* results six derivatives showed greater activity than TZ and were selected for *ex vivo* studies. Three of these derivatives were shown to enhance the macrophage killing activity and one of the derivatives was even more active than TZ. From the animal studies it was possible to select the conditions to apply in further studies with the most active derivatives. From all the data obtained during this Thesis it was possible to develop a model based on the macrophage interaction with the bacteria and the subsequent action of the compounds. The macrophage model can elucidate what takes place inside the human macrophage when these compounds are added to the medium.

Due to the uniqueness of the approaches developed during the Thesis research, other potential sources of anti-tubercular compounds were explored: plants and organosilicon compounds (SILA). These studies demonstrated that extracts from the nuisance plant *Carpobrotus edulis* could enhance the killing of intracellular bacteria such as MDR-TB and MRSA. In addition, the extract was shown to modulate the immune system thereby

indicating its potential use for cellular immune deficient disorder as well as render MDR mouse lymphoma cells carrying the human *mdr*1 gene completely susceptible to cytotoxic drugs to which they were initially resistant. The results of this component of my research have resulted in the design of new experiments which are now being carried out by another Ph.D. student in our Unit and at the Medical University of Szeged, Hungary.

In conclusion, the results obtrained from my Thesis research has paved the way for clinical trial consideration of the many compounds studied shown to have significant intracellular activity against XDR-TB/MDR-TB at concentrations that are non-toxic and that can be readily achieved in the infected human.

RESUMO

O objectivo principal desta tese foi o de avaliar a acção da tioridazina (TZ), bem como de compostos derivados desta, obtidos por manipulação química, como agentes com actividade contra *Mycobacterium tuberculosis*, em particular contra *M. tuberculosis* multi-resistente (MDR-TB). Desta forma foram obtidos vinte e dois derivados e efectuados testes de toxicidade e mutagenicidade pelo método de exclusão com azul de trypan (realizado em linfócitos humanos) e o teste de Ames, respectivamente. Todos os derivados não tóxicos e não mutagénicos foram testados *in vitro* contra estirpes de *Staphylococcus aureus* resistente à meticilina (MRSA), que foi o microrganismo modelo utilizado durante todo o trabalho, e estirpes de MDR-TB. Visto que a tuberculose é uma infecção do macrófago alveolar, estes estudos foram subsequentemente aplicados a macrófagos infectados. Desta forma, é importante analisar a actividade que estes compostos apresentam dentro do macrófago, local onde normalmente a micobactéria se encontra. Iniciaram-se estudos animais com a TZ, de forma a verificar a eficácia deste composto em curar murganhos Balb/C infectados com *M. tuberculosis* H37Rv ATCC27294. Desta forma foi possível optimizar parâmetros, tais como, a via de infecção, a concentração de composto a administrar, entre outros. Os resultados obtidos demonstraram que dos vinte e dois derivados nenhum apresentava toxicidade ou efeitos mutagénicos, nas condições testadas. Desta forma, os vinte e dois derivados foram seleccionados para estudos *in vitro* contra estirpes de *S. aureus* e de *M. tuberculosis*. Dos estudos *in vitro* foi possível verificar que seis derivados apresentaram uma maior actividade do que a TZ e desta forma foram seleccionados para os estudos *ex vivo*. Quando testados em macrófagos infectados três derivados demonstraram um efeito marcado na activação das células fagocitárias (“enhancement of the killing activity”), sendo um dos derivados ainda mais activo do que a TZ. Dos estudos em animais, foi possível seleccionar as condições a serem implementadas em estudos futuros com os derivados mais activos. De todos os resultados obtidos durante esta tese foi possível desenvolver um modelo baseado na interacção do macrófago com a bactéria e a subsequente acção destes compostos. O modelo desenvolvido (“macrophage model”) pode assim contribuir para clarificar o que ocorre a nível intracelular aquando da adição dos compostos ao meio de cultura.

Devido à inovação das abordagens desenvolvidas durante esta tese, outras potenciais fontes de compostos tuberculostáticos foram exploradas, tais como, plantas e compostos SILA. Os estudos desenvolvidos demonstraram que extractos da planta *Carpobrotus edulis* podem apresentar actividade antibacteriana contra bactérias intracelulares, tais como, MDR-TB e MRSA. Adicionalmente, foi demonstrado que este extracto é um imuno-modulador do sistema imunitário, o que reflecte a sua potencial utilização em disfunções imunes verificadas a nível celular. Outra das potenciais aplicações é a sua utilização em linhas celulares murinas de linfoma multi-resistentes (que apresentam o gene *mdr*1), tornando-as completamente susceptíveis a compostos citotóxicos, aos quais estas células eram inicialmente resistentes. Os resultados desta componente da minha tese contribuíram para o "desenho" de novas experiências que se encontram de moemento a ser desenvolvidas por outra aluna de doutoramento, na Unidade de Micobacterias e na Universidade Médica de Szeged, Hungria.

Em conclusão, o trabalho desenvolvido durante esta tese veio abrir caminho para a aplicação dos vários compostos analisados em ensaios clínicos, dado que estes demonstraram ter actividade intracelular contra estirpes de XDR-TB/MDR-TB a concentrações não tóxicas e que podem ser facilmente atingidas num indivíduo infectado.

PUBLICATIONS

Publications that have resulted from this thesis and which are presented in the text are listed below. Copies of these publications as well others which resulted from the Thesis research are collated in the Appendix which due to the largesse of the text, is separately bound.

In International Scientific Journals

1. Martins, M., M. Viveiros, and L. Amaral. 2008. Inhibitors of Ca^{2+} and K^{+} transport enhance intracellular killing of *M. tuberculosis* by non-killing macrophages. In Vivo **22**: *In press*.

2. Martins, M., M. Viveiros, and L. Amaral. 2008. 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 Medicine: *In press*.

3. Martins M., S. G. Dastidar, S. Fanning, J. E. Kristiansen, J. Molnar, J. M. Pagès, Z. Schelz, G. Spengler, M. Viveiros, and L. Amaral. 2008. Potential role of non-antibiotics (helper compounds) in the treatment of multidrug-resistant Gram-negative infections: mechanisms for their direct and indirect activities. Int. J. Antimicrob. Agents **31**:198–208.

4. Martins, M., M. Viveiros, and L. Amaral. 2008. Enhanced killing of intracellular pathogenic bacteria by phenothiazines and the role of K^{+} efflux pumps of the bacterium and the killing macrophage. Anti-Infective Agents In Medicinal Chemistry **7**:63–72.

5. Amaral, L., M. Martins, and M. Viveiros. 2007. Phenothiazines as Anti-Multi-Drug Resistant Tubercular Agents. Infect. Disord. Drug Targets **7**:257–265.

6. Amaral, L., M. Martins, and M. Viveiros. 2007. Enhanced killing of intracellular multi-drug resistant *Mycobacterium tuberculosis* by compounds that affect the activity of MDR efflux pumps: a review. J. Antimicrob. Chemother. **59**:1237–1246.

7. Martins, M., M. Viveiros, J. E. Kristiansen, J. Molnar, and L, Amaral. 2007. The curative activity of thioridazine on mice infected with *Mycobacterium tuberculosis*. In Vivo **21**:771–776.

8. Martins, M., Z. Schelz, A. Martins, J. Molnar, G. Hajös, Z. Riedl, M. Viveiros, I. Yalcin, and L. Amaral. 2007. *In vitro* and *ex vivo* activity of thioridazine derivatives against *Mycobacterium tuberculosis*. Int. J. Antimicrob. Agents **29**:338–340.

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11. Martins, M., D. Ordway, M. Kristiansen, M. Viveiros, C. Leandro, J. Molnar, and L. Amaral. 2005. Inhibition of the *Carpobrotus edulis* methanol extract on the growth of phagocytosed multidrug-resistant *Mycobacterium tuberculosis* and methicillin-resistant *Staphylococcus aureus*. Fitoterapia **76**:96–99.

12. Viveiros, M., M. Martins, I. Couto, J. E. Kristiansen, J. Molnár, and L. Amaral. 2005. The *in vitro* activity of phenothiazines against *Mycobacterium avium*: potential of thioridazine for therapy of the co-infected AIDS patient. In Vivo **19**:733–736.

13. Martins, M., W. Bleiss, A. Marko, D. Ordway, M. Viveiros, C. Leandro, J. Molnar, J. E. Kristiansen, J. Wecke, and L. Amaral. 2004. Clinical concentrations of thioridazine enhance the killing of intracellular methicillin-resistant *Staphylococcus aureus*: an *in vivo*, *ex vivo* and electron microscopy study. In Vivo **18**:787–794.

14. Ordway, D., M. Viveiros, C. Leandro, R. Bettencourt, J. Almeida, M. Martins, J. E. Kristiansen, J. Molnar, and L. Amaral. 2003. Clinical concentrations of thioridazine kill intracellular multidrug-resistant *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **47**:917–922.

Articles Submitted

- **Martins, M., M. Viveiros, J. Ramos, I. Couto, J. Molnar, and L. Amaral.** 2008. SILA compound 421, an inhibitor of efflux pumps of cancer cells, enhances the killing of intracellular MDR-TB. *Submitted to J. Antimicrob. Chemother.*

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LIST OF ABREVIATIONS

AFB	Acid-fast-bacillus
AhpC	Alkyl hydroperoxide reductase subunit C
AIDS	Acquired Immune Deficiency Syndrome
AMK	Amikacin
AMPLICOR MTB	Roche AMPLICOR® *Mycobacterium tuberculosis*
AS	Additional Species
ATCC	American Type Cell Culture
ATP	Adenosine triphosphate
ATS	American Thoracic Society
B.C.	Before Christ
BCG	Bacille Calmette-Guérin
CaM	Calmodulin
CaMKII	CaM-dependent protein kinase II
CCCP	Carbonyl cyanide *m*-chlorophenylhydrazone
CDC	Centers for Disease Control and Prevention
CELME	*Carpobrotus edulis* leaves methanolic extract
CFP-10	Culture Filtrate Protein-10
CFU	Colony Forming Units
CIP	Ciprofloxacin
CLSI	Clinical Laboratory Standards Institute
CM	Common Mycobacteria
CPZ	Chlorpromazine
CR	Complement Receptors
DGS	Direcção Geral de Saúde
DNA	Desoxirribonucleic Acid
DOT	Directly Observed Treatment
DTH	Delayed-Type Hypersensitivity
EB	Ethidium Bromide
EEA1	Early Endosome Autoantigen 1
ELISA	Enzyme-Linked Immunosorbent Assay
ELISpot	Enzyme-Linked Immunospot Assay

EMB	Ethambutol
EPIs	Efflux Pump Inhibitors
ESAT-6	Early Secretory Antigenic Target-6
EU	European Union
Fc	Fragment, crystallizable
FDA	Food and Drug Administration
G.I.	Growth Index
GLC	Gas Liquid Chromatography
GTP	Guanosine-5'-triphosphate
HBSS	Hank’s Balanced Salt Solution
his	Histidine
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
hVPS34	PI-specific PI 3-kinase
i.p.	Intraperitoneally
i.v.	Intravenous
ICL	Isocitrate Lyase
IFN	Interferon
Ig	Immunoglubolin
IL	Interleukin
INH	Isoniazid
IUATLD	International Union Against Tuberculosis and Lung Disease
KAN	Kanamycin
kb	Kilo-base
LAM	Lipoarabinomannan
LPS	Lipopolysaccharides
LZD	Linezolid
ManLAM	Glycosylated phosphatidylinositol LAM
MAP	Mitogen-Activated Protein
MATE	Multidrug And Toxic Compound Extrusion
MBC	Minimum Bactericidal Concentration
MBP	Mannose Binding Protein
MCP-1	Monocyte Chemoattractant Protein-1

MDM	Monocyte Derived Macrophages
MDR	Multi-Drug Resistance
MDR-TB	Multi-Drug Resistant Tuberculosis
MH	Mueller-Hinton
MHA	Mueller-Hinton Agar
MHC	Major Histocompatibility Complex
MIC	Minimum Inhibitory Concentration
MOTT	Mycobacteria Other Than Tuberculosis
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MTD	Gen-Probe AMPLIFIED^{TM} *Mycobacterium tuberculosis* Direct
NADH	Nicotinamide Adenine Dinucleotide (reduced form)
NICE	National Institute for Clinical Excellence
NK	Natural Killer
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
NOS2	Nitric Oxide Synthase 2
NTM	Non-Tuberculous Mycobacteria
O.D.	Optical Density
OFX	Ofloxacin
OHS	Office of Health and Safety
OXA	Oxacillin
PAβN	β-naphthylamide
PANTA	Antimicrobial mixture that contains Polymyxin B, Amphotericin B, Nalidixic acid, Trimethropim and Azlocillin
PAS	Para-amino Salicylic acid
PBMC	Peripheral Blood Mononuclear Cells
PBMDMs	Peripheral Blood Monocyte-Derived Macrophages
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI	Phosphatidylinositol
PI3P	Phosphatidylinositol 3-phosphate
PIM	Phosphatidylinositol mannoside
PknG	Protein kinase G

POES	Polyoxyethylene stearate
PPD	Purified Protein Derivative
PZA	Pyrazinamide
QFT	QuantiFERON®-TB test
QFT-G	QuantiFERON®-TB Gold Test
r.p.m.	Rotations per minute
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
RD	Region of Difference
RIF	Rifampin
RNA	Ribonucleic acid
RND	Resistance-nodulation-cell division
RNI	Reactive Nitrogen Intermediates
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal RNA
SapM	Secreted acid phosphatase of *M. tuberculosis*
SDS	Sodium Dodecyl Sulfate
SHP-1	Tyrosine phosphatase
SILA	Organosilicon compounds
SILA 409	1,3-Dimethyl-1,3-bis(4-fluorophenyl)-1,3-bis(3-morpholino-propyl)-disiloxan-dihydrochlorid
SILA 421	1,3-Dimethyl-1,3-bis(4-fluorophenyl)-1,3-bis{3-[1(4-buthyl-piperazinyl)]-propyl}-disiloxan-tetrahydrochlorid
SNARE	Soluble N-ethylmaleimide-sensitive factor-attachment protein receptor
SP-A	Surfactant Protein A
STR	Streptomycin
TACO	Tryptophan aspartate rich coat protein
TB	Tuberculosis
TCH	Thiophene-2-carboxylic acid hidrazide
TEM	Transmission electron microscopy
TET	Tetracycline
TGFβ	Transforming growth factor beta
TLC	Thin-Layer Chromatography

TLRs	Toll-like receptors
TNF	Tumour necrosis factor
TSA	Trypticase Soy Agar
TSB	Tryptic Soy Broth
TST	Tuberculin skin test
TZ	Thioridazine
UNICEF	United Nations Children's Fund
US	United States
UV	Ultraviolet
V-ATPase	Vacuolar-type proton ATPase
WHO	World Health Organization
XDR-TB	Extensively-Drug Resistant Tuberculosis
XXDR-TB	Extremely-Drug Resistant Tuberculosis
γδ T cells	Gamma-delta T cells

LIST OF UNITS

G	Gravitacional force
Kg	Kilograms; g - grams (10^{-3}Kg)
L	Liter; mL - mililiter (10^{-3}L), μL - microliter (10^{-6}L)
m	Meter; mm - milimeter (10^{-3} m)

INTRODUCTION

THESIS OUTLINE AND OBJECTIVES

This Thesis includes the *in vitro, ex vivo* and *in vivo* studies of TZ against *M. tuberculosis* strains resistant to antibiotics. It is the intent to provide data about new anti-tubercular compounds and novel approaches that could be used for the treatment of MDR-TB and XDR-TB infections. The general aims of this thesis are:

- Screening of thioridazine derivatives obtained by chemical manipulation for toxicity and mutagenicity;

- Determination of the activity of the selected derivatives on antibiotic resistant and susceptible *M. tuberculosis in vitro*;

- Determination of the activity of derivatives against *M. tuberculosis* that has been phagocytosed by the human macrophage, the amount of derivative that is concentrated by the phagocytic cell and if it is altered by the human macrophage;

- Determination of selected thioridazine derivatives to clear the mouse pulmonary tree from MDR-TB infection and to prevent the infection of the mouse by MDR-TB.

The organization of this Thesis is in accordance to the Bologna Agreement Guidelines which employs as a basis the publications that have resulted from the Thesis research. The sections of the Thesis have been organized so that each one can stand on its own as a full report that refers to the methods used in that section, the results, its discussion and conclusions as well as all references cited. The sequence of Result sections has been designed so that the reader can be led from discovery to discovery in a developmental manner. However, this sequence does not reflect the chronological order of the work done since many of the experiments were conducted while some other experiments were "cooking" (mycobacteria do grow rather slowly).

This thesis is divided into 11 chapters, as follows:

Chapter I. State of the Art. This section provides a review of the current knowledge on Tuberculosis and the emergence of MDR and XDR resistance. The issues focused on this Thesis were put into context to highlight the relevance of the studies presented.

Chapter II. Materials and Methods. This section describes all the methodology used during the work performed in these studies.

Chapter III. The *in vitro* and *ex vivo* effects of clinical concentrations of TZ against Methicillin-resistant *Staphylococcus aureus* are described. *In vitro, ex vivo* and electron microscopy studies were conducted.

Chapter IV. This section presents all the results obtained from the *in vitro* and *ex vivo* activity of TZ and its derivatives against *Mycobacterium tuberculosis*. It also describes the *in vitro* effects of several phenothiazines tested against *M. avium*.

Chapter V. Describes the results of the curative activity of TZ on mice infected with *Mycobacterium tuberculosis*. Animal studies were conducted using TZ for the treatment of the infection.

Chapter VI. This chapter focuses on the effect that other efflux pumps inhibitors such as, the methanolic extract of *Carpobrotus edulis*, reserpine, ouabain and verapamil have on the growth of phagocytosed multidrug-resistant *M. tuberculosis* and methicillin-resistant *S. aureus*.

Chapter VII. This chapter provides data on new patented and synthesized SILA compounds and their effect on infected human monocyte-derived macrophage, as a special example relationship between efflux inhibitors and its effect on macrophage infected with MDR *M. tuberculosis* strain. In this chapter a new and innovative method used to screen efflux pump activity and efflux pumps inhibitors is also described.

Chapter VIII. The future of chemotherapy is the main subject presented. The discovery of new anti-microbial agents that can be used in the therapy of MDR bacteria as well as a new approach that uses these agents in conjunction with the classical antibiotics will be analysed and discussed.

Chapter IX. The Tuberculosis laboratory of the Future. In this section it will be discussed the role of the macrophage in the selection of agents that can be employed in the therapy of an MDR infection.

Chapter X. From all the studies performed a hypothetical model (macrophage model) was developed in order to explain the enhancement of the killing activity of the infected macrophages when TZ, its derivatives or other inhibitors of Ca^{2+} and K^{+} transport are added to the cell cultures. The **Macrophage Model** will be described in detail to clarify the intracellular processes that take place inside the infected and treated macrophage.

Chapter XI. Concluding Remarks and Future Perspectives. This section highlights the main conclusions obtained in each of the previous chapters as well as future recommendations and guidelines to be developed and implemented in order to treat MDR- and XDR-TB.

CHAPTER I.

State of the Art

I.1 History and Epidemiology of Tuberculosis (TB)

Tuberculosis (TB) continues to be considered the major cause for worldwide morbidity and mortality caused by an infective organism. This disease is caused by the steadfast human pathogen *Mycobacterium tuberculosis*, the bacterium known to infect the man since the birth of civilization (Gernaey *et al.*, 2001; Morell, 1994). The *M. tuberculosis* complex presently includes: *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. microti*, *M. pinnipedii*, *M. caprae*, and *M. canettii* (Ernst *et al.*, 2007). However, in some studies, *M. canettii* is not considered in this complex (Gutierrez *et al.*, 2005). This difference is due to the molecular approaches employed in the strains identification (difference in the number of genes analysed, *etc*.). The development of new molecular genetics tools and the sequencing of the genome of several *M. tuberculosis* strains allow a more accurate estimation of the time of origin of mycobacteria (Gutierrez *et al.*, 2005). It is expected that all the members of the *M. tuberculosis* complex had a common African ancestor about 35,000-15,000 years ago (Daniel, 2006), since an early progenitor of *M. tuberculosis* was discovered to be present in East Africa as early as 3 million years ago (Gutierrez MC *et al.*, 2005).

M. tuberculosis infections can involve any organ or tissue of the human body (Hopewell, 1995; Lillebaek *et al*., 2002). Because of this extensive involvement, it has been possible to determine that this infection has been with man since the dawn of civilisation (Zink *et al*., 2003) as evident from typical skeletal abnormalities produced by TB that have been found in Egyptian mummies. The frequency of skeletons with apparent tubercular deformities and use of DNA amplification of *M. tuberculosis* obtained from tissues suggests that the disease was common among that population (Nerlich *et al.*, 1997). In recorded history, Assyrian clay tablets describe patients coughing blood in the 7th century B.C. In classical Greece, Hippocrates writes of patients with phthisis (the Greek term for TB), which means consumption, *i.e.*, wasting away associated with chest pain and coughing, frequently with blood in the sputum. Hippocrates thought the disease was largely inherited, while Aristotle (4th century B.C.) stressed its contagious nature, as did Galen, in the 2nd century A.D. (Sakula, 1983). By this time, the frequency of descriptions of patients with TB-like symptoms indicates that the disease was already well entrenched. Figure 1 describes some of the more representative events that took place during the history of TB.

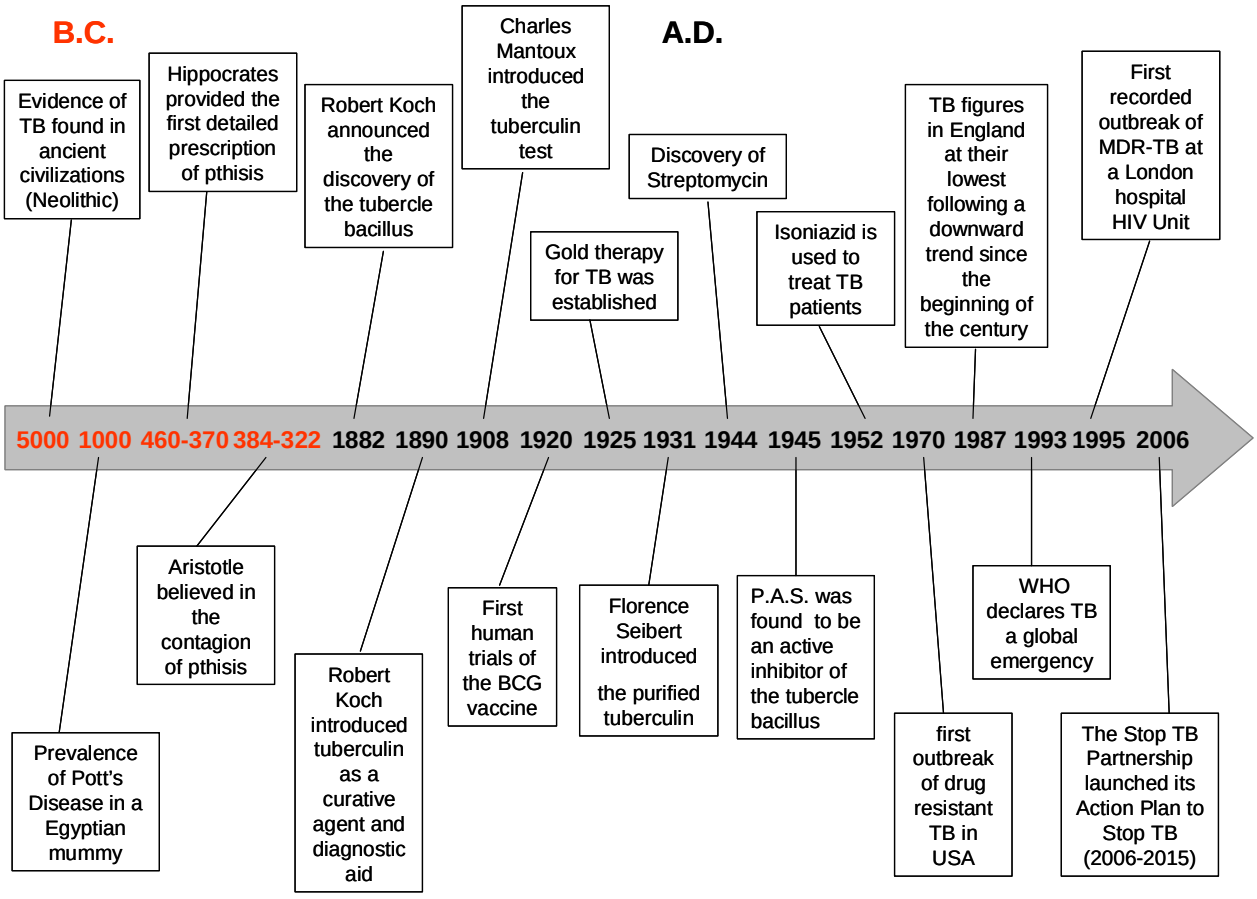


Figure 1. Time-line of TB. Some of the most important events that occurred in the history of TB until the present time (sources: Daniel, 2006; Di Perri and Bonora, 2004; Dietrich *et al.*, 2006; Murray, 2004; Myers, 1967; Nerlich *et al.*, 1997; Sakula, 1983) .

In the 19th century TB was well established in East Africa by the time Europeans reached that area. Analysis of human phenotypes, like lactose tolerance, that is associated with the raising of cattle and selection for the ability to utilize milk, as well as the resulting exposure to *M. tuberculosis* suggests that Indo-Europeans cattle herders that entered East Africa spread the disease to Europe and Asia during their migrations into these regions (Smith, 2003). In studies recently published (Ernst *et al.*, 2007), this theory is not supported and the transmission of the disease from cattle to human is considered to be impossible. However, this is not a consensual subject since some studies based on genetic analysis of the strains refute this belief. The actual representatives of the common ancestor of the *M. tuberculosis* complex include the ancestral and modern strains of *M. tuberculosis*. The latter are characterized by the deletion of a 2.1-kb fragment termed TbD1, account for most current TB cases, and include the Beijing family of strains that are prevalent in Asia and have caused

outbreaks worldwide. A separate lineage, marked by deletion of region of difference 9 (RD9), diverged from an ancestral *M. tuberculosis* and sequentially gave rise to *M. africanum, M. microti, M. pinnipedii, M. caprae*, and *M. bovis*. It is thought that *M. bovis*, which has the greatest number of deletions, is the most recent member of this lineage. Based on these results the popular belief that *M. tuberculosis* evolved from *M. bovis* through the adaptation of a bovine strain to the human host is clearly refuted. Since *M. tuberculosis* contains chromosomal segments that have been deleted from *M. bovis*, *M. tuberculosis* could not have evolved from *M. bovis* (Ernst *et al*., 2007). Based on these new studies and genetic approaches the manner of transmission between humans and cattle is still not completely clarified.

I.1.1 Evolution of TB in Europe and diagnosis of infection

As Europe entered the middle ages, the written record of TB is almost non-existent. However, this does not mean that the disease was not present. In modern times, TB reached epidemic proportions in Europe and North America during the 18th and 19th centuries. The understanding of the pathogenesis of TB began with the work of Theóphile Laennec in the 19th century. He clearly elucidated the pathogenesis of TB and unified the concept of this disease. At that time, TB surged across Europe with death rates in London, Stockholm and Hamburg that approached 800-1000 cases/100,000 inhabitants/year (Daniel, 2006). The demonstration of the transmissibility of *M. tuberculosis* infection was accomplished by Jean-Antoine Villemin in 1865. He inoculated a rabbit with a purulent liquid obtained from an individual who had died of TB (Murray, 2004). Although the animal remained healthy, it was found to have extensive TB when sacrificed and autopsied (Daniel, 2006). This was an important achievement; however, the history of TB was markedly changed in 1882, with Robert Koch's discovery of the tubercle bacillus. In his presentation “Die Aetiologie der Tuberculose”, Koch identified the tubercle bacillus as the etiologic agent and presented his famous postulates, which continue to set the standard for the demonstration of infectious etiology (Kaufmann and Schaible, 2005). In 1890, Koch started the isolation of tuberculin (Myers, 1967). The tuberculin skin test was developed in 1907 by Clemens von Pirquet and 3 years later used to demonstrate latent infection in asymptomatic children. Charles Mantoux introduced the use of a cannulated needle and syringe to inject tuberculin intra-cutaneously in 1908, and Florence Seibert developed

purified protein derivative (PPD) during the 1930s. With the availability of well-standardized PPD, several studies of tuberculin reaction sizes were conducted. In 1952 Carroll Palmer and Leroy Bates published a large study of reactions to one test unit of PPD-S (equivalent to 0.4 units of PPD-RT23) (Daniel, 2006). Three years later, a World Health Organization (WHO) study group published a report that expanded these skin testing studies to school children in various populations. This report showed that in high TB prevalence countries, a substantial number of healthy school children reacted with reaction sizes similar to those in TB patients (Daniel, 2006). These children had latent TB infections. In some populations, reaction sizes in these children were skewed towards smaller reactions. Edwards and Palmer using guinea pigs demonstrated that these smaller reactions were due to cross reacting hypersensitivity to the antigens of environmental mycobacteria (Daniel, 2006). They also established conclusively that latent TB infection is common in the United States (US) and presumably in other low TB prevalence countries; that latent infection could be recognized by tuberculin testing; and that non-specific tuberculin reactivity was common in certain geographic regions. During the early to mid-19th century the number of new cases of TB in Europe and North America and its associated mortality rates began to decline. The improved social and living conditions, as well as the improved nutrition status, contributed to the strengthening of immunity resulting from natural selection of humans resistant to TB. However, this postulated cause of the decrease, doesn't explain the rapid decline in TB rates. This decrease was accompanied by an apparent shift of disease occurrence to older individuals (Davies *et al*., 1999; Frost, 1939).

I.1.2 Approaches developed for treating TB, its control and prevention

In the late 19th and early 20th centuries sanatoria were developed for the treatment of patients with TB. Pulmonary collapse therapy became popular, especially for cavity closure (Daniel, 2006). A more direct approach to the public health challenges of TB was taken by Albert Calmette and Camille Guérin. They developed the Bacille Calmette-Guérin (BCG) vaccine, by attenuating an *M. bovis* strain and the vaccine was ready to be tested in 1921 (Dietrich *et al*., 2006). Over the next few years, the vaccine was readily accepted in much of Europe and more than 100,000 children were immunized. TB rates fell rapidly in Europe during the first half of the 20th century, but the ravages of World War I led to a resurgence of the disease. During World War I, both

the allies and Germans screened their military recruits for TB using chest radiographs. After the war, TB detection in the population was accomplished by mass radiographic screening (Myers, 1967). In 1948, a campaign to control TB sponsorship by United Nations Children's Fund (UNICEF) and the Danish Red Cross was based on tuberculin testing followed by BCG vaccination of non reactors. During the next 3 years, nearly 30 million persons were tuberculin tested and nearly 14 million vaccinated with BCG. The program was the first disease control program undertaken by an agency of the WHO. In 1974 the WHO Expert Committee on TB provided policy guidelines for TB control for the next 2 decades (Daniel, 2006). It discouraged both radiographic screening and tuberculin testing and promoted sputum microscopy of symptomatic individuals and those at risk. It advocated ambulatory therapy and recommended against hospitalization of TB patients, strongly encouraging BCG vaccination. Today, the WHO continues to mount programs for TB control, but it no longer recommends BCG vaccination except for newborns. In the US, where BCG has not been widely used, TB control programs emphasize treatment of latently infected individuals (WHO, 2006d).

I.1.3 Evolution of the Therapy of TB

The discovery of para-amino salicylic acid (PAS) by Jorgen Lehmann in 1943 and of thiosemicarbazone by Gerhard Domagk yielded the first therapeutic agents with efficacy in the treatment of TB (Di Perri and Bonora, 2004). In 1944 Albert Schatz, Elizabeth Bugie, and Selman Waksman reported the isolation of streptomycin (STR), the first antibiotic and first bactericidal agent effective against *M. tuberculosis* (Daniel, 2006). Isoniazid (INH), the first oral mycobactericidal drug, followed in 1952 and this agent was soon followed by rifamycins in 1957. A new era of TB treatment started allowing effective public health measures. Treatment was increasingly expanded to include those with latent TB infections. The history of TB control entered a new chapter. However, the underdeveloped world is still suffering from TB. It is estimated that over one-third of the World's population is currently infected; thirty million new cases were reported for 2003; TB infections caused three million deaths (WHO/IUATLD, 2004). Due to these facts, TB has been declared by the WHO a Global Emergency. The annual number of global cases is expected to continue to escalate. In the third world countries, the rates of new cases of TB are extremely worrisome. The number of infected persons continues to increase due to famine, war, over-crowding, as

well as to the cost of anti-tubercular drugs which for many countries, is beyond their affordability (Amaral *et al*., 2001b). In order to stop and control this infection, there is the need to understand the pathogenesis of TB, develop rapid methods for the detection of infection and active disease, identify the areas in which the disease continues unabated, develop controls that will prevent the dissemination of the disease and develop new treatments which are affordable to the affected population. In short- eliminate the global presence of TB.

I.1.4 BCG vaccination

M. tuberculosis infects only man and because it cannot survive in the environment it is considered a steadfast human pathogen. Therefore, all pulmonary infections of TB emanate from human sources. In order to prevent the rate of infection, the vaccination policy was applied and still persists. In some countries, such as is the case for Portugal, newborns are vaccinated with the BCG. This vaccine has been the subject of intensive research during the past decades. However, from the numerous studies performed and published it is evident that the efficacy of BCG vaccination is highly variable, ranging from 0% to 80% (Infuso *et al.*, 2006). It is more likely that BCG vaccine prevents against disseminated forms of TB in children and is less effective for pulmonary TB in adolescents or adults. BCG vaccination is recommended in asymptomatic children with or at risk for Human Immunodeficiency Virus (HIV) infection, but it should not be used in persons with symptomatic HIV infection or Acquired Immune Deficiency Syndrome (AIDS) (WHO, 2001). In healthcare workers exposed to *M. tuberculosis*, including Multi-Drug Resistant TB (MDR-TB), BCG vaccination generally is not recommended (Cohn, 1997). Re-vaccination with BCG does not confer more benefit than initial vaccination and in the opinion of some experts the repeated vaccinations should be discontinued (Cohn, 1997). The recent advances in technology and the better understanding of the immunopathogenesis of TB lead to continuous efforts to develop a more potent and specific vaccine. However, vaccination has become an obstacle in the detection of new cases of infection. The fact that BCG vaccination “masks” the results implies that new infections cannot be detected and therefore all the new cases that are reported correspond to an active disease state. This feature should be the subject of attention and there is the urgent need for every one to understand two simple, but very important concepts: infection *versus* active disease.

I.1.5 Infection: processes and sites

A TB infection is acquired via airborne microdroplets of sputum that contain the organism *M. tuberculosis* and which are generated when a person that has progressed to active disease, coughs, sneezes or yells. The inhaling of these microdroplets brings the organism in contact with pneumocyte type II cells of the alveolar sac, also known as pulmonary macrophages (Tomioka, 2003). These cells are efficient in the phagocytosis of the bacteria; however, they are unable to destroy them. It is important to note that 90% of all such infections are resolved without any noticeable pathology and are therefore sub-clinical (Cardona, 2007). However, the remaining 10% of TB infections progress to active disease. In this state of the disease there is the release of the bacterium from the macrophage, concomitantly with the destruction of the lung parenchyma (Russell, 2007). By this manner, while pulmonary TB is considered an intracellular infection, active disease is an extracellular one. Active disease is therefore the means by which the infective organism is transmitted from individual to individual and because a new infection cannot be readily distinguished by skin tests or radiological procedures, all the new cases of infection that are reported correspond to active disease.

I.1.6 Global TB today

The global distribution of **new cases of TB** per 100,000 inhabitants is illustrated by Figure 2. The incidence of TB ranges from less than 10 per 100,000 inhabitants in North America to 100 to 300 per 100,000 inhabitants in Asia and Western Russia to over 300 per 100,000 inhabitants in Southern and Central Africa. There is one death from TB every 15 seconds (over two million per year), and eight million people develop TB every year (Chakrabarti and Davies, 2007). Without treatment, up to 60% of people with the disease will die. Essentially all these cases are in the third world countries, reflecting the poverty and the lack of healthy living conditions and adequate medical care. This global crisis is compounded by the emergence of multi-drug resistance (MDR) in countries like the former Soviet Union, South Africa, and India, where some antibiotics are available but are of inferior quality or are not used for a sufficient time to control the disease according to the recommended regimens (Dorman and Chaisson, 2007).

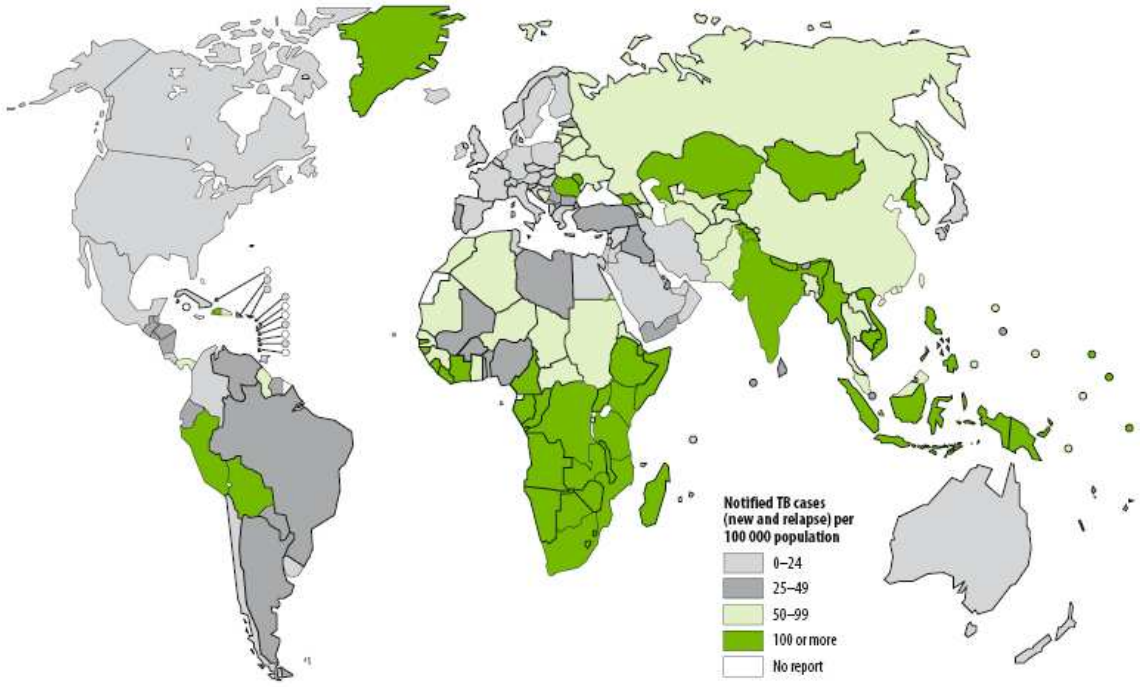


Figure 2. TB notification rates (2005). The 199 countries reporting to WHO notified 5.1 million new and relapsed cases, of which 2.4 million (47%) were new smear-positive cases (reproduced from WHO, 2007a).

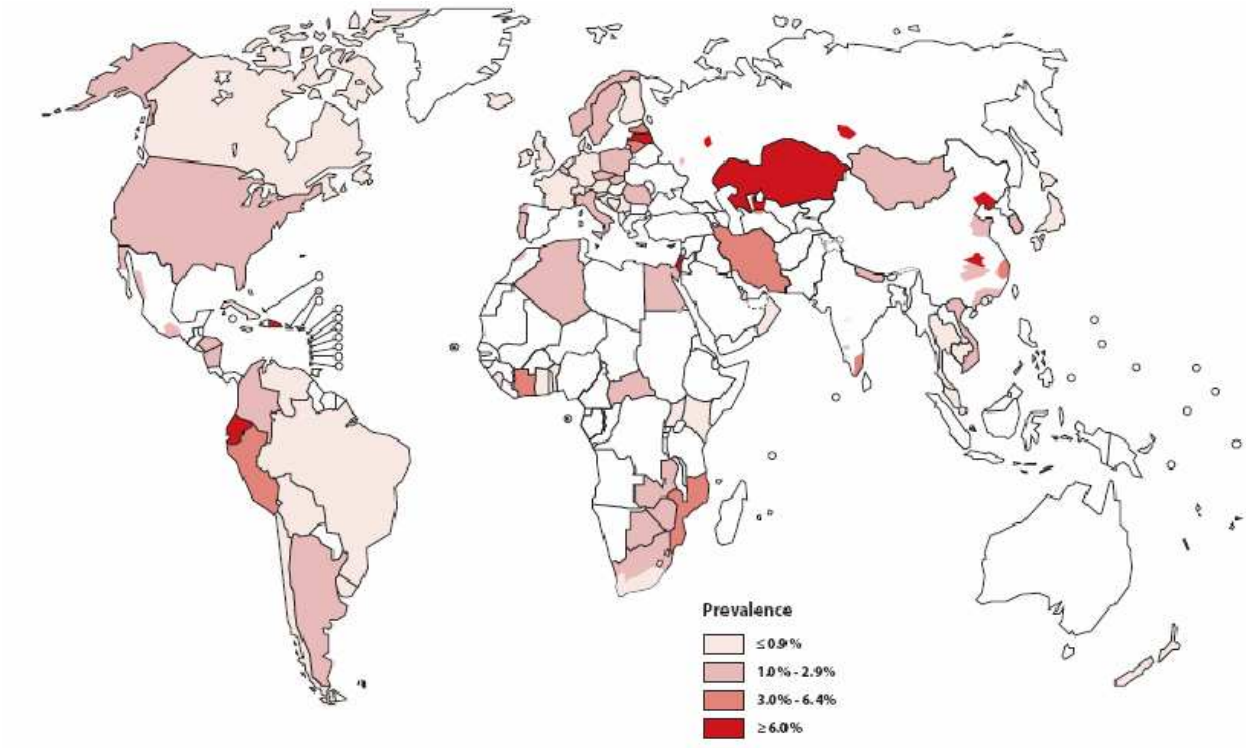
I.2 Multi-Drug Resistance (MDR)

Due to the increasing number of individuals infected with HIV and the problems associated with the current regimens of antimicrobial therapies, the appearance of MDR-TB strains, *i.e.*, strains resistant to at least INH and rifampin (RIF) took place wherever HIV/AIDS was frequent (Di Perri and Bonora, 2004; Munsiff *et al.*, 2002). The development of MDR-TB was due to incompetent therapy and patient non-compliance (Mitchison, 2004; Ormerod, 2005; Zhang, 2007) and took place as follows; initially susceptible strains to antibiotics that became resistant to one antibiotic by spontaneous mutation were selected as a consequence of ineffective therapy and these in turn could infect patients who would also be managed poorly with these agents thereby favouring a second spontaneous mutation that caused resistance to the second antibiotic (Rao, 1998). These MDR-TB strains that emerged during the 1990s posed a threat to the control of TB, both in the US (Dooley *et al.*, 1992) and worldwide (WHO/IUATLD, 2004). The therapy of MDR-TB regardless of whether all five first line drugs of defence (INH, RIF, STR, ethambutol (EMB), pyrazinamide (PZA)) or in combination with

second line of defence compounds is extremely problematic (Amaral *et al*., 2004). Because the use of these therapeutic modalities produce significant morbidity, hence the problem of non-compliance (Drobniewski and Balabanova, 2002), new cases of MDR-TB have continued to increase in many of the urban centres of western and third world countries (WHO, 2005). Nevertheless, recommended treatment of MDR-TB infections requires second-line drugs (WHO, 2006c). These drugs in general are less effective, more toxic, and costlier than first-line INH- and RIF-based regimens (Di Perri and Bonora, 2004; Gupta *et al*., 2001; Shah *et al*., 2007). Furthermore, individual resistance to these agents is many fold greater than resistance to INH and RIF (Johnson *et al*., 2006; Shi *et al*., 2007). In 2000, the Stop TB Partnership's Green Light Committee was created to increase access to second-line drugs worldwide while ensuring their proper use to prevent increased drug resistance. MDR-TB also requires a two- to four-fold longer period of treatment compared with drug-susceptible TB (Di Perri and Bonora, 2004). In the treatment of MDR-TB there are three major issues that should be addressed: rapid diagnosis, selection of drugs to which the strain is susceptible and adherence of the patient to therapy. The starting point should be the access to good diagnostic services. Rapid methods to reduce time of diagnosis of MDR-TB and of susceptibility testing are important measures to adopt. The radiometric BACTEC 460 technique has been proved to be reliable for second-line drug-susceptibility testing compared with the standard proportional method with solid media. Other improved automated non-radiometric systems, such as the MGIT 960 or the MB/BACT, however, have been only evaluated for the first-line of anti-tuberculous drugs. Among tools for rapid detection, the reverse hybridization-based probe assay and other amplification methods are available for the rapid detection of mutations to RIF (Di Perri and Bonora, 2004). These methods are discussed in the next section. The choice of an anti-MDR-TB regimen should give preference to the first-line drugs shown to still be active, such as pyrazinamide, STR and EMB. Depending on local resources, parenterally administered drug such as amikacin, capreomycin or kanamycin could eventually be included in association with second-line oral agents such as fluoroquinolones, ethionamide, PAS, cycloserine, clarithromycin, linezolid, *etc*. The duration of the treatment should be determined on an individual basis and should be prolonged for at least 18 months after sputum conversion (sputum free of acid fast mycobacteria). Hospital-based treatment should be performed at least until sputum conversion occurs. Because adherence of the patient to treatment continues to be a major issue due to the morbidity produced by the

combination of first line and second line of defence drugs, directly observed treatment (DOT) must be provided, particularly when there is prior evidence of patient non-compliance. The prevention of the development of further drug resistance and epidemics in high prevalence regions must remain a major concern (Di Perri and Bonora, 2004). Furthermore, as formulated by the programmes of the WHO for the control and prevention of TB and MDR-TB, surveillance is an absolute necessity. Therefore, data describing the global distribution of MDR-TB on an annual basis and its comparison to that obtained for previous years as shown by Figure 3 provide an understanding of where the MDR-TB problem continues and where, as a consequence of effective recently introduced TB control programs, the MDR-TB problem is seen to abate. As can be seen by the data provided by Figure 3 resistance to anti-TB drugs is present worldwide. The global number of MDR-TB cases reported between 1994 and 2002 showed enormous gaps in many areas, especially in countries with a large TB burden or where available data strongly suggest that there may be a much larger problem, particularly in Asia and countries of the former Soviet Union. From 1994-1997 particularly high prevalence of MDR-TB were found in the Baltic republics of Estonia and Latvia, parts of Russia, China, Iran, the Dominican Republic, Argentina, Nepal, Gujarat, India, New York City, Bolivia and Korea (Figure 3A) (Cohn *et al*., 1997; Pablos-Méndez *et al*., 1998). The increased case rates in Sub-Saharan Africa were largely due to the HIV epidemic and in the former Soviet republics, mostly due to the social-economic crisis that followed the collapse of the Soviet Union (Raviglione *et al*., 1993). This increased number of MDR-TB cases led the WHO and the International Union Against Tuberculosis and Lung Disease (IUATLD) to establish a global project of drug resistance surveillance based on standard epidemiological methods and quality control through an extensive network of reference laboratories. From 1994-2002 until 2004 it is notorious the increase in the number of MDR-TB cases reported especially, in Russia. Analysing the data reported by the WHO in 2006, the 3 areas with significantly different proportions of MDR-TB cases among new cases were the Central Europe, Latin America and Africa (both with low and high HIV prevalence, respectively), Eastern Europe and the Eastern Mediterranean region, Southeast Asia and the Western Pacific region (Figure 3B). The regions with the highest burden of MDR-TB were found to be Eastern Europe, Southeast Asia, and the Western Pacific region.

A



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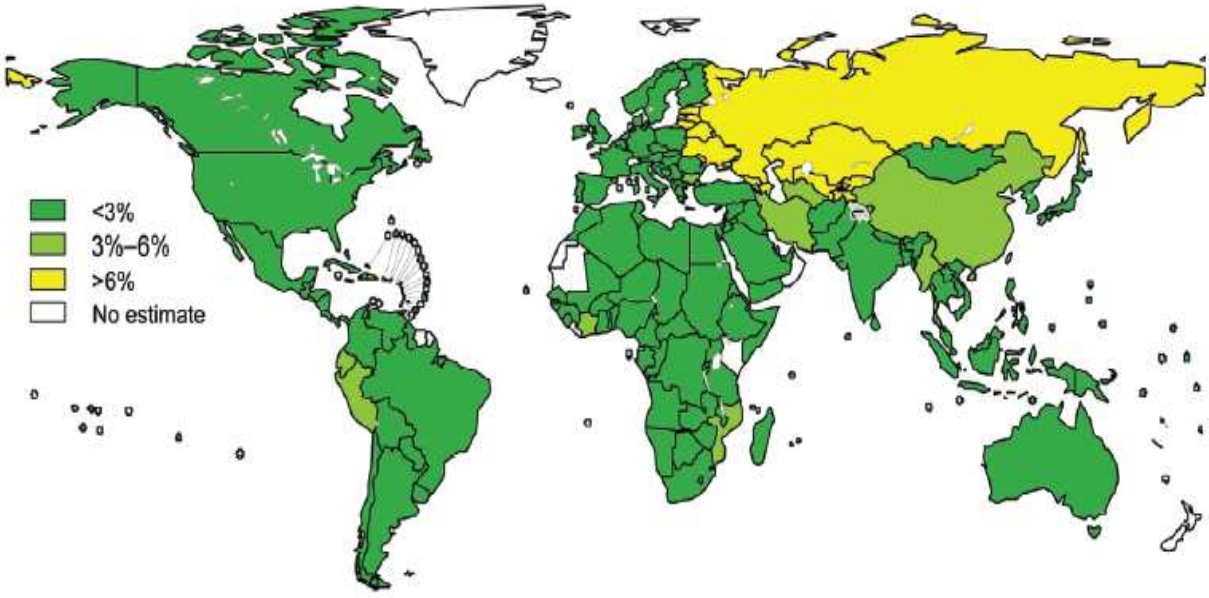


Figure 3. Global distribution of MDR-TB rates. Prevalence among new TB cases (1994-2002) (A); (2004) (B) (reproduced and adapted from WHO/IUATLD, 2004; Zignol *et al.*, 2006).

The vast majority of patients with MDR-TB in low-resource countries do not have access to proper diagnostic services and to the quality-assured second-line drugs needed to treat these infections. By this manner, plans to expand appropriate diagnostic and treatment services for patients with MDR-TB are urgently needed, especially in countries where the burden of MDR-TB is greatest, such as the case of China, India and the Russian Federation and in areas with the highest MDR-TB rates (such as Eastern Europe) (Zignol *et al*., 2006).

I.2.1 Extensively Drug Resistance TB (XDR-TB)

The term Extensively Drug Resistance TB (XDR-TB) was used for the first time in March of 2006, in a report jointly published by the US Centers for Disease Control and Prevention (CDC) (CDC, 2006) and the WHO (WHO, 2006b). In that report, XDR-TB was defined as a disease caused by *M. tuberculosis* resistant to INH and RIF (MDR-TB) and to at least three of the six classes of second-line anti-TB drugs (aminoglycosides, polypeptides, fluoroquinolones, thioamides, cycloserine and PAS). However, due to difficulties in performing drug susceptibility testing for "second line of defence" drugs and also because some forms of drug resistance are less treatable than others, this definition was revised at a meeting of the WHO XDR-TB Task Force that occurred in Geneva (Switzerland) in 2006. Thus, currently XDR-TB is defined as: *M. tuberculosis* strains resistant to at least INH and RIF, in addition to any fluoroquinolone, and to at least one of the three injectable drugs used in anti-TB treatment: capreomycin, kanamycin and amikacin (WHO, 2006b). Re-evaluating the definition of XDR-TB led to incomplete information about the actual number of cases of the disease (Migliori *et al*., 2007). The results presented by the CDC (CDC, 2006) revealed that XDR-TB strains had been reported in all continents prior to 2000 with the highest known rates in Eastern Europe and Asia. The first study on the prevalence of XDR-TB was reported on 2006 in Tugela Ferry, a rural town in the South African province of KwaZulu-Natal (Gandhi *et al*., 2006), the epicentre of South Africa's HIV/AIDS epidemic (Singh *et al.*, 2007). This area which presents a very high number of cases of HIV was assessed for prevalence of XDR-TB by Gandhi and co-workers from January of 2005 to March of 2006. The results showed that from the 1539 patients monitored, 221 had MDR-TB and from these, 53 were infected with XDR-TB (Gandhi *et al*., 2006). After the initial cases reported in South Africa other countries also started to report at least one case of XDR-

TB. Among those countries are: Argentina, Armenia, Bangladesh, Brazil, Chile, Czech Republic, Ecuador, France (one case imported), Georgia, Germany, Italy, Republic of Korea, Latvia, Mexico, Peru, Portugal, Russian Federation, South Africa, Spain, Thailand, United Kingdom and the United States of America (Figure 4**)**. Indeed, in the 1997 WHO Report on Global MDR-TB, the frequency of strains resistant to 4 or more first line of defence drugs were identified as a global problem. Admittedly, these strains do not satisfy the current definition of XDR-TB; it does suggest that these XDR-TB strains have been present much earlier than recognised.

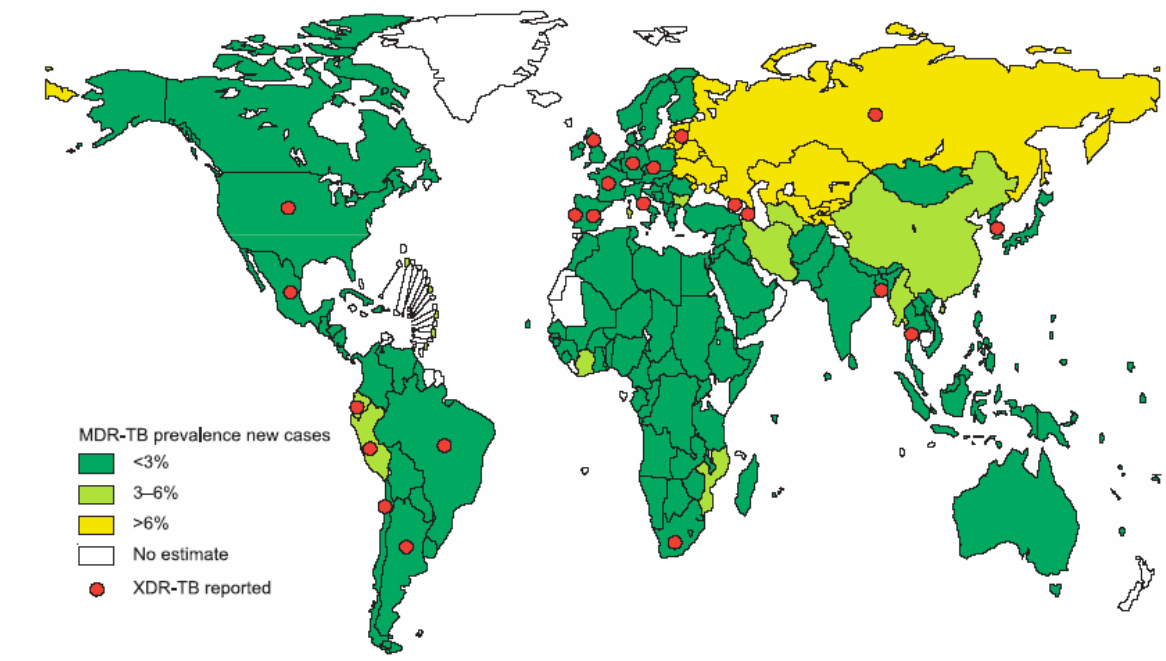


Figure 4. Global distribution of MDR-TB among new cases and countries that reported XDR-TB. The countries with red circles reported at least one case of XDR-TB (reproduced from Migliori *et al*., 2007).

At least 10 of the countries that reported XDR-TB are in, or bordering, Europe. Preliminary anecdotal evidence suggests that some XDR-TB strains in Europe (from Italy, for example) may, in fact, be "XXDR-TB" or "extremely drug-resistant", *i.e*. resistant to all first- and second-line drugs available. This increased resistance to second-line drugs implies losing the fluoroquinolones and the injectable agents that are the last resource measures available at the present. It also means losing the most potent and least toxic options for second-line drug therapy. Fluoroquinolones are some of the few potent antibiotics still active against most MDR-TB strains and their use has been identified as a favourable prognostic factor in cases of MDR-TB, with very few cases of

fluoroquinolones resistance reported (Veziris *et al*., 2007). Unfortunately, a significant number of XDR-TB strains go beyond the definition of XDR-TB and are essentially untreatable, being resistant to numerous other second-line drugs (Hamilton *et al*., 2007). This constitutes a big concern since the number of cases is almost for sure higher than the ones reported. In that manner, some strategies should be developed to control or prevent the spread of the disease. The WHO Global Task Force on XDR-TB developed several main recommendations to prevent and control XDR-TB (WHO, 2007b). These recommendations consist in the prevention of XDR-TB through basic strengthening of TB and HIV control. These are priority interventions which main guidelines are based on the new Stop TB Strategy and the Global Plan to Stop TB. Other important recommendation requires the improvement in the management of patients suspected to have XDR-TB. These interventions have to consider accelerating the access to the laboratory facilities. However, this could be difficult to implement since in most resource-limited settings TB laboratory services are inadequate and laboratory-based diagnosis of TB typically relies on microscopy of stained sputum. This method has poor sensitivity (missing more than 50% of cases), especially in HIV-positive individuals, and does not provide information about drug susceptibility. Culture is more sensitive than microscopy and can be used for drug susceptibility testing, but *M. tuberculosis* takes several weeks to grow. New diagnostic modalities that could improve TB detection and the identification of drug resistance include: nucleic acid amplification tests to detect *M. tuberculosis* in sputum; phage-based methods to detect *M. tuberculosis* and drug resistance in sputum; and rapid molecular genetic tests to detect RIF resistance in sputum or in cultured bacteria (see next section for more detailed information). These tests, however, have generally been regarded as too costly for widespread use in most TB endemic settings (Dorman and Chaisson, 2007). Besides these rapid tests of drug susceptibility one should consider the improvement on the detection of cases suspected of presenting MDR-TB strains in HIV infected individuals and the strengthened management of XDR-TB and treatment design in HIV negative and positive individuals. The basis of this intervention is the correct application of the new WHO guidelines for programmatic management of drug-resistant TB using second-line drugs properly. Also a new approach centred on the patient in order to establish adequate support and supervision. One most important recommendation is the standardization of the definition of XDR-TB. It is important that all the countries adopt the new definition to allow the comparability of data obtained through ongoing

surveillance in low and high TB incidence countries. One main area of concern is the health care workers. There is the need for an increased control and protection of all the health care workers; the main goal should be the reduction of the transmission of MDR strains especially among HIV positive individuals in congregate care settings presenting high HIV prevalence. Therefore, there is the need to implement immediate XDR-TB surveillance programs that should be coordinated by the Supranational and National Reference Laboratories and should be focused on the complete assessment of the XDR prevalence worldwide. One important aspect that should never be forgotten is the initiation of advocacy, communication and social mobilization activities. It continues to be extremely important to inform the general community and raise awareness about TB and XDR-TB (Migliori *et al*., 2007). In order to achieve control of MDR or XDR-TB, basic research has to be conducted to develop new diagnostics, new drugs, treatments and prevention tools. Exactly 125 years have passed since Robert Koch's discovery of *M. tuberculosis* (the milestone of modern control of TB) and the ''white plague'' is still affecting mankind. Lastly, consideration must be given to the use of all methods that provide the means for control of TB, MDR-TB and XDR-TB. The argument that it is costly does not stand up in the face of the costs that are incurred in terms of human suffering and economics such as loss of wages, increased dependence of patient and family on governmental assistance, and not to say the least, the cost of treating (cost of drugs) and maintaining a patient within hospital grounds (Muniyandi *et al*., 2006). If TB can be obviated, the costs required for this task are infinitely less than the incurring of costs due to increasing frequency of TB, MDR-TB and XDR-TB.

I.3 TB in Portugal

Portugal presents a very peculiar situation since it has an incidence of TB that is approximately 10 times greater than the countries of Western Europe (DGS, 2006). The understanding of the global evolution of TB and the new tools of epidemiologic analysis is forcing responsible specialists to develop more ambitious goals for the control of TB in the next decades. This attention is now centred on the DOT strategy and in the detection of latent cases. With the appearance of the HIV and the development of MDR, the strategy has to suffer a rearrangement in order to solve this continuous case of infection. Portugal has an incidence of 33 cases/100,000 inhabitants (new cases in 2005)

(DGS, 2006). However, the data reported to the WHO is relative to the year of 2005 and since there is no available data for 2006 the situation cannot be accurately discussed. The actual incidence of TB means that the risk of being infected with TB suffered a reduction to half in the last 17 years. In terms of notified number of cases, only the three Baltic countries have higher numbers, namely, Lithuania (63), Latvia (63) and Estonia (43) (WHO, 2007a). In a global context the highest incidences of cases in the EU are considered intermediates (between 20 and 80 cases/100,000 inhabitants). In this group are included Poland (26) and Hungary (22) (WHO, 2007a). However, if we consider the differences in the detection rates to this calculus of estimated cases, Spain will also be included in this group with an estimated rate of 27/100,000 inhabitants. In Portugal the distribution of TB cases is very different. According to the number of cases reported by the Direcção Geral de Saúde (DGS) in 2006 (relative to the year of 2005), the areas that have higher number of cases reported are Porto (696), Lisboa (569), followed by Setúbal (276), Braga (182), Faro (132) and Aveiro (129) (DGS, 2006). As can be seen, the big metropolitan areas of Lisboa and Porto have the highest rates of incidence (Figure 5) (DGS, 2006). In the opposite, areas like Alto Alentejo, a big part of the centre of the country and Bragança district have a very few number of cases notified.

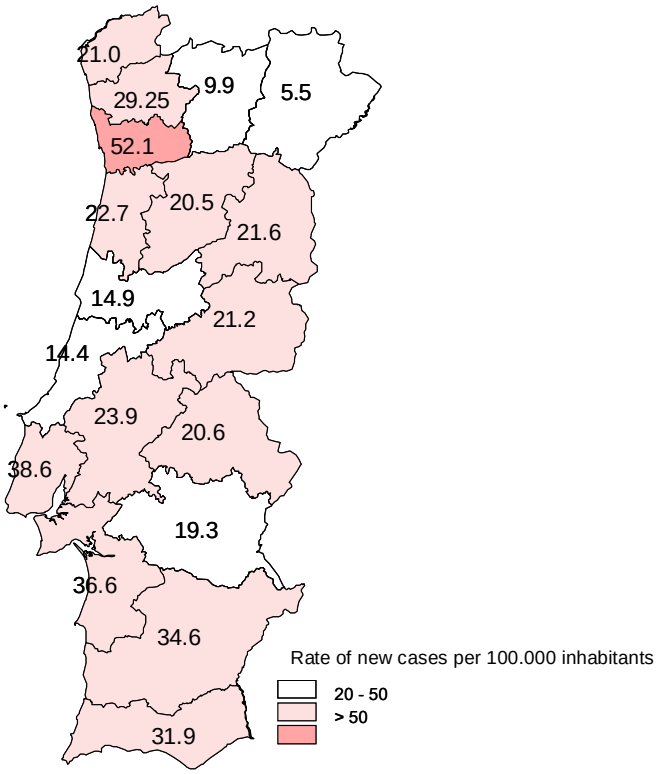


Figure 5. Rate of new cases of TB per district of Portugal. The islands account for 17.6 and 14.1 for Madeira and Açores respectively (reproduced and adapted from DGS, 2006).

One important aspect is the immigration. In Portugal, the proportion of cases in immigrants is of 12%, much lower than the one in the EU (28% in 2004) (DGS, 2006). The proportion of TB cases in immigrants is small compared to other EU countries like Belgium, Cyprus, Denmark, Malta, Holland, Sweden and United Kingdom, were there is an incidence of more than 50% of cases in immigrants. The incidence rates of TB in immigrants in the EU are 12 times higher in the foreign population (WHO, 2006b). In Portugal, this rate is 3 times higher relatively to the incidence in the national citizens (DGS 2006). These differences are also due to the origin of the immigrants. In the EU the majority of cases are from citizens from Africa (32%), Asia (30%) and Central and East Europe (25%) (WHO, 2006b). In Portugal, the vast majority is from the Sub-Saharan Africa (70%). From Asia and East Europe there is the notification of 6 and 9% of the cases, respectively (DGS 2006). One of the most important factors is undoubtedly the number of HIV cases. The HIV infection contributes to the risk of reactivation of TB, being an important demographic factor to consider (WHO, 2006a). In comparison with the EU, Portugal is the country with more cases of TB/HIV (15% in 2005), what it is correlated with the highest incidence of AIDS (9.9/100,000 inhabitants in 2004) (DGS 2006). In general, in the other countries of the EU the number of cases of TB/HIV has been stable with the exception of Estonia and Latvia (WHO, 2006b). In Portugal, the absolute number of cases has been reduced to 30% in the last years. However, in the population infected with HIV the estimated risk of TB is 15 times higher. In 2004, in 11.6% of all TB cases, this was the disease considered has initial indicative of AIDS (against 2.9% in the EU) (DGS 2006). Despite the progress recorded in the last years, the HIV testing in the TB cases is still a poor marker of performance (around 50%) (Post and Emerson, 2008). The co-infection with TB/HIV is very high in the foreign populations (26%) and in the district areas of Lisbon (23%) and Setúbal (24%) (DGS, 2006). However, other demographic factors contribute to this epidemiology, such as the numbers of individuals that are in prisons (estimated risk of 26 times) (Marques *et al*., 2006), the drug addicts (estimated risk of 15 times) (March *et al*., 2007), the health care workers (estimated risk of 1.5 times) (Banhart *et al*., 1997) and the homeless (no estimated risk available) (DGS 2006). The individuals that are in prisons constitute a group with a very increased risk, because of the dynamic of the transmission (Post and Emerson, 2008). This population infected with TB constitute 3.2% of the total of notified cases in 2005 with medium ages of 30 years and presenting a high incidence of MDR-TB (12%), high percentage of foreigners (20%), a big

proportion of co-infection with HIV (55%) and consumers of injectable drugs (61%) (DGS, 2006). The proportion of pulmonary TB in Portugal is 73% and similar to the EU, that is 77%. In terms of distribution of TB the extrapulmonary form more notified is the pleural TB (13%), followed by the lymph node TB (7%) (Marques *et al*., 2006). The meningeal (1.3%) and disseminated (3%) forms are concentrated mainly in the group of 25 to 44 years and they are associated with the HIV infection in 50 and 65% of the cases, respectively. The proportion of cases of pulmonary TB with positive microscopy in the sputum is much higher in Portugal than in the other countries of the EU (65% against 43%) (DGS, 2006; WHO, 2006b). The confirmation of pulmonary cases by culture, is in the total of the EU (60%), slightly lower than in Portugal (64%). The major difference is the extrapulmonary forms with 18% (against 37% in the EU). Relatively to the identification of the *M. tuberculosis* complex, this is just known in 42.4% of the cases in our country, against 74% in the EU (DGS, 2006).

I.4 TB diagnosis

Rapid and accurate diagnosis of symptomatic patients is a major goal for the TB control strategies (Perkins, 2000). To help control the spread of TB, rapid diagnostic is desirable (Bergmann and Woods, 1996). Therefore, the clinical laboratory has a major role in the control of TB, inasmuch as an effective management of TB patients from areas that have high rates of MDR-TB is dependent on the rapid identification of *M. tuberculosis* complex strains as well as their antibiotic susceptibility profiles (Ridderhof *et al*., 2007). Deficiencies in current case-findings tools in disease endemic countries have made it difficult to ensure access to good diagnostics at all health service levels, leaving many patients undiagnosed. Additionally, in well-established TB control programs where diagnostic access has been ensured, efforts to interrupt disease transmission have been hampered by the insensitivity and late detection of smear microscopy (Perkins, 2000). Some methods are being developed and others remain in usage and will be described in the following sections.

I.4.1 Smear microscopy

The primary laboratory tool supporting case detection in the vast majority of cases in disease endemic countries is still the microscopic examination of the stained sputum smear (Perkins, 2000). The advantages of the acid-fast staining of smears are well known. It is relatively inexpensive to perform; is a rapid technique; and detects the most infectious subset of patients (Perkins, 2000). However, it has a low sensitivity since it needs approximately 10^4 bacteria/mL of specimen for a positive result. Other disadvantage is the fact that it does not differentiate between species of *Mycobacterium* (Bergmann and Woods, 1996). The differentiation of *M. tuberculosis* from other mycobacteria represents an important health issue, since only TB is transmissible from individual to individual (Heifets and Good, 1994). It also requires equipment that is difficult to maintain in field settings and yields results that depend upon a trained technician. An additional problem aroused with the increase in the number of cases of smear-negative disease in patients infected with HIV (Perkins, 2000).

I.4.2 Culture

Culture remains the gold standard for the diagnosis of TB (Tortolli and Marcelli, 2007). The growth of the organism on solid or liquid media can take up to 6 to 8 weeks, depending on the method used (Bergmann and Woods, 1996). Radiometric liquid culture systems have been currently used in level II or III Mycobacteriology laboratories for more than a decade due to their speed and sensitivity (Perkins, 2000), being BACTEC 460 TB system one of the best known successful examples. However, the difficulty in working with radioactive materials, the necessity of expensive apparatus for the detection of radioactive gas and the cost of materials constitute primary obstacles (Perkins, 2000). Alternative growth detection methods for liquid culture employing oxygen quenching and redox reagents have been commercialized and demonstrated performances comparable to BACTEC 460 TB, such as the case of BACTEC MGIT 960 system (Gumber and Whittington, 2007; Perkins, 2000; Piersimoni *et al*., 2006). It is unquestionable the importance of the routine culture of acid-fast bacilli since it is still necessary for its higher sensitivity and ability to identify mycobacterial species other than *M. tuberculosis* complex and for the recovery of isolates for antimicrobial susceptibility testing (Zheng *et al*., 2001). However and despite all the improvements

made in recent years it still requires, on average, 2 to 3 weeks for positive samples to be detected (Tortolli and Marcelli, 2007). Amplification techniques can therefore, be used in order to support and accelerate an earlier diagnostic, enabling rapid implementation of treatment and minimizing the risk of contagion (Tortolli and Marcelli, 2007).

I.4.3 Biochemical methods

Biochemical methods were, for many years, the only available tool to identify *Mycobacterium* species after isolation in culture. The development of these methods was focused on the identification of the *M. tuberculosis* complex. When the determination of *Mycobacterium* species is required after a preliminary report of *M. tuberculosis* complex, a few tests are necessary for differentiation from other species in the complex. A combination of biochemical tests may be used to identify and differentiate mycobacteria within the *M. tuberculosis* complex. These include: the niacin test, the nitrate reduction test, the pyrazinamidase test, the thiophene-2-carboxylic acid hidrazide (TCH) and the heat-stable catalase test (Heifets and Good, 1994). The niacin test is based on the detection of niacin in the medium, not in the bacteria. The niacin in the medium is extracted and subsequently detected by the use of a commercial strip used to detect this compound. After an incubation period the development of colour is obtained. The test is considered positive if the liquid turns yellow. Formerly the niacin test was developed for identification of *M. tuberculosis* but it can be positive with other mycobacterial species such as *M. simiae* and some BCG strains among others (Burgos *et al*., 2004). Other biochemical test that can be used is the nitrate reduction test. This test is based on the identification of the enzyme nitrate reductase that is produced by a variety of species like *M. tuberculosis*, *M. kansasii*, *M. fortuitum*, among others. This is useful after the initial identification of the *M. tuberculosis* complex. The test is based on the addition of several reagents with the main goal of colour development with the addition of N-napthylethylenediamine that can be compared to a series of colour tube standards and reactions from 3 quality controls. The pyrazinamidase test detects the presence of the enzyme that converts PZA to pyrazinoic acid. Pyrazinamidase can be found in cultures of *M. tuberculosis* strains susceptible to PZA but PZA-resistant *M. tuberculosis* strains do not possess detectable amounts of this enzyme. *M. bovis* strains including BCG are resistant to PZA as well as other non-tuberculous mycobacteria. The principle is the addition of biochemical

reagents in order to observe the development of coloured reactions, indicatives of a positive result. Only *M. bovis* strains (including BCG) are susceptible to TCH. This test is performed in the same way as an indirect susceptibility test and all mycobacterial species other than *M. bovis* produce growth in the presence of TCH (Heifets and Good, 1994). The heat-stable catalase test identifies strains of the *M. tuberculosis* complex but it has no role in differentiating *M. tuberculosis* from *M. bovis* since both species produce negative results. This test is useful to differentiate between *M. gastri* and *M. malmoense* from other slowly growing non-chromogenic mycobacteria. When the primary isolate is identified as *M. tuberculosis* complex by one of the rapid methods, the differentiation between members of the complex can be limited to the other four biochemical tests described. Although these are simple and rapid tests, in fact they are not accurate and sometimes present false-positive results or crossed results with other mycobacterial species. This has contributed to the replacement of these tests by other identification methods (Burgos *et al*., 2004; Hazbón 2004; Millar *et al*., 2007).

I.4.4 Chromatographic methods

Chromatographic analysis of cell wall lipids of *M. tuberculosis* can be performed by High Performance Liquid Chromatography (HPLC), Gas Liquid Chromatography (GLC) or Thin-Layer Chromatography (TLC). These methods are able to differentiate between *M. tuberculosis* and other mycobacteria and also expedite determination of the species of the non-tuberculous mycobacteria (Burgos *et al*., 2004). HPLC uses a liquid mobile phase at high pressure to carry a sample through a column packed with particulate material or stationary phase, where the separation into components take place (Heifets and Good, 1994). Mycolic acids extracted from saponified mycobacterial cells are converted to the p-bromophenacyl esters, and the unique mycolic acid pattern associated with each species is detected by chromatographic separation of the esters. The method has been used for accurate identification of eight slowly growing mycobacterial species: *M. tuberculosis, M. bovis, M. kansasii, M. szulgai, M. gordonae, M. asiaticum, M. marinum* and *M. gastri* (Heifets and Good, 1994). The analysis is based on a comparison of retention time of the peaks and of their height ratios. Despite all the simplifications of the method to examine a large number of cultures in one working day, approximately 10^6 colony forming units (CFU) are required to perform the test. BACTEC cultures cannot be used directly in HPLC, since that the usual harvest of

M. tuberculosis from 12B broth cultures may not be sufficient for this procedure (Siddiqi *et al*., 1981). In comparison with other detection systems like the nucleic acid probe systems it requires specialized instrumentation and technicians as well as a longer period of time. GLC uses hydrogen in the mobile phase and liquid in the stationary phase. The analysis of the microbial short-chain fatty acids (methyl esters) is based on the comparison of the retention time of the tested sample to the retention times of known standards. GLC analysis of the cell wall lipids for mycobacterial identification has been successfully used in several laboratories by employing a commercially available chromatograph and a computer (Heifets and Good, 1994). The library of aerobic bacteria includes the cell wall lipid patterns of 26 or more mycobacterial species. Profiles of the tested cultures are compared with those in the library, and the results are presented by a computer-generated report giving the actual parameters of the chromatogram as well as percentages of probability that the isolate belongs to a certain species. The main disadvantages of these methods are that usually they require a substantial bacterial harvest from solid media. To have a positive identification usually takes 3 weeks or longer (Heifets and Good, 1994) and it is demanding the use of specialized instrumentation and skilled technician, most of which conditions are not available in a Mycobacteriology laboratory.

I.4.5 Phage systems

Clinical diagnostic applications have been spawned by basic mycobacteriophage research. One is a phage replication system that detects live mycobacteria in clinical samples or in fresh liquid cultures using phages that infect and replicate in mycobacterial cells, as indicators. However, the technical skills requirement of such tests, which can be used both for case detection and drug susceptibility testing are likely to be at least as high as those for conventional culture, which will limit the number of laboratories in which this might be applied. Luciferase reporter phages have also been engineered which may be pressed into service for rapid drug susceptibility testing (Perkins, 2000).

I.4.6 Nucleic acid – based methods

I.4.6.1 Probe hybridization and Polymerase Chain Reaction (PCR)

One of the most important technical advances for TB laboratories in industrialized countries has been the development of nucleic acid – based identification assays. Most of these assays use hybridization and/or amplification procedures. The fist one is based on the hybridization between a particular region of the mycobacterial DNA or RNA and a single-stranded probe, which targets specific region(s) of the genome (Heifets and Good, 1994). The principle of this reaction - the complementarities between the nucleotide sequences of nucleic acids - is the same behind the annealing of primers to target regions needed for nucleic acid amplification by the polymerase chain reaction (PCR). While the hybridization procedures introduced speed, but most important, high reliability to the identification methods by identifying accurately mycobacteria present in the culture, the PCR procedure, which exponentially amplifies the amount of targeted DNA, further allowed detecting mycobacteria directly from specimens. PCR as a diagnostic tool for identification of mycobacteria is being developed in various technological modifications. The major advantage of this technique is that PCR can detect just a few bacterial cells in a raw specimen, which means that a molecular diagnosis of TB can be completed in a few days or even hours after the arrival of the specimen to the laboratory instead of the period required currently. Another advantage is the possibility of diagnosis with specimens having a number of bacteria below the levels of sensitivity of culture methods.

I.4.6.2 Commercial available tests

Until the present date, US Food and Drug Administration (FDA) have approved two tests for the direct detection of *M. tuberculosis* in respiratory specimens: the Gen-Probe AMPLIFIED™ *Mycobacterium tuberculosis* Direct (MTD; Gen-Probe Inc., San Diego California) Test and the Roche AMPLICOR® *Mycobacterium tuberculosis* (AMPLICOR MTB) test (Roche Molecular Systems Inc.; Branchburg, N.J.). In 1998, a modified version of the MTD test that is faster and more sensitive than the previous version was approved by the FDA. The MTD test utilizes transcription-mediated amplification to detect *M. tuberculosis* complex rRNA directly in respiratory specimens

(Zheng *et al*., 2001). According to the manufacturer's it can detect *M. tuberculosis* complex rRNA at a concentration equivalent to 5 CFU per test and does not react with 150 species of microorganisms (including respiratory flora) at the level of 5×10^7 organisms per reaction. It is intended for use with specimens from patients that show signs and symptoms consistent with active pulmonary TB. The test should be used in addition to acid-fast bacillus smear and culture but it can provide results in 2.5 to 3.5 hours. The AMPLICOR MTB test is also based in nucleic acid amplification, allowing the evaluation of respiratory specimens (Bergmann and Woods, 1996). It identifies the presence of genetic information unique to *M. tuberculosis* complex directly in pre-processed clinical samples. The nucleic acid amplification technique is used to obtain sufficient nucleic acid so that, within a few hours, these tests can distinguish between *M. tuberculosis* complex and non-tuberculous mycobacteria in an acid-fast bacilli positive specimen. These tests are recommended only for respiratory specimens, smear positive for acid-fast bacilli, from persons who have never received anti-TB treatment or have received less than 7 days of anti-TB treatment during the past year (ATS, 2003). A positive direct amplified test in conjunction with an acid-fast bacilli positive smear is highly predictive of TB disease. However, the results of the nucleic acid amplification tests are preliminary, since the mycobacterial culture is still needed for species identification and confirmation as well as for drug-susceptibility testing. Negative nucleic acid amplification with an acid-fast bacilli positive smear indicates that the acid-fast bacilli are probably non-tuberculous mycobacteria. However, they present some limitations. The MTD and MTB tests are highly sensitive and specific for *M. tuberculosis* on specimens that are smearing positive for acid-fast bacilli. Occasionally they can give false negative or false positive results. The result can be falsely negative if there are substances in the specimen itself inhibiting the amplification so that the test does not detect the *M. tuberculosis* complex. The falsely positive result can occur in case of contamination. In addition, because the nucleic acid amplification test can amplify DNA from both viable and non-viable organisms, a positive result may be misleading. For example, the test may detect non-viable tubercle bacilli that are coughed up by individuals who have been receiving anti-TB treatment; the test result may be positive even though the treatment has decreased the likelihood that the TB is infectious. By this manner, the test should be interpreted in a clinical context and the final decision as to the use and interpretation of the MTD should be defined by the physician.

The test most widely used for identification of the *M. tuberculosis* complex is the commercially available Accuprobe (Gen-Probe, San Diego, California) system, which is based on hybridization with nucleic acid probes. This test is based on the ability of complementary nucleic acid strands to align and associate with bacterial rRNA to form a stable, specific, double-stranded complex. The chemiluminescent acridinium ester-labelled single-stranded DNA probe is complementary to the rRNA of the target species, so that the association of the two strands forms a stable hybrid. Chemiluminescence is developed by the addition of hydrogen peroxide, and the response is measured in a luminometer (Heifets and Good, 1994). Regarding identification of cultures, other probes are also available for identification of *M. avium*, *M. intracellulare*, *M. kansasii* and *M. gordonae* (Heifets and Good, 1994).

I.4.6.3 Genotype® Mycobacterium CM and Genotype® Mycobacterium AS

Within the genus *Mycobacterium* it can be distinguished between *M. tuberculosis* complex and Non-Tuberculous Mycobacteria (NTM or MOTT – Mycobacteria Other Than Tuberculosis) (Falkinham III, 1996). In view of the varying pathogenicity of some species a fast and certain differentiation is extremely important. From a medical point of view it is indispensable to make a clear and safe distinction between the *M. tuberculosis* complex and NTM and to apply adequate therapies. A late diagnosis and hence a delay in the beginning of appropriate treatment is detrimental to the successful of the treatment and facilitates transmission of the disease. Non-tuberculous mycobacteria can cause chronic mycobacterioses. Infectiousness and symptoms vary in a broad range and depend both on the pathogen as well as on the immunocompetence of the affected individuals. If the immune system is weakened, such as in the case of HIV infection or patients presenting cancer, mycobacteria can spread into the organism and lead to infections. This is a serious threat and requires rapid and appropriate treatment of atypical mycobacterioses. Consequently, the clinical significance of the NTM group members also has to be differentiated in order to avoid under- or over-treatment. On the basis of a positive solid or liquid culture the combination of two test systems Genotype® Mycobacterium CM (Common Mycobacteria) (Hain Lifescience GmbH) and Genotype® Mycobacterium AS (Additional Species) (Hain Lifescience GmbH) allow a safe and fast differentiation of more than 30 clinically relevant mycobacteria species. Both tests are based on the DNA•STRIP® Technology including an

amplification step and a subsequent reverse hybridization. The high sensitivity achieved by this combination enables the detection of even weak-positive cultures and the identification of mixed cultures from fast- and slow-growing mycobacteria. The Genotype® Mycobacterium CM allows the detection and discrimination of 13 non-tuberculous mycobacteria species and of the *M. tuberculosis* complex as a whole. In addition, a genus-specific probe identifies the presence of other mycobacterial species. In this case, the Genotype® Mycobacterium AS offers a further differentiation. Some examples of species identified other than the ones belonging to the *M. tuberculosis complex* are: *M. avium*, *M. chelonae*, *M. fortuitum*, *M. intracellulare*, *M. kansasii*, *etc*. When applying the Genotype® Mycobacterium AS, another 16 non-tuberculous species can be distinguished. The use of this test system is particularly advantageous when only the genus-specific probe is developed in the Genotype® Mycobacterium CM. The amplicon generated in the Genotype® Mycobacterium CM may also be used for the Genotype® Mycobacterium AS; it is thus not necessary to perform a second PCR. Some species that can be detected by this test are *M. simiae*, *M. smegmatis*, *M. lentiflavum*, *M. phlei*, *M. szulgai*, *etc*.

I.4.6.4 INNO-LIPA Rif. TB

A variety of diagnostics have capitalized on the recent elucidation of molecular mechanisms of drug resistance, especially to RIF, which in most settings is a good marker for MDR-TB detection and which is almost always caused by a limited number of mutations in a single gene, the *rpoB* (Perkins, 2000). Ninety-five percent of *M. tuberculosis* strains with resistance to RIF contain distinct mutations located within an 81-pb (27 codon) region of the beta (β) sub-unit of the RNA polymerase (*rpoB*) gene (Somoskovi *et al.*, 2001; Telenti *et al.*, 1993). Several methods can rapidly detect these specific mutations and thereby identify RIF-resistant *M. tuberculosis* complex strains (Kapur *et al.*, 1994; Piatek *et al.*, 1998; Telenti *et al.*, 1993; Victor *et al.*, 1999). INNO-LIPA Rif. TB (Innogenetics, Zwijndrecht, Belgium) is one of these methods. This commercial kit has not yet been approved by the US FDA, however, it has been on the market since 1997 (Rossau *et al.*, 1997). The assay is simple, convenient, cost-effective, and highly reliable when run in parallel with a conventional TB laboratory diagnostic algorithm. It can provide an answer between 24 to 48 hours after the arrival of the specimens, when adapted to direct detection from clinical samples. It is based in a

reverse-phase DNA probe system that identifies *M. tuberculosis* complex strains, detecting RIF resistance within a very short period of culture time (Rossau *et al.*, 1997). It has ten probes, from which: one probe is specific for *M. tuberculosis* complex strains, five-partially overlapping probes hybridize exclusively with the *rpoB* gene wild-type sequence and four probes hybridize with amplicons carrying the mutations R2:D616V, R4a:H526Y, R4b:H526D and R5:S531L (Rossau *et al*., 1997; Viveiros *et al.*, 2005b). The absence of hybridization of one or more of the wild-type probes indicates a mutation that can be identified by hybridization with one of the mutation probes. When adapted to the direct amplification of *M. tuberculosis* complex from clinical specimens is a useful adjunct to microscopy and culture and is used worldwide with the goal of reaching an earlier diagnosis of active TB (Tortolli and Marcelli, 2007). The availability of a system that combines the direct detection of *M. tuberculosis* complex in clinical specimens with the prediction of RIF resistance is therefore very useful, particularly because RIF resistance is, in the large majority of cases, accompanied by INH resistance (Tortolli and Marcelli, 2007). However, the major limitations found in this system are the false-negative results due to the inhibition of amplification, the absence of an internal amplification control and the fact that this assay, as well as other nucleic acid amplification assays, identifies DNA from dead mycobacteria from patients undergoing therapy (Viveiros *et al*., 2005b). The possible presence of PCR inhibitors may explain the failure of the system to amplify a number of *M. tuberculosis* complex-positive isolates, but, since INNO-LIPA Rif. TB does not include any system for monitoring inhibitors, no quantification is possible. Although the INNO-LIPA Rif. TB does not seem sufficiently sensitive to be used solely as a diagnostic tool on unselected specimens it may represent, thanks to the added value of prediction of RIF susceptibility, a valid alternative to other amplification methods on smear-positive samples (Tortolli and Marcelli, 2007).

I.4.7 The TB-Task Force – The Lisbon experience

One of the WHO recommendations for the control of TB is the rapid diagnosis of the disease, namely of MDR cases (WHO, 2006a). Following these guidelines, the **"TB Fast-Track Program"** for the rapid diagnosis of TB was implemented at the laboratory of the Unit of Mycobacteriology (IHMT/UNL) in Lisbon. This program was modelled after that of the New York State Department of Health (Frieden *et al.*, 1993; Hale *et al.*,

2000; Salfinger, 1977) and is under the supervision of the TB Task Force of Greater Lisbon. The TB Task Force of Greater Lisbon is a cooperative joint venture involving a structured net of 12 hospitals of the Greater Lisbon area and it was further extended to a second phase (now named **"Faster-Track Program"**), being employed with success to perform the rapid detection of the MDR cases (Watterson *et al.*, 1998). In Portugal, as is the case worldwide, the vast majority of *M. tuberculosis* complex strains with resistance to RIF are also resistant to INH and although monoresistance to INH is common (Ridzon *et al.*, 1998) monoresistance to RIF is rare (CDC, 1993, 2005b, 2005c; DGS, 2004; Mitchison, 1998; Portugal *et al.*, 1999; Somoskovi *et al.*, 2001). Thus, RIF resistance can be used for the identification of MDR-TB infections (Hale *et al.*, 2000; Somoskovi *et al.*, 2001). This makes possible the aggressive treatment of MDR-TB patients (with four or five drugs) while sparing non MDR-TB patients from areas with high MDR-TB frequencies from said therapy (CDC, 1993; Hale *et al.*, 2000; Parsons *et al.*, 2004); a marked reduction in the frequency of non-compliance would consequently be expected (CDC, 1993; Hale *et al.*, 2000; Mitchison 1998; Parsons *et al.*, 2004). At the laboratory of the Unit of Mycobacteriology the line probe assay INNO-LiPA Rif.TB was evaluated for the identification of *M. tuberculosis* complex strains and the detection of mutations in the *rpoB* gene linked to RIF resistance directly from acid-fast smear positive respiratory specimens obtained from patients who presented with active TB (clinical symptoms and radiological evidence). The assays were performed in parallel with conventional isolation, identification and susceptibility testing procedures routinely used in the Mycobacteriology clinical laboratory. From September 2002 to September 2003, a total of 360 acid-fast positive respiratory specimens consisting of sputa (n=318), bronchoalveolar lavage fluids (n=23), and bronchial secretions (n=19) from patients presenting with presumptive active TB were received at the laboratory; each specimen was accompanied by a physician-completed questionnaire that included pertinent patient demographics, clinical history, and MDR-TB risk factors. The patients, all from the Greater Lisbon area, ranged in age from 14 to 89 years (average, 42 years) and were mainly male (73.8%). The three major MDR-TB risk factors reported were, in order of importance, prior anti-TB treatment, contact with other MDR-TB patients, and origin from an area with a known high incidence of MDR-TB. The HIV status was determined for only 150 patients (41.7%), and of these, 82 patients were co-infected with HIV. The identification of *M. tuberculosis* and *M. avium* complex strains present in full-grown cultures in the BACTEC MGIT 960 culture system was conducted with the Accuprobe

system. A positive identification of *M. tuberculosis* was followed by susceptibility assays for RIF, INH, EMB, STR, and PZA afforded by the BACTEC MGIT 960 system. The ability to rapidly identify an MDR-TB-type infection afforded the effective management of MDR-TB, reduced the frequency of non-compliance since patients that do not have MDR-TB were treated less aggressively, and therefore contributed to the control of TB.

I.4.7.1 The contribution of the Unit of Mycobacteriology to the control of TB in the Greater Lisbon Area

As of this writing the Unit of Mycobacteriology of the IHMT via its Faster TB Track Programme has processed more than 100 MDR-TB specimen (since 2002) most of which are reported within one day of receiving the specimen. The ability to reduce the turn-around time of reporting and identifying an MDR-TB patient to one day has, in my opinion, been the main reason as to why the rates of new cases of MDR-TB have decreased from an excess of 28% in 2003 to less than 8% in 2006. Further reductions in the rate of new cases of MDR-TB are expected in the years to come. Unfortunately, because Porto does not use the system that has been developed in Lisbon, its rate of new cases of MDR-TB continues to escalate at an alarming rate (DGS, 2006).

I.4.8 Tests to detect latent infection

I.4.8.1 Tuberculin skin test (TST)

Tuberculin (also known as “old tuberculin”) was discovered by Robert Koch in 1890. The original tuberculin was a glycerine extract of the tubercle bacilli and was initially developed as a treatment for TB. However, the fact of being an unrefined product, contributed to its lack of sensitivity in the diagnosis of infection with *M. tuberculosis*. By this manner, it was considered ineffective in the treatment of TB. Clemens von Pirquet discovered that patients who had previously received injections of horse serum or smallpox vaccine had quicker and more severe reactions to a second injection. Soon thereafter von Pirquet discovered that the same type of reaction took place in individuals infected with TB, and he thus found the utility of what would become the tuberculin skin test (TST), also referred, in some countries, as the Mantoux test. The

TST continues to be used as the standard method for detecting latent infection with *M. tuberculosis*. It is widely used to support clinical and radiological findings in the evaluation of patients with suspected TB. A positive TST result can help in the decision to start treatment while bacteriological confirmation is awaited or lacking (Cardona, 2007). However, the greatest utility of a test of *M. tuberculosis* infection lies in its potential to exclude a diagnosis of TB when other tests are negative. This requires a test with high diagnostic sensitivity to minimise the possibility of falsely ruling out TB. The poor sensitivity of TST in young children and immunosuppressed people makes it impossible to interpret negative TST results in these groups. Moreover, up to 25% of immunocompetent adults with active TB may have a negative or ambiguous result (Gooding *et al.,* 2007). Multiple puncture tests should not be used to determine whether a person is infected, as this test is unreliable (because the amount of tuberculin injected intradermally cannot be precisely controlled) (WHO 2006d). The Mantoux test is now standardised by the WHO and uses purified protein derivative (PPD). PPD was obtained from the refinements of the "old tuberculin" preparation and is a combination of mycobacterial antigens obtained from *M. tuberculosis* culture and that share a large number of antigens both with BCG and with environmental mycobacteria. The test is based on the intradermal injection of 0.1 mL of tuberculin (100 Units/mL). This combination, elicits delayed-type hypersensitivity (DTH) response or type IV hypersensitivity reaction that is mediated by specific T lymphocytes. This denomination is due to the fact that these responses, caused by pre-existing T lymphocytes, take hours or days to develop. Such effectors cells function in essentially the same way as during a response to an infectious agent. When small amounts of PPD are injected, a T cell mediated local inflammatory reaction evolves in individuals who have previously responded to *M. tuberculosis*. This indicates the presence of antibodies or lymphocytes that are specific for PPD. This cell-mediated immunity can then be detected as a local response when the individual's skin is injected with a small amount of PPD. The response typically appears a day or two after the injection and consists of a raised, red and hard (or indurated) area. This induration can be measured two to seven days afterwards and disappears as the PPD is degraded (Janeway *et al.,* 1999). Since hypersensitivity responses are relatively non-specific, PPD skin testing is plagued by errors due to reader interpretation and by the need for return patient visits (Perkins, 2000). Due to all the problems involving the TST other alternative tests have been pursued and are discussed below.

I.4.8.2 Interferon-gamma (IFN-γ) detection assays

Individuals infected with *M. tuberculosis* complex strains have lymphocytes in their blood that recognize mycobacterial antigens. This recognition process involves the production of interferon-gamma (IFN-γ), a specific cytokine for cell mediated immune response (Smith *et al*., 2002). The QuantiFERON®-TB test (QFT) (Cellestis Limited, Carnegie, Victoria, Australia) was developed to measure the IFN-γ produced by T lymphocytes in whole blood after stimulation with PPD (Cellestis, 2005). This test was approved in 2001, by the US FDA, as an aid for diagnosing latent TB infection. This test is no longer commercially available (CDC, 2005a). Tests using the same methodology that the one employed for PPD hypersensitivity are problematic, since that tuberculin reactivity is such an imperfect marker, as already seen with the TST (Perkins, 2000). In 2005, the US FDA approved a new test: the QuantiFERON®-TB Gold Test (QFT-G, Cellestis Limited, Carnegie, Victoria, Australia). This test was developed to aid in the diagnosis of *M. tuberculosis* infection, whether latent or active. However, it does not distinguish between latent TB infection and active disease (CDC, 2005a). QFT-G was based on a new design for the replacement of the first-generation version of QFT tests. This enzyme-linked immunosorbent assay (ELISA) test detects the release of IFN-γ in fresh heparinized whole blood from sensitized persons. Whereas the TST and QFT measure the response to PPD, QFT-G measures the response to a mixture of synthetic peptides simulating two specific antigenic proteins that are present in *M. tuberculosis*: the early secretory antigenic target-6 (ESAT-6) and the culture filtrate protein-10 (CFP-10) (CDC, 2005a). ESAT-6 and CFP-10 are secreted by all *M. tuberculosis* and pathogenic *M. bovis* strains (Brodin *et al*., 2004). Because these proteins are absent from all BCG vaccine strains and from commonly encountered non-tuberculous mycobacteria, except *M. kansasii*, *M. szulgai*, and *M. marinum*, QFT-G is expected to be more specific for *M. tuberculosis* than tests that use PPD as the antigen (CDC, 2005a). QFT-G results can be available in less than 24 hours after testing without the need for a second visit. As a laboratory-based assay, QFT-G is not subject to biases and errors of TST placement and reading. However, errors in collecting or transporting blood specimens or in running and interpreting the assay can decrease its accuracy (Cellestis, 2005). Each of the three tests (TST, QFT, and QFT-G) relies on a different immune response and differs in its relative measures of sensitivity and

specificity. The TST assesses *in vivo* DTH (Type IV), whereas QFT and QFT-G measure *in vitro* release of IFN-γ. QFT-G is not affected by prior BCG vaccination and is expected to be less influenced by previous infection with non-tuberculous mycobacteria. In the opposite, TST are variably affected by these factors. QFT-G does not trigger an anamnestic response (*i.e.*, boosting) because it does not expose persons to antigen. Injection of PPD for the TST can boost subsequent TST responses, primarily in persons who have been infected with non-tuberculous mycobacteria or vaccinated with BCG. Compared with the TST, QFT-G might be less affected by boosting from a previous TST. QFT-G represents one type of IFN-γ release assay (CDC, 2005a). Other type is the enzyme-linked immunospot assay (ELISpot) that enumerates cells releasing IFN-γ after mononuclear cells recovered from whole blood are incubated with similar antigens. The commercially available ELISpot assay, T-SPOT.TB (Oxford Immunotec Ltd, Abingdon, UK) have been approved by the National Institute for Clinical Excellence (NICE) for diagnosis of latent TB infection and as adjunctive tests for the evaluation of patients with active TB (de Joncheere *et al*., 2006). The assay detects T cells specific for antigens in *M. tuberculosis*, which are absent from BCG and most environmental bacteria. It is therefore not confounded by prior BCG vaccination and is more specific than TST. ELISpot was found to have a sensitivity of 96% in HIV-negative adults with culture confirmed active TB, and 92% in HIV-positive patients with pulmonary TB (Meyer *et al*., 2005). These attributes suggest that this test could improve and accelerate the diagnostic evaluation of patients with suspected TB. However, clinical data on the performance of this test in TB patients and how they will impact on clinical decision-making is limited. The most relevant target population is that in which the test will actually be used in practice: patients presenting with suspected TB whose diagnoses are unknown at the time of testing (Gooding *et al.*, 2007). Despite all the evidences there is still the need for more information about these tests and time will dictate if they are sensitive and contributing to the rapid detection of TB. Consideration of the role of new diagnostics should start with the recognition that more than one diagnostic test type is needed.

I.4.8.3 Antibody detection tests

Despite all the research conducted on the humoral response to TB, serologic tests haven't demonstrated sufficient performance to be implemented in the routine of a Mycobacteriology laboratory. The accomplished knowledge about the humoral response in the last few years has been threatened by the HIV epidemic and its impact on immunity. The existing tests use well-described antigens to detect immunoglobulin G (IgG) or other Ig classes, usually in an ELISA assay format (Abebe *et al.*, 2007). However, the data available sustains that none of the commercial tests evaluated perform well enough to replace sputum smear microscopy. Thus, these tests have little or no role in the diagnosis of pulmonary TB. Lack of methodological rigor in these studies was identified as a concern. The co-infection with HIV makes most of these assays perform very poorly, detecting less than 1/3 of patients with active disease (Steingart *et al.*, 2007). Promising research developments should include the availability of highly purified and recombinant antigens, improved understanding of the heterotypic nature of the humoral response to TB and the development of multi-antigen tests that maintain high specificity; novel *M. tuberculosis* proteins identified and characterized with the assistance of the newly available genome sequence; the characterization of a number of non-protein antigens, some of which appear promising in HIV-infected patients and the development of improved and simplified test formats, including those accepting whole-blood or non-invasively collected specimens (Abebe *et al.*, 2007; Perkins, 2000).

I.5 Antimycobacterial agents

The overall goals for treatment of TB are to cure the patient and to minimize the transmission of *M. tuberculosis* to other persons. Thus, successful treatment of TB has benefits both for the individual as also to the community in which the patient resides (ATS, 1993, 2003). The emergence of strains of *M. tuberculosis* resistant to existing drugs has focused attention on the urgent need for development of new anti-mycobacterial agents. Such agents have not been perceived as a high priority by pharmaceutical companies over the last thirty years and a coordinated effort to screen general antimicrobial compound developed during this time for activity against *M. tuberculosis* may well prove worthwhile. However, as long as spontaneous resistance by

mutation to new compounds results as a consequence of selection due to faulty therapy, *etc*., the problem will remain unless the mechanism of action of new drugs bypasses mutational events leading to resistance (Di Perri and Bonora, 2004). The recent development of genetic tools for monitoring the viability of *M. tuberculosis* provides a rapid approach for this type of screening. From a broader perspective, molecular genetic tools for study and manipulation of mycobacteria provide access to a vast amount of new information about the biochemistry and metabolism of *M. tuberculosis*, and exploitation of this information has important potential in the rational development of a new generation of anti-mycobacterial agents. To avoid disruption of normal microbial flora during the prolonged course of TB therapy and to limit possible transfer of resistance factors from other bacterial genera, it is preferable that new drug targets be specific for mycobacteria. Drugs must act on a target that is essential for bacterial survival, and ideally, they should be effective against bacteria throughout their growth cycle both inside and outside mammalian cells during infection.

I.5.1 First-line and second-line anti-TB drugs

Currently, there are 10 drugs approved by the US FDA for treating TB (http://www.fda.gov/). These include the first-line drugs, INH, RIF, Rifapentine, EMB, PZA and the second-line drugs, cycloserine, ethionamide, PAS, STR and capreomycin. In addition, the fluoroquinolones, although not approved by the FDA for TB, are used relatively commonly to treat TB caused by drug-resistant organisms or for patients who are intolerant to some of the first-line drugs. The same happens with amikacin and kanamycin (WHO, 2003). Of the approved drugs, INH, RIF, EMB and PZA are considered first-line anti-TB agents and form the core of initial treatment regimens (Di Perri and Bonora, 2004). Rifabutin and rifapentine may also be considered first-line agents under specific situations. STR was formerly considered to be a first-line agent and in some instances, is still used in initial treatment. However, an increasing prevalence of resistance to STR in many parts of the world has decreased its overall usefulness (ATS, 2003). The other drugs are reserved for special situations such as drug intolerance or resistance. INH is used for treatment of all forms of TB caused by organisms known or presumed to be susceptible to the drug. It has profound early bactericidal activity against rapidly dividing cells. RIF is used for treatment of all forms of TB and it has activity against organisms that are dividing rapidly (early bactericidal

activity) and against semi-dormant bacterial populations, thus accounting for its sterilizing activity (WHO, 2003). RIF is an essential component of all short-course regimens. Rifabutin is used as a substitute for RIF in the treatment of all forms of TB. The drug is generally reserved for patients who are receiving any medication having unacceptable interactions with RIF or have experienced intolerance to RIF. Rifapentine may be used once weekly with INH in the continuation phase of treatment for HIV-seronegative patients with non-cavitary disease and/or drug susceptible pulmonary TB patients who have negative sputum smears at completion of the initial phase of the treatment. EMB is used to treat all forms of TB. It is included in the initial treatment regimens primarily to prevent emergence of RIF resistance when primary resistance to INH may be present. PZA is a nicotinamide analog prodrug which is converted into the active bactericidal form, pyrazinoic acid, by the bacterial enzyme pyrazinamidase. Its mode of action and mechanism of resistance is still not completely understood, since PZA does not appear to have a specific cellular target. Instead, exerts its bactericidal effect by disrupting the membrane energetic and acidification of the cytoplasm. However, its sterilizing activity against semi-dormant tubercle bacilli makes it one of the most important front line anti-TB drugs. And when used in combination with RIF and INH, it has a remarkable role in shortening the treatment duration from 9 months to 6 months (Singh *et al*., 2006; Hu *et al*., 2006). Relatively to the second-line drugs, cycloserine is used to treat patients with drug-resistant TB caused by organisms with known or presumed susceptibility to the agent. Ethionamide is used for patients with drug-resistant TB disease caused by organisms that have demonstrated or presumed susceptibility to the drug (WHO, 2003). STR and EMB have been shown to be approximately equivalent when used in the initial phase of the treatment with 6-month regimens. However, among patients likely to have acquired *M. tuberculosis* in a high-incidence country, the likely high rate of resistance to STR limits is usefulness (ATS, 2003; WHO 2003). Amikacin and kanamycin are two closely-injectable second-line drugs that are used for patients with drug-resistant TB whose isolate has demonstrated or presumed susceptibility to the agents. There is nearly always complete cross-resistance between the two drugs, but most STR-resistant strains are susceptible to both (WHO, 2006a). Because it is used to treat a number of other types of infections amikacin may be more easily obtained, and serum drug concentration measurements are easily available. Capreomycin is a second-line injectable drug that is used for patients with drug-resistant TB caused by organisms that have known or presumed susceptibility

to the drug. PAS is an oral agent used in the treatment of drug-resistant TB caused by organisms that are susceptible to the drug. Of the fluoroquinolones, levofloxacin, moxifloxacin, and gatifloxacin are the most active against *M. tuberculosis*. On the basis of cumulative experience suggesting a good safety profile with long-term use of levofloxacin, this drug is the preferred oral agent for treating drug-resistant TB caused by organisms known or presumed to be sensitive to this class of drugs, or when first-line agents cannot be used because of intolerance. Cross-resistance has been demonstrated among ciprofloxacin, ofloxacin, and levofloxacin and presumably is a class effect. Fluoroquinolones should not be considered first-line agents for the treatment of drug-susceptible TB except in patients who are intolerant to first-line drugs (ATS, 2003).

I.5.2. Phenothiazines

The antimicrobial activity of phenothiazines has been known for over a century. At the end of the 19th century, Paul Ehrlich described the properties of methylene blue (Kristiansen and Amaral, 1997). This tricyclic compound would later become the nucleus of a group of derived compounds that constitute the phenothiazines (Figure 6). The application of this dye at dilute concentrations was described by Ehrlich to render motile bacteria and protozoa immobile as well as inhibit the *in vitro* growth of some Gram-positive bacteria (Kristiansen and Amaral, 1997). The antimicrobial properties of this dye were not immediately exploited because of Ehrlich's formula for the treatment of syphilis and the use of sulphur-containing compounds for the management of bacterial infections. But interest in the antimicrobial properties of methylene blue remained and the compound was used to manage urinary tract infections and parasitic infections of sheep (De Eds *et al*., 1939). However, the antimicrobial properties were overshadowed by its neuroleptic properties after the demonstration that the dye caused cats to become lethargic (McMahon and Kadowitz, 1992). The effects that methylene blue presented on the central nervous system stimulated a large number of studies.

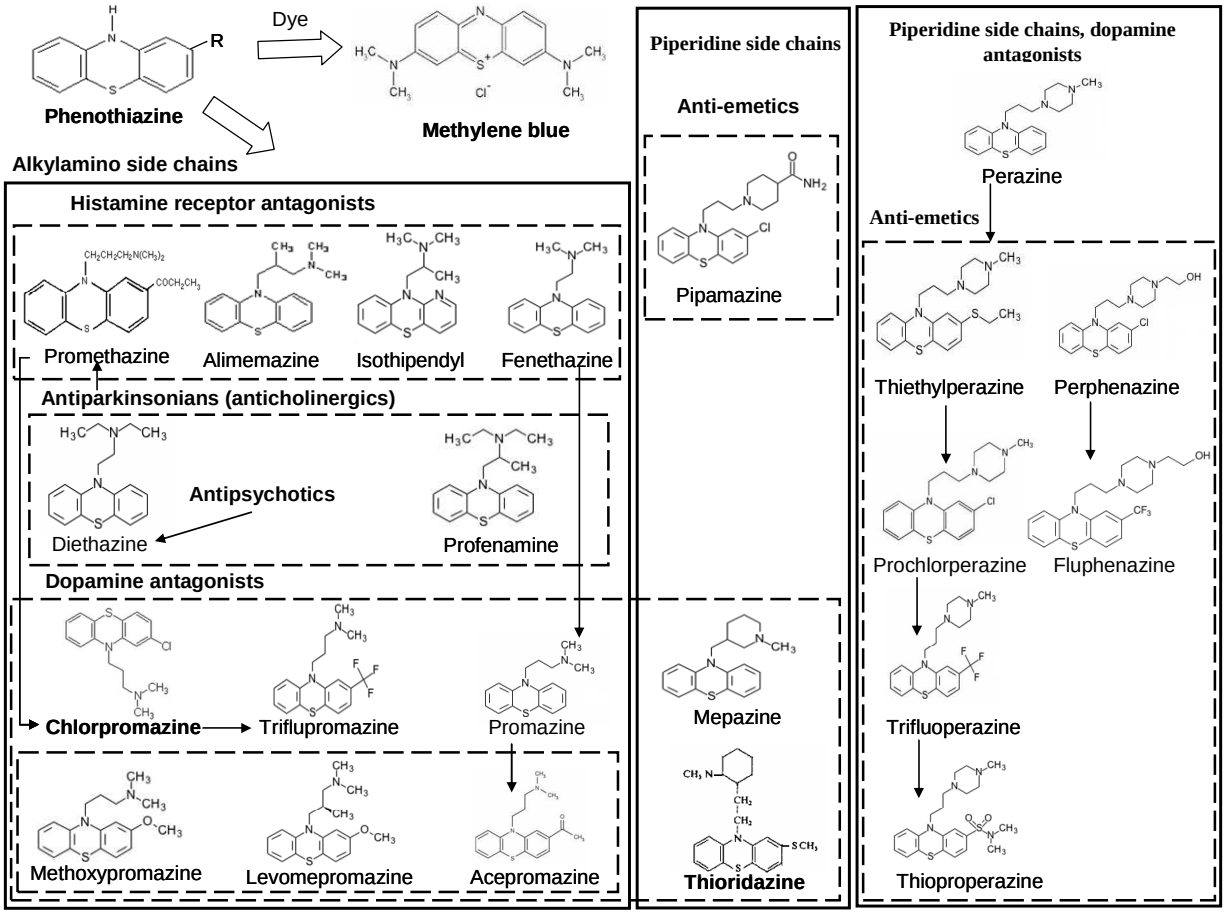


Figure 6. Molecular structures of phenothiazines (adapted from http://apropos.mcw.edu/kegg_pathways/show/267, consulted in January, 2008).

In 1952, Charpentier *et al*. associated to the French pharmaceutical company Rhone-Poulenc, developed the first antipsychotic drug obtained from a phenothiazine-derived methylene blue, chlorpromazine (CPZ). The availability of CPZ in 1957 resulted in its widespread use for the therapy of psychoses and severe neuroses. Its use in patients presenting a variety of infections was confirmed by numerous studies, sporadically reported on literature. Simultaneously, the number of antimicrobial agents grew almost exponentially. Soon became clear that CPZ had antibacterial properties, including activity against *M. tuberculosis* (Bourdon, 1961; Levaditi *et al*., 1951; Molnar *et al*., 1977). However, that time was the "Golden Age of Antibiotics" and other effective anti-TB compounds such as STR and INH were available (Amaral and Kristiansen, 2000). The extensive use of antibiotics however made the problem of resistance to emerge (Amaral *et al*., 2001b). The number of cases was so significantly increased that for many bacterial infections treatment became problematic (Amaral *et al*., 2006; Nielsen

and Black, 1999; Viveiros and Amaral, 2001). The pharmaceutical companies initially responded by making available new antibiotics. However, this was a short-time solution since that soon it started to appear strains resistant to these newly introduced antibiotics. The failure of conventional anti-tubercular therapy experienced early in the 1990s contributed to the search for new anti-tubercular drugs. This search however, didn't result in new effective compounds. Because the problem of MDR-TB primarily took place in countries that were economically disadvantaged (Loddenkemper *et al*., 2002; Raviglione *et al.*, 1993) the required incentive was not present for the creation of new and effective compounds, given the high cost of drug development and the poor market conditions present in the affected countries. This brought attention to new compounds that could be used to manage these infections and phenothiazines were again the subject of interest.

I.5.2.1 Antimicrobial activity

Phenothiazines were shown to be active against a wide range of microorganisms such as: Gram-negative, Gram-positive bacteria (Kristiansen and Mortensen, 1987) and mycobacteria regardless of their antibiotic susceptibility status (Amaral *et al*., 1996; Crowle *et al*., 1992; Gadre and Talwar, 1999; Kristiansen and Vergmann, 1986; Molnár *et al*., 1977). A wide gamut of parasitic organisms such as amoeba (Josefsson *et al*., 1975; Ockert, 1984; Schuster and Mandel, 1984; Schuster and Visvesvara, 1998), plasmodia (Chen and Hu, 1993; Kristiansen and Jepsen, 1985; Miki *et al*., 1992; Oduola *et al*., 1998; Ohnishi *et al*., 1989; Satayavivad *et al*., 1987; Tanabe *et al*., 1989), *Leishmania* (Banerjee *et al*., 1999; Benaim and Romero, 1990; Berman and Gallalee, 1985; Berman and Lee, 1983; Chan *et al*., 1998), chlamydamona (Detmers and Condeelis, 1986; Hirschberg and Hutchinson, 1980; Schuring *et al*., 1990), trichinella (Stewart *et al*., 1985), trypanosomes (Chan *et al*., 1998; Chang and Pechère, 1989; Loiseau *et al*., 1996; Paglini-Oliva *et al*., 1998), toxoplasma (Pezzella *et al*., 1997) and many other parasites was also reported. It has also been shown that phenothiazines reduce the adherence of Gram-negative bacteria to epithelial cells (Molnar *et al*., 1983, 1987). The phenothiazine promethazine prevents the recurrence of pylonephritis caused by *Escherichia coli* in pediatric patients (Molnar *et al*., 1990). Because the concentration of this phenothiazine required to inhibit the growth of bacteria is well beyond that clinically relevant, the success of the therapy is probably due to the effect of

promethazine on the adherence of *E. coli* to the epithelium of the urinary bladder. The *in vitro* as well as *ex vivo* antibacterial activities of phenothiazines most probably account for cures of bacterial infections treated with phenothiazines. Mice infected with *Salmonella enterica* serovar Typhimurium can be cured with trifluoperazine (Jeyaseeli *et al*., 2006; Mazumder *et al*., 2001) or fluphenazine (Dastidar *et al*., 1995), by a combination of trimethoprim and trimeprazine (Guha *et al*., 2000). It has been shown by Komatsu *et al*. that pre-treatment with 10-[n-(phthalimido)alkyl]-2-substituted-10H-phenothiazines or 1-(2-chloroethyl)-3-(2-substituted-10H-phenothiazines-10-yl)alkyl-1-ureas protected the animals from lethal infection of *E. coli* to various extents (Komatsu *et al*., 1997) and mice infected with mycobacteria could be cured with methdilazine (Chakrabarty *et al*., 1993). It was also demonstrated that when exposed to light, phenothiazines yield a variety of derivatives that have greater activity against bacterial infectious agents than the parent molecule (Wainwright, 1998; Wainwright *et al*., 1998).

I.5.2.2 Mode of action in prokaryotic and eukaryotic cells

Until the present date the mechanism by which phenothiazines exert their antimicrobial activity is not completely understood. However, some studies show that phenothiazines inhibit bacterial growth by inhibiting calcium transport or by interfering with the influx and efflux of potassium into bacterial cells (Kristiansen *et al*., 1982; Molnar *et al*., 1992a, 1997; Ren *et al*., 1993; Sinha and Dick, 2004). Phenothiazines may also increase the permeability of the cell wall envelope (Amaral and Kristiansen, 2000; Amaral and Lorian, 1991; Amaral *et al*., 1992; Chattopadhyay *et al*., 1998) and interfere with DNA synthesis/transcription by intercalating with DNA (de Mol and Busker, 1984; Guth *et al*., 1964; Hagmar *et al*., 1992; Izbirak, 1989; Kelder *et al*., 1991; Kochevar *et al*., 1998; Lalwani *et al*., 1995). Although this may be the case for microorganisms that do not have a cell wall, it is doubtful that these molecules can reach beyond the cell envelope of the affected cells. In bacteria, the morphological responses of phenothiazine-affected cells are remarkably similar to those produced when such bacteria are exposed to concentrations of β-lactams below their minimum inhibitory concentration (MIC). This suggests that they inhibit one or more of the penicillin-binding proteins of the plasma membrane of the bacteria (Amaral and Lorian, 1991; Kristiansen and Amaral, 1997). In mycobacteria, it was proposed that the mechanism by which phenothiazines exert their anti-tubercular activity is by inhibiting the activity of Type-II nicotinamide adenine

dinucleotide (reduced form) (NADH)-menaquinone oxidoreductase, which is an essential respiratory enzyme of *M. tuberculosis* (Yano *et al*., 2006). Although the majority of the studies reported have been primarily *in vitro*, there are a number of reports describing the antimicrobial activity of phenothiazines in humans and animals. It is documented that phenothiazines and their derivatives bind to calmodulin (CaM), the Ca^{2+}-binding protein, to prevent the influx of Ca^{2+} into eukaryotic cells (Hidaka and Naito, 1998; Ishikawa and Hidaka, 1993; Motohashi, 1991; Roufogalis *et al*., 1983). Soon after CPZ was widely used, psychotic patients presenting with cavitary infections of *M. tuberculosis* were cleared of this infection (Kaminska, 1967). The phenothiazine methdilazine was also shown to cure mice of experimental infections of TB (Chakrabarty *et al*., 1993).

I.5.2.3 Secondary side-effects

The resistance to clinical use of phenothiazines is mainly due to the side-effects associated with the long-term use of some earlier phenothiazines, such as CPZ. Severe side-effects such as hepatotoxicity and cardiac dysrhythmia do occur in some patients (Chitturi and George, 2002; Moore, 2002). Because the chronic administration of CPZ is known to produce a wide range of mild-to-severe side-effects, the use of this compound for the therapy of TB was not seriously considered (Amaral *et al*., 2007c). Long-term use of CPZ inhibits the *in vitro* phagocytosis of infectious agents (Elferink, 1979) by preventing Ca^{2+} transport (Kodovanti *et al*., 1990). The toxicity associated with CPZ further discouraged its use for antimicrobial purposes (Kaplowitz *et al*., 1986; Kodovanti *et al*., 1990). However, toxic side-effects occur infrequently with new phenothiazine neuroleptics. Thioridazine (TZ), a specific derivative of CPZ that is milder and less toxic, is also used for the treatment of psychosis. In fact, the major side effect is somnia although it is now known to produce Torsades de Pointes (Vieweg and Wood, 2004), albeit, at a very low frequency (1 sudden death over 10,000) man years of use (Glassman and Bigger, 2001).

I.5.2.4 The use of phenothiazines to treat intracellular infections

TZ has *in vitro* antimycobacterial properties, being as effective as CPZ against all encountered clinical strains of *M. tuberculosis* regardless of their antibiotic susceptibility status (Amaral *et al*., 1996). Nevertheless, the concentrations required for *in vitro* inhibition of mycobacterial growth are well beyond those achievable in the patient (Amaral *et al*., 2007c). *In vitro* activity of these compounds takes place at concentrations higher than 1 mg/L (Amaral *et al*., 1996; Courmont and Derries, 1951; Crowle *et al*., 1992). However, because phenothiazines are concentrated by tissues and organs containing large populations of macrophages (Daniel and Wojcikowski, 1999a, b, 1997b; Daniel *et al*., 2001), Crowle *et al.* demonstrated that physiological concentrations of CPZ present in the medium could enhance the killing of phagocytosed *M. tuberculosis* (Crowle *et al*., 1992). Macrophages that have phagocytosed *M. tuberculosis* can concentrate phenothiazines more than 10-fold (Daniel and Wojcikowski, 1999a, 1999b). Thus, clinically acceptable dosing of a TB patient might result in an inhibitory effect *in situ* at the intracellular level similar to that observed *in vitro* (Viveiros and Amaral, 2001). This is of great importance because a TB infection is essentially an intracellular infection of the macrophage. As the effective *in vivo* concentration against *M. tuberculosis* is the result of the drug being concentrated by the macrophage, levels of the drug employed for the management of TB would be much lower than those clinically employed for the management of psychosis, and hence no significant toxicity would be anticipated. Other problem that should be considered is the development of resistance by the bacterial strains. Because CPZ and TZ lack specificity and thus affect a large number of targets lethal to *M. tuberculosis* (Gadre *et al*., 1998; Ratnakar and Murthy, 1993; Ratnakar *et al*., 1995; Salih *et al*., 1991), the problem of resistance to these compounds may be quite small. By this manner, TZ might be useful as an adjuvant to current regimens used for the management of freshly diagnosed TB as its *in vitro* activity is equal to that of CPZ against *M. tuberculosis* (Viveiros and Amaral, 2001). Although TZ may serve a useful anti-tubercular function, its use should be limited to specific situations that limit the effectiveness of current front-line anti-tubercular drugs. For example, if a patient is newly diagnosed with a recent TB infection, and hence cavitary disease would be nominal or moderate and if the patient is most likely to be infected with a strain of *M. tuberculosis* that is MDR, and if antibiotic susceptibility data is not forthcoming within 3 or 4 weeks, then the use of this

compound is justifiable. However, although the case for the use of TZ is quite strong, and in light of the fact that it may cause no harm, it must not be used indiscriminately. Studies to provide a definition of the compound and recommendations for its use in human patients are urgently needed. Alternative strategies such as TZ derivatives should be considered. Often the chemical manipulation of the parent phenothiazine yields a molecule that is more active than the parent compound (Csiszar and Molnar, 1992; Molnar *et al*., 1991). This could constitute a good approach to obtain more efficient compounds that lack the problems initially stated for the parent molecule.

I.5.3 Other inhibitors of efflux pumps

Mycobacteria display marked intrinsic resistance to a variety of antimicrobial agents (Piddock, 2006). This property is caused by their unique cell wall structure, since the mycobacterial cell wall functions as an efficient protective barrier and limits the access of drugs to their cellular targets (Nikaido, 1994; 1998; Piddock, 2006; Poole, 2007). However, the cell wall barrier alone is not sufficient to explain the intrinsic drug resistance of these bacteria. Drug efflux is also known to contribute to intrinsic or acquired resistance (Li and Nikaido, 2004; Li *et al*., 2004; Nikaido and Zgurskaya, 1999; Piddock, 2006; Poole, 2005). In this manner, assessment of efflux pumps of a clinical isolate has been for the last few years the subject of intense research. The main goal has been the discovery and/or creation of new agents which have the capacity to inhibit MDR efflux pumps and hence, render the organism susceptible to the antibiotics to which it was once resistant (Lomovskaya and Bostian, 2006; Zgurskaya and Nikaido, 2000). Given the significance of multi-drug efflux mechanisms, as regards antimicrobial resistance in important human pathogens, such as *M. tuberculosis*, there is a need to search for new and active efflux pump inhibitors (EPIs).

EPIs isolated from plant extracts are one important source of compounds with antimycobacterial activity (Marquez, 2005). One of these examples is reserpine. This indole alkaloid was first isolated in 1952 from the dried root of *Rauwolfia serpentine* (Ruyter *et al*., 1991), which had been used for centuries in India for the treatment of insanity, as well as fever and snakebites (Mashour *et al*., 1998). Due to its antipsychotic and antihypertensive properties, reserpine has also been used for the control of high blood pressure. However, because of the development of better drugs for these purposes

and due to its toxicity, it is rarely used today (Michalet *et al*., 2007). The antihypertensive properties are a result of its ability to deplete catecholamines from peripheral sympathetic nerve endings. It almost irreversibly blocks the uptake and storage of norepinephrine, serotonin, and dopamine, normally involved in controlling heart rate, force of cardiac contraction and peripheral resistance (Buu, 1989; Verheij and Cools, 2007). When used as an EPI, reserpine potentiates the activity of fluoroquinolones on MDR Gram-positive bacteria and of tetracycline (TET) on MRSA strains (Piddock, 2006). It also decreases the emergence of MDR *S. aureus* and *S. pneumoniae* strains *in vitro* (Marquez, 2005). Unfortunately, it cannot be used in combination with antibiotics for the treatment of staphylococcal infections, since the concentrations required for the treatment are neurotoxic (Michalet *et al*., 2007). Other important feature is the known inhibition of the P-glycoprotein (Piddock, 2006).

Members of the plant family Aizoaceae, considered as one of southern Africa's most diverse and abundant plant families but also the least studied in terms of its medicinal potential (van der Watt and Pretorius, 2001) also contain alkaloids known to have narcotic-anxiolytic properties and strong synergism with psychomimetics (Smith MT *et al*., 1996). These properties are consistent with those presented by other neuro-active compounds whose activities reside primarily at the level of the plasma membrane (Williams JT *et al*., 2001). Although some of these alkaloids have been reported to have anticancer properties as well (Smith *et al*., 1996), they have received little attention, perhaps due to their reported toxicity. One such member of the Aizoaceae family is *Carpobrotus edulis* a plant that besides been found in southern Africa (van der Watt and Pretorius, 2001) is also found along the coast of Portugal and that is so prolific that it is considered a nuisance (Ordway *et al*., 2003a). Due to the antimicrobial activity of this family, *C. edulis* has also become the subject of research for antimicrobial activity (Ordway *et al*., 2003a; van der Watt and Pretorius, 2001).

Other compound usually found in the ripe seeds of the African plants *Strophanthus gratus* and *Acokanthera ouabaio*, is ouabain. It is used worldwide extensively for *in vitro* studies to block the Na^+ pump (Na^+/K^+-ATPase) (Clausen, 2003). The blockage of this pump is associated with high concentrations of the compound that are attainable *in vitro*. In the opposite, low concentrations stimulate the Na^+/K^+-ATPase (Gao *et al*., 2004). Interestingly, this compound was also identified in the human, as an endogenous

hormone (Schoner and Scheiner-Bobis, 2005), being probably an isomer of the ouabain found in plants (Gao *et al*., 2004). In the human, it is synthesized in the adrenal gland and in the hypothalamus, being augmented in conditions of oxygen deficiency. However, its exact mode of action and physiological significance is not yet determined (Schoner and Scheiner-Bobis, 2007). In France and Germany, ouabain has a long history in the treatment of heart failure, and some continue to advocate its use in angina pectoris and myocardial infarction (Gao *et al*., 2004).

Some phenols, present in green tea extracts, also possess numerous biological activities, including antimicrobial activity (Bandyopadhyay *et al*., 2005; Marquez, 2005), reversal of methicillin resistance in MRSA strains (Michalet *et al*., 2007) or inhibition of P-glycoprotein (Marquez, 2005; Michalet *et al*., 2007). Natural products isolated from Berberis plants have also been identified as inhibitors of bacterial efflux pump (Michalet *et al*., 2007). Interestingly, they potentiate the antibacterial activity of berberine, an alkaloid produced by the same plant, suggesting that plants may have evolved to produce weak antibacterial compounds associated with EPIs, to enhance their activity (Marquez, 2005; Michalet *et al*., 2007).

The calcium channel antagonist, verapamil, is a voltage-dependent Ca^{2+} channel blocker that inhibits several bacterial efflux pumps (Marquez, 2005). It has been used in the treatment of hypertension, angina pectoris, cardiac arrhythmia, headaches, being also used as a vasodilator during cryopreservation of blood vessels (Yedinak, 1993). It was approved by the FDA as an antiarrhythmic, being very effective in controlling the ventricular rate (Grossman and Messerli. 2004). Calcium channels are present in the smooth muscle that lines blood vessels. By relaxing the tone of this smooth muscle, Ca^{2+}-channel blockers dilate these vessels what has led to their use in treating hypertension and angina pectoris (Yedinak, 1993). Verapamil is also used as an inhibitor of drug efflux pump proteins, such as P-glycoprotein. This is useful as many tumour cell lines over-express drug efflux pumps, limiting the effectiveness of cytotoxic drugs (Nobili *et al*., 2006).

Disiloxanes are synthetic resistance modifiers that inhibit MDR efflux pumps in various cancer cell lines, as well as reverting MDR of *E. coli* strains. Among these, SILA compounds, showed a remarkable anti-proliferative effect and markedly inhibited the P-

glycoprotein-mediated efflux mechanism *in vitro* (Zalatnai and Molnár, 2006). Due to the activity of these compounds on the inhibition of Ca^{2+} and K^{+} flux, they could constitute a good basis for the synthesis of new agents. These new agents could be important in the activation of infected macrophages and the subsequent destruction of the internalized bacteria.

I.5.4 Limitations of drugs

The last effective anti-TB drug was RIF and this drug came into use in the early 1960's. Since TB has remained a serious medical problem in Africa, South-East Asia and South America (WHO, 2005) the need for new anti-TB drugs became more and more prominent. Although each year hundreds of compounds are reported that have activity against TB, with few exceptions, activities are restricted to *in vitro*. Other aspect is that the infection is most frequent in countries that are economically disadvantaged (Amaral *et al*., 2004) posing a problem in terms of monetary incentive. However, the resurgence of TB in Western urban centres, and more importantly, the appearance and escalation of MDR-TB and XDR-TB worldwide has caused sufficient concern. This was the basis for the development of several international programs, to make funds available for the development of new anti-TB drugs (TB Alliance, 2006). These programs may in time alleviate the problem but we must consider that creating effective anti-TB drugs is far more difficult than that for the therapy of other bacterial infections. To obtain an effective anti-TB drug two important criteria must be satisfied: i) it must penetrate the cell housing the trapped mycobacterium, and ii) it must be active at the intracellular level, when non-toxic concentrations of the drug are administered to the patient. This poses several problems, since that although thousands of compounds can inhibit the replication of mycobacteria *in vitro*, very few are effective at the intracellular site. All of these questions should contribute to a novel approach in drug design. Perhaps it would be wiser to develop drugs that enhance the killing activity of the phagocytic cells instead of the traditional approach that has been implemented. This new strategy is also important since that if we create drugs that act in the phagocytic cell instead of acting in the bacteria itself then, the problem of resistance, as is the case for conventional antibiotics, could be prevented.

I.6 Immunology of TB

I.6.1 Mycobacterial interaction with the macrophage

One of the most known associations in TB is the relationship established between the macrophage and *M. tuberculosis*. These mycobacteria exhibit specific cellular tropism for mononuclear phagocytes, *i.e.*, macrophages, monocytes and dendritic cells (Kusner, 2005). However, the success of this infection is due to the ability of *M. tuberculosis* to persist inside the host cells, namely the macrophages (Mueller and Pieters, 2006; Zhang, 2005). The alveolar macrophage is a phagocytic cell that as compared to neutrophils presents little killing activity of its own (Ordway *et al*., 2003b). This ability to survive and replicate in its hostile environment as well as the route of entry of *M. tuberculosis* into the alveolar macrophage constitute key components of the pathogenesis of TB (DesJardin *et al*., 2002; Fenton, 1998; Gomes *et al*., 1999; Schlesinger, 1996). During primary infection with the mycobacteria, aerosol-droplet nuclei containing small numbers of *M. tuberculosis* are deposited in the lungs alveoli and subsequently phagocytosed by the alveolar macrophages. Once in the alveoli environment *M. tuberculosis* can enter the macrophage by binding to a different number of receptors.

I.6.1.1 Macrophage receptors for *M. tuberculosis*

M. tuberculosis can infect macrophages via receptor mediated phagocytosis, a process that involves complement receptors (CR) (CR1, CR3 and CR4) (Vergne *et al*., 2004). The mechanism and consequences may be determined by features of the individual bacterial strain (complement dependent or independent), the environment of the macrophage (such as the availability of complement proteins), and the state of differentiation or activation of the macrophage (Ernst, 1998). Human monocyte-derived macrophages are also able to internalize virulent *M. tuberculosis* by binding of the ligand of *M. tuberculosis*, lipoarabinomannan (LAM) to the mannose receptors of the macrophages (Kang *et al*., 2005; Shimada *et al*., 2006). The expression of these receptors is down regulated by IFN-γ (Ernst, 1998); therefore, their role in ingestion of *M. tuberculosis* early in infection or in individuals with compromised cellular immunity may be more important than in established granulomas. The mannose receptors can also mediate the delivery of LAM to endocytic compartments that contain CD1b, thereby

facilitating presentation of mycolic acid and lipoglycan antigens to T cells (Prigozy *et al*., 1997). Other receptors involved in the phagocytosis process are the scavenger receptors. These receptors bind polyanionic macromolecules and particles, including lipopolysaccharides (LPS) of Gram-negative bacteria and lipoteichoic acid of Gram-positive bacteria (Ernst, 1998; Hirsch *et al*., 1994; Schlesinger, 1993; Stokes *et al*., 1993; Zimmerli *et al*., 1996). Some infected individuals have circulating antibodies to *M. tuberculosis* (Sousa *et al*., 2000) and one study has demonstrated that the intracellular trafficking of *M. tuberculosis* H37Rv opsonized with immune serum is distinct from that of non opsonized bacteria. The entry through Fcγ (Fragment, crystallisable) receptors (proteins found on the surface of certain cells, including macrophages) may have implications in distinct intracellular trafficking pathways for virulent *M. tuberculosis*. However, it does not affect the intracellular growth of the bacteria. Other route of entry into the macrophage is the direct binding of *M. tuberculosis* to the surfactant protein A (SP-A). This protein is the most abundant protein present in the surfactant monolayer of the lung, being a member of the collectin family of proteins, which includes serum mannose binding protein (MBP) and complement component C1q (Gold *et al*., 2004). This response is mediated through a direct interaction between SP-A and the macrophage (Beharka, 2002; Gaynor, 1995). It is suggested that SP-A can operate as an opsonin and modulate the activity of one or more receptors that are responsible for directly binding *M. tuberculosis* (Khubchandani and Snyder, 2001). A phosphatidylinositol glycan-linked membrane protein, CD14, is a high affinity receptor for LPS of Gram-negative bacteria. However, CD14 can also bind LAM of *M. tuberculosis* (H37Ra), and this binding induces macrophages to secrete interleukin-8 (IL-8) (Ernst, 1998). Toll-like receptors (TLRs) play a central role in activation of the cells of the innate immune system. They recognize conserved motifs in microorganisms, activating several signal cascades in order to destroy the internalized pathogens (Tjärnlund *et al*., 2006). Mycobacteria trigger different TLRs through different mycobacterial components. These components can activate cells through TLR1, TLR2, TLR4 and TLR6. TLR-activating compounds include mycobacterial lipoproteins that are thought to have an important role in the activation of immunity (Houben *et al*., 2006). LPS acts through TLR4, and the lipoprotein 19 kDa of *M. tuberculosis* act through TLR2. TLR2 is not only present on the surface of macrophages, but also within the phagosome. This suggests that chronic infection of macrophages could continuously down-regulate Major Histocompatibility Complex

(MHC) class II expression by stimulation of intraphagosomal TLR2 (Flynn and Chany, 2003). A particulate target such as *M. tuberculosis* that displays numerous and diverse ligands on its surface are likely to engage several receptors of multiple types simultaneously. Therefore, *in vivo*, *M. tuberculosis* is probably not internalized by macrophages using a single receptor-mediated pathway.

I.6.1.2 Mycobacterial phagosome

One of the main reasons why *M. tuberculosis* is successful as a pathogen is because of its ability to persist in an immunocompetent host. This pathogenic mycobacterium does not avoid capture by macrophages or tolerate the lysosomal environment. Instead, it survives the bactericidal milieu of the macrophage by blocking fusion of their intracellular niche, the phagosome, with late endosomes and lysosomes (Nguyen and Pieters, 2005). After sealing of the plasma membrane, the phagosome undergoes a sequence of changes in its composition. These alterations are triggered by a series of membrane fusion and fission events (Griffiths, 2004; Hackam *et al*., 1997; Mueller and Pieters, 2006; Yates *et al*., 2005). Incomplete luminal acidification, absence of mature lysosomal hydrolases and aberrations in trafficking of plasma membrane markers are the most prominent characteristics on the *M. tuberculosis* phagosome (Anes *et al*., 2003; Chua *et al*., 2004). In fact, pathogenic mycobacteria employ eukaryotic-like signal transduction mechanisms to modulate the host cell trafficking pathways (De Chastellier and Thillo, 2006; Kuehnel *et al*., 2001; Warner and Mizrahi, 2007). The intracellular survival of *M. tuberculosis* is due to its ability to inhibit the fusion of the phagosome with lysosomes, avoid direct bactericidal mechanisms and block efficient antigen processing and presentation (Deretic *et al*., 2006).

I.6.1.3 Modulation of the macrophage trafficking events by *M. tuberculosis*

Hosts infected with *M. tuberculosis* mount a strong immune response. This response is usually sufficient to prevent progression to active disease. The strong immune response can control, but not eliminate the bacilli, indicating that *M. tuberculosis* has evolved mechanisms to modulate or avoid detection by the host immune response. Recent advances have improved our understanding of how *M. tuberculosis* evades two major antimicrobial mechanisms of macrophages: phagolysosome fusion and the production

of toxic reactive nitrogen intermediates (RNI). Once the bacteria are phagocytosed, the macrophage activates a cascade of cellular and biochemical processes in order to destroy the internalized bacteria. The most studied antimycobacterial mechanism of activated macrophages is the nitric oxide synthase 2 (NOS2)-dependent pathway, which generates toxic RNI (Flynn and Chany, 2003). However, *M. tuberculosis* has evolved various mechanisms to evade RNI toxicity. *M. tuberculosis ahp*C encodes the peroxiredoxin alkyl hydroperoxide reductase subunit C (AhpC) protein. This peroxiredoxin has the ability to catalytically catabolize peroxynitrite anion ($ONOO^-$), a potent oxidant formed via the reaction between nitric oxide (NO) and superoxide anion (O_2^-), two products of activated macrophages. Other studies revealed that *M. tuberculosis* peptide methionine sulfoxide reductase catalyses the reduction of methionine sulfoxide (which is formed via the reaction of $ONOO^-$ and methionine residues of proteins). This might play a significant role in resistance against the toxic effect of this potent oxidant (Flynn and Chany, 2003). The pathogen-containing phagosomes undergo a series of fusion and fission events that modify the composition of their limiting membrane and of their contents. This process of phagosome maturation culminates in the formation of the phagolysosome (Vieira *et al*, 2002). Subsequently there will be acidification of the phagolysosome with the decrease in the internal pH of the cellular compartment, inducing the activation of hydrolases which in turn, afford the complete destruction of the bacterium (Anes *et al*., 2006; Jordao *et al*., 2008; Pillay *et al*., 2002; Vieira *et al*., 2002). However, in order to escape this killing process, mycobacteria have evolved strategies that control the ability of the macrophage to kill the bacterium. By this manner, to persist in the host, *M. tuberculosis* arrests the maturation of bacilli-containing phagosomes into phagolysosomes, thereby evading the antimicrobial effects of the lysosome. In addition, *M. tuberculosis* maintains an intraphagosomal environment that allows its persistence. It is thought that this is accomplished by the exclusion of vacuolar H^+-ATPases (acquired from endosomes) from bacilli-containing phagosomes that by this manner avoid an acidic environment (Hestvik *et al*., 2005; Nishi and Forgac, 2002;). Studies on the molecular basis for the trafficking of intracellular vesicles have identified the soluble N-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) proteins and the small Guanosine-5'-triphosphate (GTP)-binding protein of the Rab family capable of regulating membrane fusogenicity, involved in phagolysosomal biogenesis (Flynn and Chany, 2003). Mannosylated LAM from *M. tuberculosis* attenuates the activity of

hVPS34, a phosphatidylinositol 3(OH) kinase and inhibits the recruitment of early endosome autoantigen 1 (EEA1), a molecule that interacts with SNARE and is implicated in endosome–endosome fusion. Another means by which mycobacteria can affect phagosome maturation is through interaction with tryptophan aspartate rich coat protein (TACO). This molecule is specific for phagosomes containing live bacilli and the sequestration of the molecule by *M. tuberculosis* prevents the fusion with lysosomes (Flynn and Chany, 2003). Other mechanisms proposed for the intracellular survival of the mycobacteria are the production of high amounts of ammonia and the interference with presentation of MHC class II-restricted antigens (Flynn and Chany, 2003).

I.6.1.4 Mycobacterial components modulation of the phagosome maturation

Both host cell and mycobacterial lipids are known to modulate the intracellular fate of pathogenic mycobacteria. The lipid-rich cell wall of *M. tuberculosis* has been recognized as providing the bacilli with an effective innate immune defence against lysosomal degradation (Nguyen and Pieters, 2005). Mycobacteria produce copious amounts of complex lipids, which constitute approximately 60% of the cell wall (Vergne *et al*., 2004). Several classes of mycobacterial lipid products have been considered as potential modulator of membrane trafficking in the host cell. The acyl trehalose family includes the sulphatides and the cord factor. Sulfolipid plays a very little if any, role in mycobacterial survival in macrophages. However, a characteristic product of *M. tuberculosis*, called cord factor, which is chemically a trehalose dimycolate has been reported to inhibit Ca^{2+}-induced fusion between liposomes *in vitro*, although its mechanism of action is not known. Another mycobacterial lipid family is represented by the phosphatidylinositol derivatives that include phosphatidylinositol mannoside (PIM) and LAM. The cell wall gycolipid LAM, activates the host tyrosine phosphatase, SHP-1, in human monocytic cells. Stimulation of SHP-1 is coupled to inhibition of mitogen-activated protein (MAP) kinases and may be linked to other inhibitory functions of LAM, including reductions in levels of tumour necrosis factor-alpha (TNF-α), IL-12, and MHC class II molecules (Kusner, 2005; Roach *et al*., 2005). LAM inhibits the recruitment of EEA1 to the phagosome and subsequently its maturation. The mycobacterial Phosphatidylinositol 3-phosphate (PI3P) analog, glycosylated phosphatidylinositol LAM (ManLAM) probably blocks the PI 3-kinase dependent pathway involved in the transport of cargo between the trans-Golgi network

and the phagosome, a transport step required for phagolysosome biogenesis (Kusner, 2005; Mueller and Pieters, 2006; Nguyen and Pieters, 2005). ManLAM also inhibits the recruitment of EEA1 to phagosomes by inhibiting a cytosolic Ca^{2+} rise. This inhibitory effect of LAM on membrane fusion raises the question of how mycobacteria maintain interactions with early endosomes. Since this pathway is postulated to deliver transferrin loaded with iron, an essential nutrient to the mycobacteria that is located in the phagosomes, there must be an alternative pathway for the iron acquisition. It has been reported that PIM specifically stimulates early endosomal fusion in an adenosine triphosphate (ATP) and cytosol dependent manner. The PIM stimulated fusion shows an absolute requirement for Rabs proteins. These are small GTPases that play an important role in phagosome maturation process by recruiting and facilitating fusion events between early and late organelles of the endocytic pathway. Fusion with early endosomes is required for mycobacterial retention in early phagosomal compartments and an inadequate supply of iron is one factor in mycobacterium's inability to prevent the normal maturation process in macrophages expressing inactive Rab5 (Hestvik *et al*., 2005). The stimulator effect of PIM is insensitive to PI3K inhibition. Thus, PIM does not simply counteract LAM, but it compensates for LAM inhibition of PI3K-dependent trafficking pathways by generating a bypass membrane fusion neo-pathway independent of PI3P generation (Vergne *et al*., 2004). Additionally, there are three mycobacterial proteins that have been shown to play an important role in the survival of intracellular bacteria: i) the secreted acid phosphatase of *M. tuberculosis*, SapM; ii) the isocitrate lyase (ICL) and iii) protein kinase G (PknG). SapM could be responsible for the depletion of PI3P at the mycobacteria phagosomes. ICL is upregulated following uptake of mycobacteria and catalyzes an essential reaction of the glyoxylate shunt pathway, which is mobilized when bacteria are grown in fatty acids as the limiting carbon source (Ehrt and Schnappinger, 2007; Mueller and Pieters, 2006). PknG is a mycobacterial virulence factor that is delivered into the cytosol of host cells and might interfere with the regulation of phagosome-lysosome transfer. In the absence of this enzyme pathogenic mycobacteria are readily delivered to lysosomes and degraded. This could be considered for the development of future drugs (Nguyen and Pieters, 2005).

I.6.2 The role of Ca^{2+} signalling in the macrophage

Upon ingestion of complement or antibody opsonized organisms, macrophages respond with a significant increase in cytosolic Ca^{2+} (Kusner, 2005). This signal is necessary for subsequent events, including the respiratory burst that generates reactive oxygen species and the maturation of phagosomes to phagolysosomes. However, live, virulent *M. tuberculosis* infect human macrophages without triggering an increase in cytosolic Ca^{2+}. This block in macrophage Ca^{2+} signalling requires mycobacterial viability since virulent *M. tuberculosis* that have been killed by heat treatment or gamma irradiation induce normal elevations in the level of cytosolic Ca^{2+} and their encompassing phagosomes mature to phagolysosomes (Kusner, 2005). Usually, the phagosome maturation is determined by acidification and acquisition of lysosomal protein markers (Pillay *et al*., 2002). Cytosolic Ca^{2+} signalling has a causative role in the pathogenesis of TB. Addition of extracellular ATP results in the maturation of phagosomes to phagolysosomes and decreased viability of intracellular mycobacteria (Stober *et al*., 2001). Characterization of the biochemical mechanisms responsible for this enhancement of macrophage immunity to *M. tuberculosis* revealed an absolute requirement for increases in the cytosolic Ca^{2+} triggered by stimulation of plasma membrane P_2X_7 receptors for ATP. Since this class of ATP receptors is largely restricted to macrophages and dendritic cells it may be possible to physiologically enhance the host immune response either alone or in conjunction with antibiotic therapy (Kusner, 2005). Killed *M. tuberculosis* triggers a complement receptor-dependent rise in cytosolic Ca^{2+} via activation of the macrophage enzyme, shingosine kinase. Through catalyzing the conversion of shingosine to the bioactive lipid, shingosine kinase functions to increase levels of cytosolic Ca^{2+} stimulating its release from the endoplasmic reticulum, where it is usually stored. This stimulation is accompanied by translocation of the enzyme from the cytosol to the region of the nascent phagosome. Live virulent *M. tuberculosis* inhibits macrophage shingosine kinase in human macrophages, *ex vivo*. This inhibition of shingosine kinase activity is accompanied by failure of the enzyme to be recruited to phagosomes containing live tubercle bacilli (Thompson *et al*., 2005). By this manner, sphingosine kinase could be considered as a novel molecular target of pathogenesis in TB, whose inhibition by live virulent *M. tuberculosis* results in evasion of Ca^{2+}-dependent phagosomal maturation and bactericidal activity. Increases in the level of macrophage cytosolic Ca^{2+} during

phagocytosis of complement-opsonized particles, including killed *M. tuberculosis*, results in activation of the major Ca^{2+} effectors protein, CaM. This activated Ca^{2+}-CaM complex translocates from the cytosol to the membrane of the nascent phagosome. CaM is a necessary component of Ca^{2+} dependent antimicrobial activity in human macrophages. The Ca^{2+}-dependent increase in phagosomal CaM is coupled to localized activation of Ca^{2+}-CaM-dependent protein kinase II (CaMKII) on the phagosome surface. Similar to blockade of CaM, specific inhibition of CaMKII activation results in decreased phagosome maturation. In fact, inhibition of either CaM or CaMKII blocks phagosome maturation despite physiologic or pharmacologic increases in cytosolic Ca^{2+}, demonstrating that the CaM/CaMKII pathway is a major regulator of phagosome-lysosome fusion. In the case of live *M. tuberculosis*, inhibition of macrophage Ca^{2+} signalling results in a lack of activation of cytosolic CaM and impairment of its recruitment to the phagosome membrane. In the absence of the activated Ca^{2+}-CaM complex, there is a lack of conversion of CaMKII from its inactive, non phosphorylated state to the activated, phosphorylated form of the enzyme that is required for subsequent phagosome maturation. This implicate specific inhibition of a phagosome localized Ca^{2+}-CaM-CaMKII signalling complex in the pathogenesis of TB leading to promotion of the intracellular survival of *M. tuberculosis* within human macrophages. The mechanism by which the Ca^{2+}-CaM-CaMKII pathway regulates the maturation of phagosomes to phagolysosomes is by linking it to components of the phosphoinositide-dependent signalling cascades. A phosphatidylinositol (PI)-specific PI 3-kinase, hVPS34, is recruited to the membrane of nascent phagosomes. This kinase catalyzes the production of the lipid second messenger, PI 3-phosphate, on the early endosomal and phagosomal membrane (Nguyen and Pieters, 2005). PI 3-phosphate is required for the recruitment of proteins, including EEA 1 that regulate fusion of phagosomes with vesicles of the endosomal-lysosomal pathway. Inhibitors of PI 3-kinase, which block the generation of phagosomal PI 3-phosphatase and the recruitment of EEA1, inhibit phagosome maturation. Normal functioning of the Ca^{2+}-CaM-CaMKII pathway is required for the hVPS34/PI 3-P/EEA1 cascade since inhibitors of CaM or CaMKII cause loss of phagosomal PI 3-P and EEA1. The phagosomes containing the mycobacterial vaccine strain BCG, or latex beads coated with LAM from *M. tuberculosis*, do not accumulate EEA1. Since *M. tuberculosis* infection modulates the macrophage transcriptome, it can be hypothesized that mycobacterial-induced alterations in Ca^{2+}-mediated signal transduction contribute to these effects on gene

regulation. A second physiologic process in which Ca^{2+} normally plays a major regulatory role, and which is modulated in infected macrophages by *M. tuberculosis* is apoptosis. Thus mycobacterial-induced deregulation of cytosolic Ca^{2+} signalling may contribute to the bacilli's inhibition of macrophage apoptosis, which promotes the intracellular survival that is characteristic of *M. tuberculosis*-macrophage interactions. Finally, inhibition of host Ca^{2+} responses may impair macrophage anti-tuberculous mechanisms that operate synergistically with phagosomal maturation, including the generation of reactive oxygen and nitrogen intermediates (Kusner, 2005; Nguyen and Pieters, 2005).

I.6.3 Cellular immune responses to *M. tuberculosis*

The interaction of *M. tuberculosis* with the cells of the immune system is a complex process. However, the sequence of events that follows the infection is relatively well defined. It all starts with the inhalation of droplets containing the infectious bacilli. Once in the pulmonary milieu, alveolar macrophages ingest the bacilli and often destroy them. However, this destruction usually depends on two factors: the intrinsic microbicidal capacity of host phagocytes and the virulence factors of the ingested mycobacteria (van Crevel *et al*., 2002). If this process is not controlled, mycobacteria will replicate inside the alveolar macrophages. The subsequent spread of bacilli to regional lymph nodes in the lungs and presentation of antigens to the host immune system cells will occur. How this develops still remains understood, but it may involve the migration of macrophages containing *M. tuberculosis* across the alveoli to lymph nodes (Saunders and Cooper, 2000). This induces the attraction of blood monocytes and other inflammatory cells to the lung. These monocytes will differentiate into macrophages which again readily will ingest but not destroy the mycobacteria. In this stage, mycobacteria grow logarithmically and blood-derived macrophages accumulate, but little tissue damage occurs (van Crevel *et al*., 2002). In the majority of cases, the host develops a protective response to *M. tuberculosis* infection that involves containment of the tubercle bacilli by the formation of granulomas that contain aggregated and fused (giant cells), apoptotic, infected macrophages (Saunders and Cooper, 2000). Two to three weeks after infection, T-cell immunity develops, with antigen-specific T lymphocytes arriving and proliferating within the early lesions or tubercles. Posterior activation of macrophages to kill the intracellular mycobacteria will

occur and as a consequence of that the early logarithmic bacillary growth stops. Central solid necrosis in these primary lesions inhibits extracellular growth of mycobacteria. As a result, infection may become stationary or latent. In most cases the individual is asymptomatic and non-infectious. This latency often extends for the lifetime of the individual. In latent infections, mycobacteria could be in a dormant non-replicating state, actively replicating but killed off by the immune response or metabolically altered with limited or infrequent replicative cycles (Flynn and Chan, 2001). However, perturbations of the immune system such as infection with HIV, poor nutrition, treatment with corticosteroids, aging, stress, alcohol and drug abuse, increase the potential for reactivation of latent TB (Flynn and Chan, 2001; Russell, 2007). More mature-phase granulomas show marked neovascularization and develop an extensive fibrotic capsule that delineates the margin between macrophages, granulocytes, foamy macrophages and giant cells, and the lymphocytic infiltrate. In the last stage, the centre of the granuloma loses its vascular appearance and becomes necrotic. Disease may progress and haematogenous dissemination may take place after primary infection, as well as months or years afterwards, as defined as post-primary TB, under conditions of failing immune surveillance. In a progressive lesion, the necrosis precedes and probably facilitates caseation (Russell, 2007). Both host and mycobacterial factors contribute to caseation. Under intense cytokine and direct cell-cell activation, macrophages and epithelioid cells undergo necrosis and/or apoptosis to form this material (Saunders and Britton, 2007). Liquefied caseous foci provide excellent conditions for extracellular growth of *M. tuberculosis*. Cavity formation may lead to rupture of nearby bronchi, allowing the bacilli to spread through the airways to other parts of the lung and the outside environment.

I.6.3.1 Experimental models of TB infection

Animal infectious disease models are fundamental to provide insight into the pathogenesis of TB. The animal model allows the identification of active drugs, access the effectiveness of drug combinations, and demonstrate the prevention of the selection of resistant mutants as well as to define the duration of the treatments (Lounis *et al*., 2003). Other important application is the analysis of the cytokine profile in inflammatory cell populations as well as the evaluation of antimicrobial agents relatively to their prophylactic or therapeutic efficiency. Testing of antimicrobials using

animal models is essential to establish the link between *in vitro* studies and clinical application. In experimental pulmonary infections, a variety of animal's species can be used. Examples are: mice, rats, rabbits, guinea pigs, hamsters and ferrets (Bakker-Wounderberg, 2003). The choice of animal species is an important determinant in the experimental studies conducted. Rabbits provide the closest facsimile of human TB in terms of tissue pathology and disease progression (Gomez and McKinney, 2004). However, this is not a model widely used probably due to its economic and logistical demanding. Other example, the guinea pigs, is important to mimic the acute disease that is obtained in TB, since these animals develop a granulomatous, extensive caseous necrosis or cavitation and death (Bakker-Wounderberg, 2003), processes that dot not occur in mice. However and despite all the advantages mentioned above, the most popular model, the inbred mouse, has a long history of research focused on the immunology and chemotherapy of infectious diseases (Gomez and McKinney, 2004). This intense research is due mainly to the cost of the animals that are relatively low, and their relatively easy maintenance. In addition, there is also a large knowledge available about mouse genetics and immunology. Reagents, such as antibodies, are also widely available (Bakker-Wounderberg, 2003). All of this has provided a large amount of information in terms of predicting mechanisms of immunity subsequently shown to be operative in humans (Orme, 2003). However, it should be highlighted that a good animal model of infection should mimic the infectious process that occurs in man. Sometimes this is not possible, since the primarily portal of entry of the infectious microorganisms are most of the times artificial and cannot be correlated with the infectious route that is observed in the human. Additionally, animals and humans differ substantially in many aspects such as the susceptibility to infectious agents, immunological status and antibiotic pharmacokinetics (Bakker-Wounderberg, 2003). Despite all the mentioned factors one of the major difference in the mouse model is a consequence of its size. The bronchial tree is far less complex in mice and there are marked differences in the vasculature. As example, mice do not present a second circulatory supply to bronchial associated lymphoid tissue. Also, the lymphatics are less complex with limited drainage into the alveolar regions (Orme, 2003). However, the mouse is a useful tool for understanding and revealing the bacterial factors that are essential in the infectious process and for probing the antigenic and cellular basis of a protective immune response (Russell, 2007).

I.6.4 Cytokines and chemokines in *M. tuberculosis* infection

One of the major problems in the persistence of infection with TB is that once inside the macrophage *M. tuberculosis* is protected from the responses induced by the immune system. Nevertheless, in order to destroy the bacteria, the infected macrophage initiates a cascade of biochemical and cellular events all of which lead to the recruitment of other cells involved in cellular immunity. The initial event subsequent to the phagocytosis of bacteria by the macrophage is the release of cytokines. This cytokine network plays a crucial role in the inflammatory response and in the outcome of mycobacterial infections (Flynn and Chan, 2001). Some examples are IL-6 and IL-12 that attract T helper (Th) lymphocytes to the site of infection. When arriving to the site of infection Th lymphocytes release cytokines, namely, IFN-γ, one of the most potent macrophage-activators. Although IFN-γ production alone is insufficient to control *M. tuberculosis* infection, it is required for the protective response to this pathogen. The main contributors to this non-specific production of IFN-γ are mainly natural killer (NK) and CD4+ and CD8+ T cells. Before adaptive T-cell immunity has fully developed, NK cells may be the main producers of IFN-γ, either in response to IL-12 and IL-18 or directly by exposure to mycobacterial products. T cells expressing γδ T-cell receptors (γδ T cells) and CD1-restricted T cells may also produce IFN-γ during early infection. γδ T cells may directly recognize small mycobacterial proteins and non-protein ligands in the absence of antigen-presenting molecules (Flynn and Chan, 2001). CD1-restricted T cells do not react with mycobacterial protein antigens in the context of MHC class I or class II molecules. Instead, these cells react with mycobacterial lipid and glycolipid bound to CD1 on antigen-presenting cells. CD1-restricted T cells display cytotoxic activity and are able to produce IFN-γ (van Crevel *et al*., 2002). This mycobacterial antigen-specific IFN-γ production *in vitro* has lead to several attempts to use this cytokine as a surrogate marker of infection with *M. tuberculosis* (CDC, 2005a; Gooding *et al*., 2007). However, this cytokine may be unreliable as an immune correlate of protection, since some studies have demonstrated that *M. tuberculosis* can prevent macrophages from responding to IFN-γ. The infection of monocytes, macrophages and dendritic cells with *M. tuberculosis* or mycobacterial products also induces the production of TNF-α. This pro-inflammatory cytokine plays a key role in granuloma formation, induces macrophage activation, and has immunoregulatory properties

(Russell, 2007). The requirement for TNF-α to control an *M. tuberculosis* infection is complex maybe due to its role as a mediator of macrophage activation. TNF-α acts synergistically with IFN-γ to induce the production of NOS2 expression. However, systemic production of TNF-α may account for unwanted inflammatory effects like fever and wasting. Clinical deterioration early in treatment is associated with a selective increase of this cytokine in the plasma of the patients (Saunders and Britton, 2007). Other pro-inflammatory cytokine involved in the host response to *M. tuberculosis* is IL-1β. In TB patients, it is expressed in excess and at the site of disease (van Crevel *et al*., 2002). IL-6 is a cytokine that presents both pro- and anti-inflammatory properties. It is produced early during mycobacterial infection at the site of infection (Flynn and Chan, 2001). However, its role may be controversial since that in mycobacterial infections it inhibits the production of TNF-α and IL-1β. One key player in host defence against *M. tuberculosis* is IL-12. IL-12 is produced following phagocytosis of *M. tuberculosis* by macrophages and dendritic cells inducing a Th1 response, with production of IFN-γ (Flynn and Chan, 2001). In TB patients, IL-12 has been detected in lung infiltrates, pleurisy, granulomas and in lymphadenitis. In humans suffering from recurrent non-tuberculous mycobacterial infections, genetic mutations in the genes encoding IL-12p40 and IL-12R have been identified. These patients display a reduced capacity to produce IFN-γ (Sahiratmadjaa *et al*., 2007). Apparently, IL-12 is a regulatory cytokine which connects the innate and adaptive host response to mycobacteria and which exerts its protective effects mainly through the induction of IFN-γ (van Crevel *et al*., 2002). In addition to IL-12, two cytokines are also important in the IFN-γ production. IL-18, a pro-inflammatory cytokine was initially discovered as an IFN-γ-inducing factor, synergistic with IL-12. It also stimulates the production of other pro-inflammatory cytokines, chemokines, and transcription factors (van Crevel *et al*., 2002). IL-15 is a pleiotropic pro-inflammatory cytokine primarily synthesized by monocytes and macrophages (Abebe *et al*., 2005). The pro-inflammatory response which is initiated by *M. tuberculosis* is antagonized by anti-inflammatory mechanisms. Soluble cytokine receptors prevent binding of cytokines to cellular receptors, thereby blocking further signalling. Three anti-inflammatory cytokines, IL-4, IL-10, and transforming growth factor beta (TGFβ), may inhibit the production or the effects of pro-inflammatory cytokines in TB. IL-10 is produced by macrophages after phagocytosis of *M. tuberculosis* and after binding of mycobacterial LAM. Release of IL-10 induces the

survival of *M. tuberculosis* in the host cells, down regulating a Th1 response (Majumder *et al*., 2006). By this manner, IL-10 antagonizes the pro-inflammatory cytokine response because it down regulates the production of IFN-γ, TNF-α, and IL-12. Mycobacterial products such as LAM induce production of TGFβ by monocytes and dendritic cells (Flynn and Chan, 2001). Like IL-10, TGFβ is produced in excess during TB and is expressed at the site of disease. In addition, TGFβ may be involved in tissue damage and fibrosis during TB, as it promotes the production and deposition of macrophage collagenases and collagen matrix (Flynn and Chan, 2001). TGFβ may also interact with IL-4 but in the presence of both cytokines, T cells may be directed to a protective Th1-type profile. The deleterious effects of IL-4 in TB have been attributed to this cytokine suppression of IFN-γ production and macrophage activation (van Crevel *et al*., 2002). In mice infected with *M. tuberculosis*, progressive disease and reactivation of latent infection are both associated with increased production of IL-4. Similarly, over-expression of IL-4 intensified tissue damage in experimental infection (Flynn and Chan, 2001). Chemokines are chemotactic cytokines responsible for the recruitment of inflammatory cells to the site of infection. Several studies have addressed the role of IL-8, which attracts neutrophils, T lymphocytes, and possibly monocytes. In TB patients, IL-8 has been found in bronchoalveolar lavage fluid, lymph nodes, and plasma and high levels of this chemokine was found in patients who died from TB. *M. tuberculosis* preferentially induces production of monocyte chemoattractant protein 1 (MCP-1). In murine models, deficiency of MCP-1 inhibited granuloma formation. In murine models, expression of Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES) (now called CCL5) was associated with development of *M. bovis* induced pulmonary granulomas. In human patients, RANTES has been detected in alveolar lavage fluid. Apart from IL-8, MCP-1, and RANTES, other chemokines may be involved in cell trafficking in TB (van Crevel *et al*., 2002). Inhibition of chemokine production may lead to an insufficient local tissue response. However, due to the complexity of the chemokine system, the contribution of individual chemokines is difficult to evaluate.

I.6.4.1 The immune system as the means for predicting who is to progress to active disease

The vast majority of countries with high burdens of TB are poorer countries in which health care facilities lack biosafety containment and implementation of obligatory preventive therapy for TB infection. In these countries, health care workers are at risk for nosocomially acquiring TB due to the repeatedly exposition to aerosolized *M. tuberculosis* (Ordway *et al*., 2005b; Raviglione *et al*., 1993). Because TB can readily result from the inhalation of few bacilli (Bloom and Murray, 1992) these health care workers besides being at proven risk (ATS, 2000), constitute sources for new infections in the community and within the hospital settings. Due to factors such as the: i) repeated exposure to *M. tuberculosis*, that most probably will result in a large number of TB infections; ii) progression of these infections to active TB disease; and iii) increased numbers of TB cases on open hospital wards and minimal or absent TB infection control, these individuals constitute a good group to study the development and progression of TB and its relationship to a variety of markers of immunity involved in the infectious process. The vast majority of studies that evaluate the immune responses in TB disease are derived from clinically diagnosed patients with active TB (Ordway *et al*., 2004). Therefore, immune responses associated with initial inhalation of tubercle bacilli, development and progression of disease are largely unknown. Studies addressing the susceptibility of individuals to TB have been conducted by several groups (Smith *et al*., 2002; van Crevel *et al*., 2000). Individuals with multiple exposures to *M. tuberculosis* develop a Th1 response (IFN-γ) associated to an early expression of a Th2 response (IL-4) and subsequently to active disease. A Th2 response confined to TCD8+ and γδ phenotype can result in reduced bactericidal function of mycobacterial infected cells. However, the components of the immune response responsible for the failure of elimination of intracellular *M. tuberculosis* that leads to active disease are not completely understood. In countries with a low incidence of TB, the results show reduced levels of IFN-γ production without an increase in IL-4 response in patients with TB (Johnson and McMurray, 1994; Sanchez *et al*., 1994). In the case of countries where TB is endemic the results show that reduced levels of IFN-γ are correlated with an increase in the IL-4 response (Bhattacharyya *et al*., 1999; Smith *et al*., 2002; van Crevel *et al*., 2000). Some of these studies have being carried out by our team at the Unit of Mycobacteriology (IHMT/UNL) in order to clarify if multiple TB exposure would lead

to induction of Th2 responses and by this manner contribute to active disease (Ordway *et al*., 2004; Ordway *et al*. 2005a, b). To address this question, we collected blood from 10 health care workers that worked with TB patients under conditions that afforded little protection to repeated exposure to air-borne *M. tuberculosis* and 10 non- health care workers with no evidence of contact with *M. tuberculosis* (Ordway *et al*., 2005a). Peripheral blood mononuclear cells obtained from these individuals were stimulated with *M. tuberculosis* and the cell proliferation and production of IFN-γ, IL-5 and IL-4 using ELISA and Flow Cytometry was evaluated. The results obtained were compared with the results from the DTH responses. This group of individuals was studied longitudinally for 5 years after the assays were completed. During this time, 6 health care workers developed active TB (Ordway *et al*., 2005a). The analysis of its cellular phenotype and cytokine production revealed an early expression of a Th1 response with increased levels of IL-4 within the CD8+ and γδ+ T cells (Ordway *et al*., 2004; Ordway *et al*., 2005a, b). These results suggest that antigen persistence from repeated *M. tuberculosis* exposure leads to early expression of an immune profile that consists of the presence of a declining Th1 response with concomitant expression of a Th2 response. This presence of a Th2 response results in reduced DTH Mantoux indurations and lack of an early clinical diagnosis. In this early phase, failing Th1 immunity, persistence of antigen and the early expression of Th2 (IL-4), leads to the eventual development of TB. The immune profile associated with these individuals was detected years prior to the onset of active disease and therefore may serve as a predictor of progression to TB disease. Health care workers that did not developed active disease showed a higher percentage of CD8+ T cells expressing IFN-γ; a higher percentage of γδ+ T cells expressing IFN-γ and low levels of IL-4 in CD8+ and γδ+ T cells (Ordway *et al*., 2005a, b). The early presence of IL-4 in T cells leads to the progression of the infection to active disease. These results are extremely important since they suggest an early immune profile that can be used to predict increased susceptibility of the human host to TB.

CHAPTER II.

Materials, Methods and Instrumentation

II.1. Determination of Toxicity of compounds to be evaluated for activity against bacteria under study

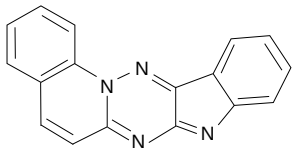
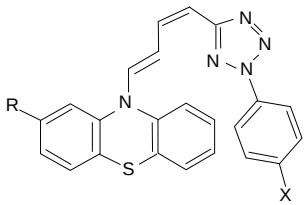
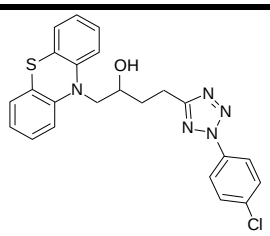
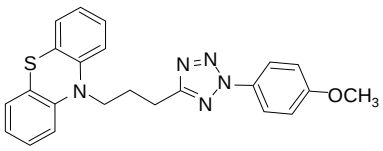
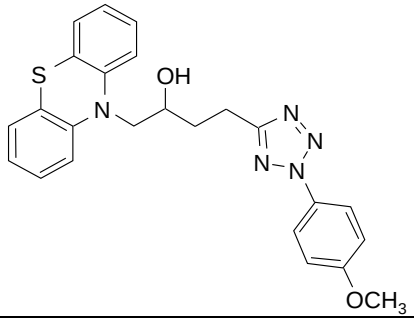
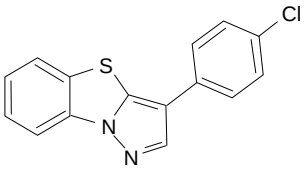
II.1.1 Materials.

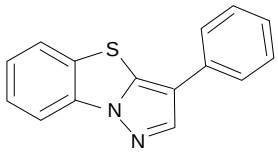
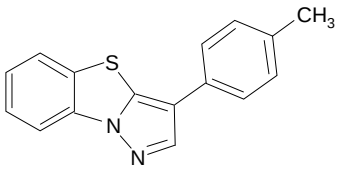
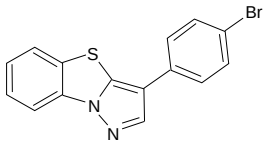
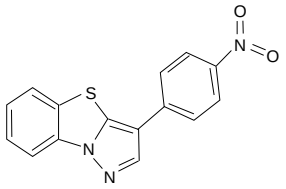
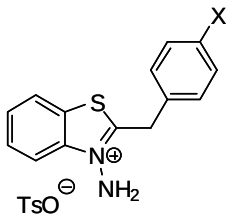
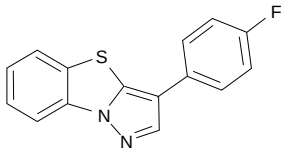
RPMI medium, Ficoll-Hypaque, Hank's Balanced Salt Solution (HBSS) and Trypan blue were purchased from Sigma-Aldrich Química SA (Madrid, Spain). 50 mL Falcon tubes and microwell tissue culture plates were purchased from Nalgene (Nunc, Thermo Scientific; Rochester, NY, USA).

Thioridazine (TZ) was purchased from Sigma-Aldrich Química SA (Madrid, Spain). Stock solutions of the phenothiazine were freshly prepared in the days of the experiment in sterile distilled water and protected from light.

Modifications of the TZ molecule to a clinically more active one were achieved by chemical manipulations and according to the mechanism of action of these neuroleptic drugs. In order to accomplish this, twenty-two TZ derivatives were synthesised by Professor György Hajós from the Institute of Chemistry, Budapest, Hungary. These derivatives were produced by chemical manipulations (new TZ derivatives attached to alkyl, aryl or hetaryl groups), the derivatives isolated (micro and semi-preparative scale; thin layer chromatography, column chromatography by normal and medium pressure, vacuum distillation), characterized (by 400 and 200 MHz Nuclear magnetic resonance (NMR) (^{1}H and ^{13}C), Fourier transfer Infra red and Raman Spectroscopy, Ultraviolet (UV) Spectroscopy, Mass Spectroscopy, X-Ray structure determination), synthesized (by ring closure and ring opening reactions, Pd(0) catalysis such as cross-coupling reactions; cyclo-additions, *etc*.) in 100 milligram quantities and then sent to Portugal. The mentioned derivatives were identified as follows: #1550, #1470, #1686, #1687, #1532-2, #1688, #1689, #1819, #1820, #1821, #1867, #1868, #1869, #1870, #1871, #1872, #1873, #1874, #1875, #1876, #1926 and #1929. The structures of these compounds are presented in Table 1.

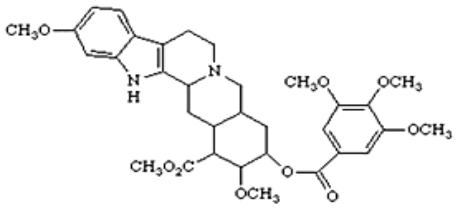
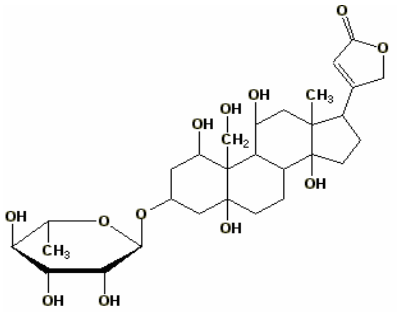
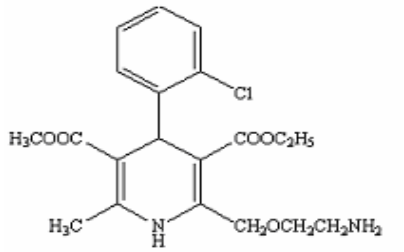
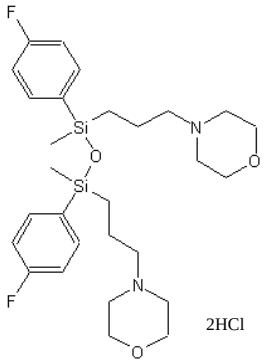
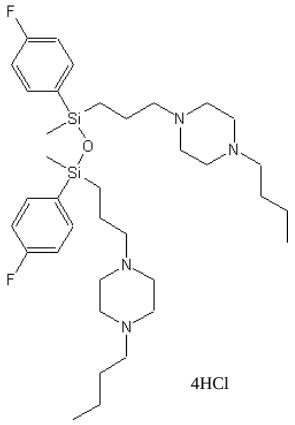
Table 1. Structure of TZ derivatives.

Compound	Structure
#1470	
#1550	Non available
#1689 (R = CF_3, X = Cl) **#1686 (**R = H, X = OCH_3) **#1687 (**R = CF_3, X = OCH_3) **#1688 (**R = Cl, X = OCH_3) **#1550 (**R = H, X = $C(NH_2)$=N-OH)	
#1819	
#1820	
#1821	
#1871	

Compound	Structure
#1872	
#1873	
#1874	
#1876	
#1875 (X = NO_2) **#1926** (X = F) **#1867** (X = H) **#1870** (X = Cl) **#1868** (X = CH_3) **#1869 (**X = Br)	
#1929	

Other compounds such as reserpine, ouabain and verapamil were purchased from Sigma-Aldrich Química SA (Madrid, Spain). Patented compounds 1,3-Dimethyl-1,3-bis(4-fluorophenyl)-1,3-bis(3-morpholino-propyl)-disiloxan-dihydrochlorid (SILA 409) and 1,3-Dimethyl-1,3-bis(4-fluorophenyl)-1,3-bis{3-[1(4-buthyl-piperazinyl)]-propyl}-disiloxan-tetrahydrochlorid (SILA 421) were synthesised by Hegyes *et al* (Hegyes *et al.*, 2000) and have received patents, Brevet Europeen nº 0099150.6, PCT/DE00/04110. Their structures are indicated in Table 2.

Table 2. Structures of other efflux pump inhibitors.

Compound	Structure
Reserpine	
Ouabain	
Verapamil	
SILA 409	
SILA 421	

II.1.2 *Carpobrotus edulis*

Carpobrotus edulis (L.) N. E. Br. (Aizoaceae) leaves were collected in December of 2003 from the cliffs of Cabo da Roca (Sintra, Lisbon, Portugal) and authenticated by Prof. António Viveiros (Professor Emeritus of Botany, Plant Biology Department, Faculdade de Ciências, Universidade de Lisboa). The plant extract was prepared as described: two hundred grams (g) of leaves were homogenised in 50 mL of water and methanol (MeOH) was added yielding a final 70% MeOH concentration (v/v). The preparation was mixed for 3 hours at room temperature and centrifuged at 10,000 rotations pre minute (r.p.m.). The residues were pooled and weighed and the supernatant filtered through a 3-mm Whatmann paper. The paper and its contents were dried and weighed, and its weight added to the pooled weight of the pellets. Total wet solids weigh approximately 194 g. Methanol was removed from the supernatant in vacuum at 55ºC to yield the aqueous solution that would be tested for its antibacterial activity. The final volume representing the aqueous phase was approximately 150 mL and this material was used at various dilutions for intracellular activity against phagocytosed bacteria. A 50 mL aliquot of the aqueous phase was further concentrated to a volume of 10 mL and used for assaying its *in vitro* activity against bacteria (Martins *et al.*, 2005).

II.1.3 Instrumentation

Analytic Balance (Explorer; Ohaus)
Vortex (Snijders Model N^{o}. 34524)
-20ºC Freezers (30L and 80L; Bosch)
Microscope (Lietz Biomed)
Neubauer chamber (Hirschmann)
Refrigerated centrifuge (Rotanta 46R; Hettich Zentrifugen)
Micropipettes (Gilson)
Laminar Flow Cabinets (NU-425-600E; Nuaire)
CO_2 Water Jacketed Incubator (Nuaire NU-4500/E)

II.1.4 Quality control

The optical microscope was adjusted for Kohler illumination (Bohnhoff, 1979) in order to certify that the optimum conditions required for sensitivity were present. Microscopy is an essential component of the laboratory work and the means by which the cells are evaluated for toxicity of compounds is an important feature since the criteria define toxicity are dependent on initial observations of the cells. It is important to ensure that the detection of stain is not due to dirt; the adjustment of the microscope was performed on a scheduled basis. All the instruments referred above are periodically checked and calibrated according to the Good Laboratory Practices employed in the Unit of Mycobacteriology (IHMT/UNL).

II.1.5 Method

Human peripheral blood (20 mL) was collected from voluntary healthy donors to a heparinized tube (300 μL, Monoparin; CP Pharmaceuticals, 1,000 Units) and the purified human mononuclear cells obtained by the Ficoll differential centrifugation method (Ordway *et al.*, 2003b), as follows: total blood was diluted in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, Madrid, Spain) 1:2 (40 mL of final volume). From this dilution, 2 sets of 20 mL were layered on the top of 12 mL of histopaque 1.077 (Sigma-Aldrich Química, S.A., Madrid, Spain) (5/3) (v/v) contained in 50 mL tubes and the tubes centrifuged at 800×G for 30 minutes. After this centrifugation 4 phases were obtained: plasma, buffy-coat containing the mononuclear cells (layer of interest), ficoll-hypaque phase and in the bottom the red blood cells, platelets and granulocytes. The plasma was carefully aspirated with a sterile Pasteur pipette to a new 50 mL tube and kept for posterior addition to RPMI medium in a 10% ratio (complete medium). The white layer (between saline and histopaque phases) was carefully aspirated, with a sterile Pasteur pipette, pooled to a new 50 mL tube and HBSS (Sigma-Aldrich, Madrid, Spain) added to the cells until a final volume of 50 mL. Cells were then washed at 800×G for 10 minutes, the supernatant discarded, 50 mL of HBSS added and a new centrifugation performed (in the same conditions mentioned above). After this, a third wash was performed. The supernatant was discarded and the pellets re-suspended in 5 mL of RPMI 1640 medium. From this, an aliquot of 10 μL of the

cellular pellet was added to 90 μL solution of trypan blue/phosphate buffered saline (PBS) for determination of cellular viability. When cells are damaged trypan blue enters the cells and stains the cytoplasm (Fong and Kissmeyer-Nielsen, 1972). Counting of viable cells was performed using a Neubauer chamber (Ordway *et al.*, 2003b). The concentration of cells was adjusted with RPMI medium plus 10% autologous human serum to yield 1×10^7 cells/mL. From this adjusted cellular suspension, 180 μL were transferred to the wells of a 96-well microplate. The cells dispersed to a 96-well microplate (1×10^7 cells/mL) were then pulsed with 20 μL aliquots of TZ and its derivatives, added in triplicate, at concentrations ranging from 0 to 0.5 mg/L. The plates were then incubated at 37ºC with 5% of CO_2 for a period of 3 days subsequent to the addition of the compounds and each day an aliquot of 10 μL was retrieved from the wells, added to 90 μL of a trypan blue solution and viability of the cells assessed. By this manner, it was possible to determine the optimum concentration of the compounds that did not present any toxicity for the human lymphocyte and select the concentrations to be used in the *ex vivo* assays.

II.1.5.1 QC for method

Human purified lymphocytes were obtained by the Ficoll differential centrifugation method as previously described (see method description). After the adjustment of cell number, lymphocytes were split into two parts. One part of the cells was killed using potassium cyanide (positive control) and the other received no compound (control). The two parts (dead and live cells) were then mixed to yield dead cells on final percentages of 10, 25, 50, 75 and 100%. Aliquots of these cell suspensions were retrieved, mixed with a 0.9% of trypan blue solution and counted in a Newbauer chamber to perform a calibration curve. By this manner, it was possible to obtain a correlation (coefficient of correlation >0.99) between the concentrations that presented toxicity to the lymphocytes and the percentage of dead cells. This assured that the percentage of stained cells (dead) after the treatment with the compounds was due to toxicity of the compounds tested.

II.2. Mutagenicity of compounds

The mutagenic effects of TZ and each of its derivatives was assessed by the modified Ames procedure (Ames *et al*., 1975). This method allows us to directly assay potential carcinogens by testing their ability to revert mutation in *Salmonella enterica* serotype Typhimurium. The most effective way to detect carcinogenic compounds is to inoculate a sample into animals and monitor for tumours development. However, this is difficult, time consuming, expensive and cumbersome (Knight and Breheny, 2002). In addition, most of the compounds that cause tumours in animal cells (carcinogens) are mutagens. Based upon this insight, Bruce Ames and colleagues developed a simple, indirect assay for potential carcinogens (Ames *et al*., 1975). The assay is based upon the reversion of mutations in the histidine (his) operon in the bacteria *Salmonella enterica* serotype Typhimurium. The operon encodes enzymes required for the biosynthesis of that amino acid. Strains with mutations in the operon are designated his auxotrophs and are unable to grow in media without addition of his. Revertants that restore his phenotype (his+) will grow on minimal medium plates without the amino acid. This provides a simple and sensitive selection for revertants of his mutants. His mutants are mixed with the potential mutagen and then plated on minimal medium with a very small amount of his. The concentration of his used is limiting, so after the cells go through several cell divisions his is used up and the auxotrophs stop growing. This is essential because many mutagens work only on replicating DNA. If the potential mutagen induces his+ revertants during the initial few cell divisions, then each of the resulting revertants will continue to divide and form a colony. The number of colonies produced is proportional to how efficiently the mutagen revert the original mutation. If these agents are shown to be mutagenic for bacteria they may also alter the DNA of eukaryotic cells.

II.2.1 Bacteria

Salmonella enterica Serotype Typhimurium TA1538 auxotrophic for histidine (his^-).

II.2.2 Materials

Glucose minimal-salts agar, histidine, biotin, sodium azide and Tryptic Soy Broth (TSB) were purchased from Oxoid (Basingstoke, England).

II.2.3 Instrumentation

37ºC Incubator (Model 600; Memmert)

II.2.4 Quality control

In order to ensure that the temperature presented by the instrument is the exact temperature inside the incubator, periodic temperature quality control was performed according to the Good Laboratory Practices implemented in the laboratories of the Unit of Mycobacteriology (IHMT/UNL).

II.2.5 Method

The experimental procedure was followed as described: glucose minimal-salts agar plates were prepared (test and control plates). After this an inoculum of *S. enterica* serotype Typhimurium (his^-), was prepared in soft agar and uniformly spread over the surface of the plates. The agar was allowed to harden for a few minutes and using sterile forceps, prepared discs were dipped into tubes containing the compounds to be tested. The excess of the liquid in the discs was removed by gently pressing the discs against the walls of the tubes. The discs were placed in the agar plates and plates were incubated at 37ºC for 48 hours. After this incubation period CFU were counted.

II.2.5.1 QC for method

In order to ensure the correct performance of the protocol, two controls were conducted in parallel. A negative control was performed using a saline solution instead of the tested compounds and the disc was placed on one half of the control plate. A positive control was also conducted using a disc emerged in a sodium azide (known mutagen) solution and the disc placed on the other half of the control plate.

II.3. Evaluation of *in vitro* activity of compounds studied against *S. aureus* strains

In vitro activity of TZ and its derivatives was initially conducted with *S. aureus* strains (ATCC25923 strain and Methicillin-resistant clinical strains) by the microplate microdilution method (CLSI, 2006) in order to determine which derivatives presented higher activity than the parental molecule (TZ). The minimum inhibitory concentration (MIC) is defined as the minimal concentration of an agent that inhibits the replication of the bacterium. The MIC of an agent against a specific bacterial strain contributes to the characterisation of that strain as well as provides the means by which the *in vitro* activity of a series of agents against the same bacterial strain can be compared and therefore, ranked (CLSI, 2006). We have found that the *in vitro* response of *S. aureus* is usually similar to that of *M. tuberculosis* (Ordway *et al*., 2003b) and defined within one day as opposed to three or more weeks for the latter species, and is far less costly. As an example of this cost differential, the cost for medium and reagents for the evaluation of *in vitro* activity against one staphylococcus strain is approximately 14 euros (for testing 22 TZ derivatives) whereas that for similar evaluation for one mycobacterial strain would be 1.237 euros (for the same 22 compounds). Therefore, we have employed the staphylococcal strains as a model for predicting which of the derivatives will be effective against *M. tuberculosis* (Ordway *et al*., 2002a, b). Derivatives which yielded MICs against staphylococcal strains that were equal or lower than that of the parental TZ compound were then selected for the evaluation of *in vitro* activity against *M. tuberculosis* strains.

II.3.1 Microplate Microdilution Method

II.3.1.1 Bacteria

S. aureus ATCC25923 (control strain) and Methicillin-Resistant *S. aureus* (MRSA) strains were selected as the experimental model for screening of compounds that will work on MDR-TB strains.

II.3.1.2 Materials

Mueller-Hinton (MH) broth and Mueller-Hinton agar (MHA) were purchased from Oxoid (Basingstoke, England). PBS was purchased from Sigma-Aldrich Química SA (Madrid, Spain). Microwell tissue culture plates were purchased from Nalgene (Nunc, Thermo Scientific; Rochester, NY, USA).

II.3.1.3 Instrumentation

37ºC Incubator (Model 600; Memmert)

II.3.1.4 Quality control

Temperature quality control was performed in a periodically basis following the Good Laboratory Practices Guidelines (UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR), 2001) implemented in the laboratory of the Unit of Mycobacteriology (IHMT/UNL).

II.3.1.5 Method by MIC and its principle

The method used with *S. aureus* strains is briefly described: MH media was prepared and aliquots of 150 µl distributed to the wells of 96-well microplates. After this, fresh stock solutions of the tested compounds were prepared and added to the wells of the microplates. A serial two-fold dilution of each compound was performed in order to test compounds in concentrations ranging from 0 to 400 mg/L. An overnight culture of *S. aureus* prepared in MH broth was diluted in a saline solution and its concentration adjusted to 0.5 of McFarland scale (according to the CLSI guidelines). Wells containing the dilutions of the compounds were inoculated with 50 µL of the adjusted bacterial suspension. In parallel, two controls were also conducted: a negative control, containing just MH broth and a positive control presenting the inoculum (no compound). The plates were incubated at 37ºC and after 16 and 18 hours the results were recorded and confirmed. The results were compared with the two controls and processed as growth (turbidity in the wells) or no growth (absence of turbidity). The MIC was defined as the lowest concentration where no growth was observed (in comparison with the absolute

control). Aliquots of 100 μL from the wells with no growth were plated in MHA plates. The plates were incubated overnight and the minimum concentration showing no colonies was considered the minimum bactericidal concentration (MBC). All the assays were conducted in triplicate and the data obtained did not vary.

II.3.1.5.1 QC for method

All the procedures were conducted according to the Performance Standards for Antimicrobial Susceptibility Testing; Sixteen Informational Supplement (CLSI, 2006).

II.3.2 Kirby-Bauer

II.3.2.1 Bacteria

S. aureus ATCC25923 (control strain) and Methicillin-Resistant *S. aureus* (MRSA) strains were selected as experimental model for screening of compounds.

II.3.2.2 Materials

MHA was purchased from Oxoid (Basingstoke, England). Phosphate buffered solution was purchased from Sigma-Aldrich Química SA (Madrid, Spain). Sterilized Petri dishes were purchased from Nalgene (Nunc, Thermo Scientific; Rochester, NY, USA). Sterilized swabs were purchased from Omnilabor (Omnilabor, Lisbon, Portugal).

II.3.2.3 Instrumentation

37ºC Incubator (Model 600; Memmert)

II.3.2.4 Quality control

Plate drying, method of storage and storage time was previously determined by the laboratory of the Unit of Mycobacteriology (IHMT/UNL) as part of the Quality Assurance Programme. Plates were storage at 4-8°C in sealed plastic bags. Tests were made in a schedule basis in order to confirm that excess moisture was not produced in a

sealed environment or that plates were not over-dried in an unsealed environment. Temperature quality control was performed periodically for all incubators used during the protocol and followed the guidelines of Good Laboratory Practices (UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR), 2001).

II.3.2.5 Method and its principle

The susceptibility profile of each strain employed in this study was conducted in accordance to the Kirby-Bauer method (CLSI, 2006). Briefly, each strain was grown in MH media for 2-3 hours of culture. After this period, the strains were diluted in PBS and the inoculums adjusted to 0.5 of McFarland scale (as recommended by the Clinical Laboratory Standard Institute (CLSI)). The adjusted suspension was used to inoculate MHA plates by dipping a sterile cotton-wool swab into the suspension and remove the excess by turning the swab against the side of the tube. The inoculum was swabbed in the surface of the plate in three directions and left stand for 3-4 minutes. After this, antibiotics disks, representative of each class, were placed on the surface of the agar containing the inoculums. Ninety mm plates were used since they can accommodate six discs without unacceptable overlapping of zones. The plates were left to stand for a few minutes and then incubated at 37ºC for 16 and 18 hours. After these periods, the diameters of the inhibition zones were measured (edge was taken as the point of inhibition judged by the naked eye) with a ruler. The zone of inhibition for the control strain was confirmed to fall within the acceptable ranges before interpreting the test. After the validation of the result obtained for the control strain, the tested strains were characterized for their antibiotic susceptibility profile as susceptible, resistant or intermediate according to the CLSI Standards (CLSI, 2006).

II.3.2.5.1 QC for method

The control organism selected was the *S. aureus* ATCC25923 as recommended by the CLSI guidelines (CLSI, 2006). To minimise the risk of mutations, the control strain was stored at -70°C, in 15% of glycerol. Two vials of the control were stored, one as an “in use” supply and the other for archiving. Every week an aliquot of the “in use” vial was sub-cultured on MH broth and checked for purity. From this pure culture, subcultures

for each of the following 7 days were prepared. The method was performed with an inoculum giving semi-confluent growth of colonies after an overnight incubation. This is important since an inoculum that yields semi-confluent growth has the advantage over that resulting in isolated colonies in that a uniform appearance is more easily be observed. Larger or a smaller inoculae will yield reduced or augmented zones of inhibition, respectively. To obtain a semi-confluent inoculum the adjustment of the cell concentration was performed accordingly with the McFarland standard. A commercial vial of 0.5 of the McFarland scale (McFarland, 1907) was used. This scale is constituted by a barium chloride ($BaCl_2$) solution with standardized turbidity. The McFarland standard was taken from the refrigerator and left to stand for a few minutes at room temperature. After this, the vial was mixed to ensure that it was evenly suspended. The commercial standard is periodically checked by measuring the absorbance in a spectrophotometer at a wavelength of 625 nm. For a 0.5 McFarland standard the acceptable range for the absorbance is between 0.08 and 0.13 (McFarland, 1907). The inoculum preparation was performed according to the CLSI guidelines. At least four morphologically similar colonies were removed with a sterile loop, transferred to MH broth and incubated with shaking at 37°C until the visible turbidity was equal to or greater than the 0.5 McFarland standard. Adjustment of the suspension was conducted by adding an aliquot of saline solution. The comparison between the test and the standard was conducted against a white background with a contrasting black line and the suspension was used within 15 minutes of preparation.

II.4. Ethidium bromide (EB)-agar method for the demonstration of efflux pump activity of bacteria

II.4.1.1 Bacteria

Escherichia coli K12 (AG100-main efflux pump acrAB intact), *E. coli* (AG100A-main efflux pump acrAB deleted-ΔacrAB) and the progeny of the AG100 strain induced to high level resistance to TET (AG100 $acrAB_{TET}$). This last strain was produced in our laboratory by serial culture in increasing concentrations of the antibiotic (Viveiros *et al*., 2005a). *S. aureus* strain ATCC25923 and MRSA strains COL (Kornblum *et al*., 1986) and HPV107 (Sanches *et al*., 1995) were generously provided by Prof. Dr. Hermínia de

Lencastre. *Enterobacter aerogenes* EA27 resistant to chloramphenicol has been maintained and characterized by our colleague, J.M. Pagès (Ghisalberti *et al*., 2005).

II.4.1.2 Materials

Luria Bertani (LB) and Tryptic Soy Broth (TSB) media employed for broth and agar based cultures for Gram-negative bacteria and *S. aureus* strains, respectively, were purchased from Difco (Madrid, Spain). Antibiotics (as powder or as Kirby-Bauer disks) were purchased from Sigma-Aldrich Química SA (Madrid, Spain). EB was purchased from Sigma-Aldrich Química SA (Madrid, Spain).

II.4.1.3 Instrumentation

37ºC Incubator (Model 600; Memmert)
Eagle Eye (Strategene, USA)

II.4.1.4 Quality control

Temperature quality control was performed in a periodically basis following the Good Laboratory Practices Guidelines (UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR), 2001) implemented at the laboratory of the Unit of Mycobacteriology (IHMT/UNL).

II.4.1.5 Method and its principle

The EB-agar method is an instrument-free method that was developed at our Unit for the demonstration of efflux pump activity of bacteria. Conventional evaluation of efflux pumps by the use of the substrate ethidium bromide (EB) or by the retention of radioactive antibiotics requires specialized instrumentation not normally found in clinical laboratories. Therefore, at the Unit of Mycobacteriology we have developed a simple, instrument free, agar based method that utilizes EB, to identify clinical isolates that have an over-expressed efflux pump activity. The assay is also shown to afford the evaluation of agents that may inhibit efflux pumps activity. This method can provide additional important information that defines the cause of MDR of the clinical isolates.

The effect of the temperature on the efflux pump system was also considered. Briefly, two sets of agar plates containing EB concentrations ranging from 0.05 to 1.5 mg/L were swabbed with the bacterial strain and incubated overnight at 37ºC. After this period, the results were recorded and one set re-incubated at 37ºC, whereas the duplicate set was transferred to 4ºC. At the end of an additional 24 hours period, the minimal concentration of EB that produced fluorescence at each temperature was compared to that evident after the first incubation (at 37ºC). To determine whether the method could distinguish a mixed culture that consisted of strains of *E. coli* or *S. aureus* that differed from each with respect to the degree of efflux pump activity, from each separate culture, 0.1 mL were transferred to a single tube containing 10 mL of saline, mixed, and from this 0.010 mL were transferred to another tube containing 10 mL of saline. These two dilutions, each containing a mixture of both strains, were plated onto a replicate series of agar plates containing concentrations of EB ranging from 0.2 to 2.2 mg/L. Evaluation of fluorescence from the excitation of EB by UV light was first made with the use of a UV transilluminator and the plates photographed with the use of the Eagle Eye system (Strategene, USA).

II.4.1.5.1 QC for method

E. coli K12 and *E. coli* AG100 $acrAB_{TET}$ strains were used as controls, due to the expression of the efflux pumps system. In the case of *S. aureus* strains, the controls used were *S. aureus* ATCC25923 and the MRSA COL strains, for the same reasons stated previously for *E. coli* strains (differences observed in the expression of the efflux pumps system).

II.5. Evaluation of *in vitro* activity of compounds studied against *Mycobacterium tuberculosis* strains

In 1969, Deland and Wagner (DeLand and Wagner, 1969) developed a technique for automated detection of the metabolism of bacteria by measuring the $^{14}CO_2$ released during the decarboxylation of ^{14}C labelled substrates present in the medium. This technique was applied successfully for the evaluation of growth of *Neisseria* sp. under different nutrient conditions and for the determination of the effects of antibiotics on said growth. Cummings and co-workers in 1975 carried out preliminary work that

showed that the same principle could be applied to detect growth of *M. tuberculosis* (Cummings *et al*., 1975). Middlebrook and his team further developed the technique and introduced 7H12 liquid medium containing a ^{14}C labelled substrate specific for mycobacterial growth (Siddiqi *et al*., 1981). He reported a significant time saving in the primary isolation of mycobacteria from clinical specimens using this improved radiometric medium. The BACTEC TB medium (12B) is an enriched Middlebrook 7H9 broth base. Mycobacteria utilize a ^{14}C labelled substrate (^{14}C palmitic acid) present in the medium and release $^{14}CO_2$ into the atmosphere above the medium. When the 7H12 medium vials with growth are tested on the BACTEC 460 instrument, the $^{14}CO_2$ is aspirated from the vial and its radioactivity is determined quantitatively in terms of numbers on a scale from 0 to 999. These numbers are designated as the Growth Index (G.I.). The G.I. numbers are displayed by the BACTEC 460 instrument and are also printed out along with identifying rack and bottle numbers. The daily increase in the G.I. output is considered to be directly proportional to the rate and amount of growth in the medium. The BACTEC instrument also introduces fresh 5% CO_2 in air into the medium head space every time a vial is tested. This enhances the growth of mycobacteria. The instrument can automatically test 60 vials at the rate of approximately one vial per minute and stops at the end of the run. If an inhibitory agent is introduced into the medium, inhibition of the metabolism is indicated by reduced production of $^{14}CO_2$ when compared to a control having no inhibitory agent. This basic principle is applied for drug susceptibility testing and also in differentiating *M. tuberculosis* from other mycobacteria. The development of 7H12 medium (BACTEC 12A) led to several studies which reported excellent recovery of mycobacteria from sputum as well as extra-pulmonary specimens (Kirihara *et al*., 1985; Morgan *et al*., 1983; Siddiqi *et al*., 1985; Takahashi and Foster, 1983). Since the BACTEC system utilizes a liquid medium, it is important to add an antimicrobial supplement to suppress growth of contaminating microorganisms which may survive the decontamination process (in the case of clinical samples processing). Siddiq *et al*. reported an antimicrobial mixture (PANTA) that contains polymyxin B, amphotericin B, nalidixic acid, trimethropim and azlocillin (Siddiqi and Rüsch-Gerdes, 2006). Although BACTEC TB medium supports rapid growth of most mycobacteria, occasional strains of *M. tuberculosis*, such as isolates from treated chronic cases, may grow poorly. By this manner, a growth promoting substance, polyoxyethylene stearate (POES) was reported by Siddiq *et al*. (Siddiqi and Rüsch-Gerdes, 2006; Siddiqi *et al*., 1981, 1985).

When added to the BACTEC TB medium, this substance enhanced growth of those strains which grew slowly or poorly. This growth-promoting substance was incorporated in the reconstitution fluid, which should be used to reconstitute lyophilized PANTA supplement. The BACTEC TB system offers a simple automated technique with significant convenience and time saving being extremely accurate for MIC determination. However, it should be stated that the results only show that the cells are respiring and that conditions which inhibit growth may not affect respiration. Hence care must be taken in interpreting the results in terms of effects of agents on the replication of the organism (Duarte *et al*., 2007). However, because of external and internal controls maintained by the Unit of Mycobacteriology, an understanding of the effects of an agent on the replication of the organism can be readily inferred. Lastly, evaluation of the BACTEC TB system involved a comparison of ^{14}C generated in the absence and presence of an agent to colony forming units obtained from the cultures themselves. This data showed without ambiguity, that in the hands of the staff of the Unit of Mycobacteriology the results obtained from the BACTEC TB system could indeed be interpreted in terms of growth and its inhibition by a given agent.

II.5.1 BACTEC 460-TB method

II.5.1.1 Bacteria

Mycobacterium tuberculosis H37Rv ATCC27294 (control strain) and Multi-drug resistant (MDR-TB) strains (resistant to INH, RIF and STR) were the strains selected for conduct studies with TZ and its derivatives.

II.5.1.2 Materials

12B BACTEC 460 vials were purchased from Quilaban (Portugal). Insulin syringes were purchased from Omnilabor (Omnilabor, Lisbon, Portugal).

II.5.1.3 Instrumentation

BACTEC 460-TB instrument (Becton-Dickinson Diagnostic Instrument Systems, Towson, Md., USA).

II.5.1.4 Quality control

A daily performance test was performed as recommended (BACTEC 460 TB SIRE method (1985) Becton Dickinson Microbiology Systems) and is described as follows. Using 1 mL syringe, 0.2 mL of a standard ^{14}C solution (provided in the kit) were injected into one of the three acid vials (provided). The vial was vigorously shaken for ten seconds to release a known amount of $^{14}CO_2$. The acid vial was then tested in the BACTEC instrument; a G.I. of 50-60 should be obtained. If the result was outside this range, a repetition was made with the same vial (as recommended by the manufacturer). Low readings usually indicate probable needle blockage requiring replacement of the needle set. High readings may indicate over-inoculation of the test vials or instrument malfunction. The maintenance schedule of the BACTEC 460 instrument was strictly followed. Good maintenance of the needle heater, filter, media trap and U.V. light was also performed. At least once a month proper disinfection of the equipment was performed as recommended by the manufacturer. Maintenance of the instrument needles was specially conducted. Needles were changed every day and used needles were placed in a larger container with proper disinfectant and autoclaved. The needles points were carefully checked and all the needles were autoclaved in individual syringe paper bags. The CO_2 supply was carefully checked in order to maintain CO_2 in 5-10% with the balance air. CO_2 pressure in the cylinder was daily checked and also the "aerobic switch" button. All the BACTEC vials were tested before the inoculation protocol, to establish the recommended 5% CO_2 atmosphere. Non inoculated vials that showed G.I. of 20 or more were discarded. The BACTEC 460 instrument contains a special TB hood (as recommended by the manufacturer). The TB hood exhaustion is made through HEPA filters and negative pressure in the test area. In addition, the TB hood is also equipped with an ultraviolet light source. The unit performs automatic testing of vials and is not used for inoculation or sub-culturing; those procedures were performed on a biological cabinet type II. BACTEC 460-TB instrument was checked and calibrated according to the annual maintenance recommended by the certified manufacturer (Quilaban, Lisbon, Portugal). Weekly performance tests were also conducted according with Becton-Dickinson Diagnostic Instrument Systems (Towson, Md., USA) recommendations.

II.5.1.5 Method

The MIC and MBC of TZ and its derivatives were determined individually by the BACTEC 460-TB method (BACTEC 460 TB SIRE method (1985) Becton Dickinson Microbiology Systems). *M. tuberculosis* strains were grown as a primary culture in Middlebrook 7H12 broth in 12B BACTEC 460 vials (Becton-Dickinson Diagnostic Instrument Systems, Towson, Md., USA), until they reach an optical density (O.D.) equal to 1 of McFarland standard (McFarland, 1907). From these cultures, aliquots were transferred to new 12B BACTEC vials and the O.D. adjusted to a 0.5 McFarland scale. Each vial received 0.1 ml of 12B BACTEC 460 medium containing an adjusted concentration of mycobacteria corresponding to approximately 10^5 to 10^6 CFU. These vials served as the source for the inoculation of triplicate BACTEC 12B vials containing the phenothiazines. A separate 1:100 dilution of the adjusted inoculum was made and transferred to one BACTEC 12B vial that contained no drug; this served as the proportional control as defined by the recommended BACTEC proportional method for *M. tuberculosis* (BACTEC 460 TB SIRE method (1985) Becton Dickinson Microbiology Systems). Inoculation of each strain into control and experimental BACTEC vials were in accordance with the BACTEC 460 proportional method procedure (BACTEC 460 TB SIRE method (1985) Becton Dickinson Microbiology Systems). Stock solutions containing each derivative were freshly prepared in water and protected from light at all times and 0.1 mL aliquots inoculated into 12B BACTEC 460 vials prior to use. The final concentrations of TZ and its derivatives in the 12B BACTEC 460 vials were 0, 0.3125, 0.625, 1.25, 2.5, 5, 10, and 20 mg/L. The vials were incubated at 37ºC and the contents daily assessed by the BACTEC 460-TB instrument (Becton-Dickinson Diagnostic Instrument Systems, Towson, Md., USA) for $^{14}CO_2$ generated from the metabolism of ^{14}C palmitic acid. Terminations of a set of cultures were in accordance to G.I. parameters to which the controls were subjected. When the absolute control reached a G.I. of 999 the vials were removed and the MIC determined. According to this method the MIC is defined as the minimum concentration where no growth is observed. The MBC of TZ and its derivatives were determined by extending the MIC curves well beyond each MIC, and 100 µl aliquots of the BACTEC 12B cultures at zero time and those after 30 days that showed no evidence of growth in the BACTEC 12B vials were subjected to determination of the numbers of CFU (Ordway *et al*., 2002a). The MBC is defined as the minimum concentration where no growth is

observed when the diluted control (1/100; vial containing 100 times less bacteria) presents a G.I. of 999 (defined as maximum growth). The MIC and MBC determinations were repeated three times, and the values obtained did not differ significantly. Derivatives that had significant activity over that exhibited by their parent compound were selected for *ex vivo* studies on antibiotic resistant *M. tuberculosis* phagocytosed by human macrophages.

II.5.1.5.1 QC for method

According to the BACTEC proportional method for *M. tuberculosis* (BACTEC 460 TB SIRE method (1985) Becton Dickinson Microbiology Systems) the diluted control (1:100) serves as the proportional control. This allows us to determine the 1% proportion of growth. The rate of increase in the G.I. or the amount of change over that of the previous day, called delta (Δ) G.I., is compared to the control vial and the vials containing the compounds. If the daily increase in the vial containing the compound is equal or greater than that in the control vial, the test organisms are considered resistant to the drug. For a susceptible population, the daily G.I. increase for the control would be higher than that of the drug vial. For example, if 1% of the mycobacterial population is resistant to INH, then 99% of the organisms would be inhibited by INH and only 1% would grow in the vial containing the antibiotic. The growth rate in the vial containing INH would be similar to the growth rate in the control vial in which the original bacterial inoculum was only 1/100 of that in the drug vial. Thus, instead of counting colonies in the control and the drug medium, the G.I. values are utilized to determine susceptibility results. The G.I. readings of a susceptibility test require more interpretation than do the primary isolation vials. For the first two to three days, the G.I. in the control vial will be low but then it will start increasing by a factor of two to three. Since the vials containing drugs were inoculated with a 1/100-fold larger inoculum, the G.I. readings are usually higher than the control for the first day or two. If the strain is susceptible to the test drug, the G.I. output levels off or decreases on the subsequent days. However, the G.I. value continues to increase for resistant strains and is much higher than the control. This is the reason that a resistant strain can be detected and reported earlier than a susceptible strain. The difference in the G.I. values from the previous day is designated ΔG.I. Negative ΔG.I. values indicate a decrease while positive ΔG.I. values indicate an increase in growth and G.I. output. When the control

vials reach a G.I. of 30 or more, the results should be interpreted as follows: if the ΔG.I. is less in the drug vial than the control, the population is susceptible; if more, it is resistant. The definition of the susceptibility profile of the strains could be summarized as follows:

ΔG.I. (Control) > ΔG.I. (drug) – "susceptible"

ΔG.I. (Control) < ΔG.I. (drug) – "resistant"

ΔG.I. (Control) = ΔG.I. (drug) – "borderline"

In all the experiments conducted this control was performed in order to assure the correct interpretation and liability of the results.

The same approach was employed to test the *in vitro* activity of phenothiazines, CPZ, TZ, PMZ, PMTZ and DSP against *Mycobacterium avium* strains CIP 14 031 002-S4 (Pasteur Institute, Paris, France) and a clinical isolated obtained from an AIDS patient. The *in vitro* effect of SILA compounds 409 and 421 against *M. tuberculosis* strain was also the same described above.

II.6. Electron microscopy (*in vitro*)

II.6.1 Bacteria

Methicillin-Resistant *S. aureus* (MRSA) and Multi-drug resistant (MDR-TB) (resistant to INH, RIF and STR) strains.

II.6.2 Materials

Thioridazine (TZ), oxacillin (OXA) paraformaldehyde, sodium dodecylsulphate (SDS), glutaraldehyde and osmium tetroxide were purchased from Sigma Aldrich Química SA (Madrid, Spain). PBS was purchased from Gibco (Paisley, United Kingdom). TSB and Trypticase Soy Agar (TSA) were purchased from Difco Laboratories (Detroit, MI, USA). TZ solutions were prepared in distilled sterile water on the day of the experiment and protect from light.

II.6.3 Instrumentation

Zeiss EM 900 electron microscope (Carl Zeiss, Oberkochen, Germany).
Ultracut S (Microtome, Leica, Vienna, Austria)

II.6.4 Quality control

The quality control was performed by Prof. Dr. W. Bleiss at his laboratory in the Institute of Molecular Parasitology of the Humboldt University, Berlin, Germany. The evaluation of the ultra-structure of bacteria and macrophages with the aid of the electron microscope was conducted jointly by Prof. Dr. Bleiss and Prof. Dr. L. Amaral.

II.6.5 Method

In order to determine the effects that TZ induced on the ultra-structure of MRSA and MDR-TB *in vitro*, bacterial cultures were prepared and TZ added at a concentration of 20 mg/L. A control with no TZ was also conducted in parallel. In the case of MRSA, 18 hours cultures were performed and in the case of *M. tuberculosis*, cultures for 24 hours. The products of *in vitro* cultures-containing or lacking TZ were centrifuged at 800×G for 30 minutes. The pellets were washed with PBS (pH 7.4), centrifuged and re-suspended in 2.5% glutaraldehyde. After being maintained for 2 hours at 4ºC, the pellets were centrifuged, washed twice with PBS, re-suspended in 2% osmium tetroxide and maintained at 4ºC for 2 hours. After this time cells were centrifuged, washed with PBS, mixed with a small volume of melted 2.5% agarose and immediately chilled on ice. The solidified agarose was cut into blocks (about 1 mm^3), stained *en bloc* for 1 hour with 1% uranyl acetate in 0.05 M sodium maleate buffer pH 5.2 at 4ºC, dehydrated in a graded ethanol series, infiltrated and embedded in Spurr's epoxy resin and polymerised for 24 hours at 70ºC. For transmission electron microscopy (TEM), ultra-thin sections of 80 nm were obtained with the aid of an Ultracut S (Microtome, Leica, Vienna, Austria) using a diamond knife. The sections were post-stained with uranyl acetate and Reynold's lead citrate and viewed by Prof. Dr. Amaral and Prof. Dr. Bleiss with the aid of a Zeiss EM 900 electron microscope (Carl Zeiss, Oberkochen, Germany).

II.7. Evaluation of *ex vivo* activity of compounds studied against *S. aureus*

II.7.1 Bacteria

S. aureus ATCC25923 (control strain) and Methicillin-Resistant *S. aureus* (MRSA) strains were selected as experimental model for screening of compounds that will work on MDR-TB. The MRSA strains were clinical isolates gently provided by Dr[a]. Teresa Pacheco (Laboratório de Microbiologia, Hospital Egas Moniz; Lisboa, Portugal).

II.7.2 Materials

RPMI medium, Ficoll-Hypaque, HBSS and Trypan blue were purchased from Sigma-Aldrich Química SA (Madrid, Spain). 50 mL Falcon tubes and tissue culture plates were purchased from Nalgene (Nunc, Thermo Scientific; Rochester, NY, USA). TSA was purchased from Oxoid (Basingstoke, England).

II.7.3 Instrumentation

Refrigerated centrifuge (Rotanta 46R; Hettich Zentrifugen)
CO_2 Water Jacketed Incubator (Nuaire NU-4500/E)

II.7.4 Quality control

The incubator was periodically checked and calibrated with a certified thermometer (Omnilabor, Lisboa, Portugal). Weekly performance tests related to temperature and percent of CO_2 inside the incubator chamber were conducted according to manufacturer recommendation.

II.7.5 Method

II.7.5.1 Isolation of Human monocyte-derived macrophages

Twenty-millilitres of blood from healthy voluntary donors was collected and transferred into tubes containing 50 U of preservative-free sodium heparin (Monoparin, CP

Pharmaceuticals Ltd, Wrexham, UK). The blood sample was processed on the same day of the collection. A 1:2 dilution was performed with RPMI 1640 medium and 20 mL were layered onto 12 mL of Ficoll (Sigma, Madrid, Spain). Blood was centrifuge at 800×G for 30 minutes. Plasma was collected to a new Falcon tube and kept for subsequent complete media preparation (RPMI medium supplemented with 10% of autologous serum). The mononuclear cells obtained from the buffy coats were transferred to new 50 mL Falcon tubes. Cells were washed with HBSS at 800×G for 30 minutes and then 2 more times at 800×G for 10 minutes. The pellet obtained was re-suspended in 5 mL of RPMI 1640 medium and an aliquot of 10 μL was retrieved and added to 90 μL of a trypan-blue solution. Viable cells were counted and the cellular concentration adjusted to 1×10^6 cells/mL in RPMI 1640 medium containing 2 mM L-glutamine (Sigma; Madrid, Spain) and supplemented with 10% of autologous serum. From this cellular suspension aliquots of 1 mL were distributed to the wells of a 24-well plate (Nalgene, Nunc, Thermo Scientific; Rochester, NY, USA). Cellular cultures were incubated at 37ºC with 5% CO_2 for 3 days. After this period, medium was retrieved from each wells and aliquots kept at -80ºC for posterior cytokines analysis. A cycle of three washes were performed with RPMI 1640 medium to remove all the non adhered cells (lymphocytes). 10% of the total cells that adhere to the wells of the plate are monocyte-derived macrophages. Aliquots of complete medium (1 mL) are added to each well and the plate incubated at 37ºC with 5% of CO_2 for 2 more additional days. After this incubation period, monocyte-derived macrophages were ready for infection with *S. aureus* strains.

II.7.5.2 Phagocytosis assay

On the day the cells were to be infected with *S. aureus*, the wells were previously washed three times with RPMI 1640 medium and new fresh medium was distributed to each well. The monocyte-derived macrophages were then infected with 0.010 mL of a bacterial suspension of 1.0×10^6 bacteria/mL and the cells incubated at 37°C, 5% CO_2 for 30 minutes. This ratio of 10:1 (bacteria:macrophage) was previously optimized and was found to result in optimum phagocytosis (Ordway *et al*., 2003b). After this period, all the supernatants were removed and three consecutive washes with RPMI were performed to remove all the non-phagocytosed bacteria. The supernatants of the last wash was pooled, plated in TSA and subjected to counting of the numbers of CFU in

order to determine if all the non-phagocytosed bacteria had been removed by the washings. The TZ and its derivatives in concentrations of 0.01 and 0.1 mg/L were added to the wells. Control wells (no compounds) were conducted in parallel. The plates were incubated at 37°C for 0, 1 minute, 2, 4 and 6 hours. 1.0 mL of 0.01% SDS was added to each well at the determined period of incubation and the wells scrapped to release all the phagocytosed bacteria. Aliquots of 0.1 ml of the lysed supernatants were serially diluted with saline to 10^{-4} and plated onto TSA plates. The plates were incubated overnight at 37°C and the CFU counted for determination of bacterial concentration (Ordway *et al*., 2002a). All the procedures were conducted in triplicate and repeated at least two times.

II.7.5.3 QC for method

All the procedures were conducted in triplicate and TZ was used in all the experiments as an internal control. The killing activity of the macrophages was also assessed by the plating of the supernatants at 0 and 1 minute of phagocytosis.

The same methodology was applied to test the effect of the methanolic extract of *C. edulis* and of the K^+ flux inhibitors, reserpine, ouabain and verapamil.

II.8. Evaluation of *ex vivo* activity of compounds studied against *M. tuberculosis* strains

II.8.1 Bacteria

M. tuberculosis H37Rv ATCC27294, which is susceptible to RIF, INH, STR, and EMB, served as the absolute control and MDR-TB strain (resistant to IHN, RIF and STR) was the strain selected for conduct studies with TZ and its derivatives. The MDR strain was isolated from a patient involved in an outbreak caused by a highly virulent MDR *M. tuberculosis* strain (cluster A or cluster Lisbon) in a Lisbon Prison Hospital in 1997 and has been typed by IS6110-based restriction fragment length polymorphism analysis as a member of this cluster (Ordway *et al*., 2003b; Portugal *et al*., 1999). This strain has been maintained in the Unit of Mycobacteriology (IHMT/UNL; Lisboa, Portugal) and characterized for their susceptibility and resistance to the antibiotics listed above with

the BACTEC 460-TB system (BACTEC 460 TB SIRE method (1985) Becton Dickinson Microbiology Systems).

II.8.2 Materials

PBS, HBSS, RPMI 1640 medium, Ficoll, and L-glutamine were purchased from Gibco (Paisley, United Kingdom). TZ, SDS and trypan blue were purchased from Sigma Aldrich Química SA (Madrid, Spain). Middlebrook 7H11 solid medium was purchased from Difco (Detroit, Mich). Microwell tissue culture plates were purchased from Nalgene (Nunc, Thermo Scientific; Rochester, NY, USA). All solutions were prepared in distilled, sterile water on the day of the experiment.

II.8.3 Instrumentation

Refrigerated centrifuge (Rotanta 46R; Hettich Zentrifugen)
CO_2 Water Jacketed Incubator (Nuaire NU-4500/E)

II.8.4 Quality control

Weekly performance tests of temperature and CO_2 were conducted at the Unit of Mycobacteriology according to the manufacturer recommendations. The incubator was also periodically checked and calibrated by a certified manufacturer (LaboControle, Lisbon, Portugal).

II.8.5 Method

II.8.5.1 Isolation of Human monocyte-derived macrophages

Twenty millilitres of intravenous blood from healthy voluntary donors was collected and transferred into tubes containing 50 U of preservative-free sodium heparin (Monoparin, CP Pharmaceuticals Ltd, Wrexham, UK). The collected blood was diluted 1:2 in RPMI 1640 medium and 20 mL was layered onto 12 mL of Ficoll (Sigma, Madrid, Spain). Blood was centrifuge at 800×G for 30 minutes and after this period monocytes were obtained from formed buffy coats and transferred to new 50 mL Falcon

tubes. HBSS was added and three washes were performed, as described: three cycles of centrifugations at 800×G for 30 minutes. The pellet obtained was re-suspended in 5 mL of RPMI medium and from these an aliquot of 10 µL was retrieved and added to 90 µL of a trypan-blue solution and displaced in a Neubauer chamber to perform a viability assay. After the cell counting, the concentration was adjusted to 1×10^6 cells/mL in RPMI 1640 medium containing 2 mM L-glutamine (Sigma; Madrid, Spain) and supplemented with 10% of autologous serum. Aliquots of 1 mL of this cellular suspension were disposed in the wells of a 24-well plate (Nalgene, Nunc, Thermo Scientific; Rochester, NY, USA), and the cultures incubated at 37ºC, 5% CO_2 for 5 days. On the third day of incubation, medium was retrieved from the wells and a cycle of three washes was performed with RPMI medium in order to remove all the non adhered cells. After 5 days of culture, monocyte-derived macrophages were then ready for infection with *M. tuberculosis*. It is important to note that all blood samples were processed on the same day of the collection.

II.8.5.2 Phagocytosis and killing activities of *M. tuberculosis* strains by peripheral blood monocyte-derived macrophages (PBMDMs)

After 18 hours of culture of PBMDMs (1.0×10^6 cells/ml) in RPMI medium containing 2 mM L-glutamine and autologous human serum, approximately 10% of the PBMDMs that adhere to the bottom of the wells are monocytes (Ordway *et al*., 2002a). A bacterial suspension of 1.0×10^6 bacteria/mL in 0.010 mL, which had previously been found to result in optimum phagocytosis (ratio of 10 CFU:1 macrophage) (Ordway *et al*., 2003b) was added to the monolayer cultures and the cultures were incubated at 37°C, 5% CO_2 for 1 hour (phagocytosis). Two consecutive washes with RPMI were performed to remove the extracellular bacteria. These washings were pooled and subjected to counting of the numbers of CFU in order to determine the efficiency of phagocytosis. A third wash was performed and subjected to plating and counting of the numbers of CFU for verification of the complete absence of non phagocytosed bacteria. New RPMI 1640 media supplemented with 10% of autologous serum was disposed in the wells of the plate. The infected macrophages were then pulsed with either TZ or its derivatives at non-toxic concentrations of 0.1 and 0.01 mg/L. The cultures were incubated at 37°C, 5% CO_2 for 0, 1, 2 and 3 days. After these periods, cells were lysed with 1.0 mL of 0.01% SDS and from these lysed supernatant, 0.1 mL aliquots were serially diluted with

saline to 10^{-4}. Aliquots (0.1 mL) from each dilution were plated onto 7H11 Mycobacteria medium and the plates incubated at 37°C for 3 to 4 weeks. After this period, CFU were counted and the bacterial concentration determined (Ordway *et al*., 2002a).

II.8.5.3 QC for method

Thioridazine was used in all the experiments as an internal control since it had been already established by us that this phenothiazine enhanced the killing activity of the macrophage in a highly reproducible manner (Ordway *et al*., 2002a; Ordway *et al*., 2003b). The killing activity of the *M. tuberculosis* infected macrophages was also assessed by periodically plating aliquots of supernatants from zero time, 1, 2 and 3 days after infection (phagocytosis of *M. tuberculosis*). All the procedures were conducted in triplicate and repeated at least two times.

The methanolic extract of *C. edulis*, SILA compounds, 409 and 421 and the EPIs, reserpine, ouabain and verapamil, were also tested for the enhancement of the macrophage killing activity, applying the same methodology described above.

II.9. Electron microscopy (*ex vivo*)

II.9.1 Bacteria

MRSA strains and MDR-TB strains were selected for studies with TZ and its derivatives. These strains have been maintained in the Unit of Mycobacteriology (IHMT/UNL; Lisboa, Portugal) and characterized for their susceptibility and resistance with the aid of BACTEC 460-TB system to the antibiotics and antimicrobial agents listed above (BACTEC 460 TB SIRE method (1985) Becton Dickinson Microbiology Systems).

II.9.2 Materials

TZ, OXA, paraformaldehyde, SDS, trypan blue, glutaraldehyde and osmium tetroxide were purchased from Sigma Aldrich Química SA (Madrid, Spain). PBS, HBSS, RPMI

medium, Ficoll and L-glutamine were purchased from Gibco (Paisley, United Kingdom). Microwell tissue culture plates were purchased from Nalgene (Nunc, Thermo Scientific; Rochester, NY, USA). TZ was prepared in distilled sterile water on the day of the experiment and protected from light.

II.9.3 Instrumentation

Refrigerated centrifuge (Rotanta 46R; Hettich Zentrifugen)
Zeiss EM 900 electron microscope (Carl Zeiss, Oberkochen, Germany)
Ultracut S (Microtome, Leica, Vienna, Austria)

II.9.4 Method

In order to determine if the effects that TZ induced on the ultra-structure of MRSA and MDR-TB *in vitro*, could also be seen *ex vivo*, macrophages cultures were prepared as previously described (see II.6. Evaluation of *ex vivo* activity of compounds studied against *Staphylococcus aureus*). Infection of the monocyte-derived macrophages was also performed in accordance with the protocols described (see sections II.6 and II.7 for *S. aureus* and *M. tuberculosis* cultures, respectively). A control with no TZ was conducted in parallel. The protocol described was conducted in the same manner for MRSA and MDR-TB strains and is described as follows: after the incubation period, cells were lysed with 0.01% of SDS and the products of *ex vivo* cultures-containing or lacking TZ, were centrifuged at 800×G for 30 minutes. The pellets were washed with PBS (pH 7.4), centrifuged and re-suspended in 2.5% glutaraldehyde. After being maintained for 2 hours at 4ºC, the pellets were centrifuged, washed twice with PBS, re-suspended in 2% osmium tetroxide and maintained at 4ºC for 2 hours. After this time, pellets were centrifuged, washed with PBS and mixed with a small volume of melted 2.5% agarose and immediately chilled on ice. The solidified agarose was cut into blocks (about 1 mm^3), stained *en bloc* for 1 hour with 1% uranyl acetate in 0.05 M sodium maleate buffer pH 5.2 at 4ºC, dehydrated in a graded ethanol series, infiltrated and embedded in Spurr's epoxy resin and polymerised for 24 hours at 70ºC. For TEM, ultra-thin sections of 80 nm were obtained with the aid of an Ultracut S (Microtome, Leica, Vienna, Austria) using a diamond knife. The sections were post-stained with uranyl

acetate and Reynold's lead citrate and viewed with a Zeiss EM 900 electron microscope (Carl Zeiss, Oberkochen, Germany).

II.10. Evaluation of *in vivo* activity of TZ against Balb/C mice infected with *M. tuberculosis*

The main goal of this thesis was to evaluate the toxicity effect of TZ and its selected derivatives as well and the effectiveness of these phenothiazines for curing Balb/C mice infected with *M. tuberculosis* H37Rv ATCC27294. This study was conducted with the parent molecule (TZ) since the parameters (toxicity, optimization of compound dosage, concentration, injection schedule, *etc*.) to be established are extremely important. The animals selected were Balb/C mice since they present an increased susceptibility to infection and progression to active disease and by this manner, can mimic the process that occur in the human exposed to *M. tuberculosis*.

II.10.1 Bacteria

Because we had already demonstrated that the response of MDR-TB is identical to that of antibiotic susceptible *M. tuberculosis* H37Rv ATCC27294 (Ordway *et al*., 2003b) the latter strain was selected for infecting the mouse. Moreover, the selection of this strain provided more safety to the Ph.D. candidate since the therapy of an infection with antibiotic susceptible *M. tuberculosis* is readily possible whereas an infection with MDR-TB can be lethal. *M. tuberculosis* ATCC H37Rv was cultured in MGIT 960 medium (Difco, Madrid, Spain) containing Tween 80 (Difco, Madrid, Spain) until an O.D. of approximately 0.6 was reached. After this, aliquots were prepared and the inoculums adjusted to 1×10^6 bacteria/mL in PBS (Sigma-Aldrich Química SA, Madrid, Spain).

II.10.2 Animals

Thirty female Balb/C mice age 2-3 weeks (approximately 30 g) were purchased to Harlan Iberica (Madrid, Spain).

II.10.2.1 Animal and Human Safety conditions

During and after the quarantine period that the animals were subjected after being delivered to the IHMT, during infection and during treatment with TZ, that is, for the duration of the experiment, mice were housed in a Class II BioSafety Cabinet maintained within a fully certifiable P3 facility (Office of Health and Safety (OHS) USA-Section VI, Recommended biosafety levels for infectious agents) at the Unit of Mycobacteriology. Licensing and certification of the Ph.D. candidate for laboratory animal handling an experimental infection of small animals was obtained after the attending of the Course in Laboratory Animal Science, FELASA, Category C, held at the IHMT/UNL (Lisboa, Portugal) and subsequent examination. The License and researcher certificate was granted by the Direcção Geral de Veterinária (DGV, Portugal).

II.10.3 Materials

Insulin syringes (Terumo) were purchased from Omnilabor (Lisbon, Portugal)
Microisolator cages (E1CCBAC056) with HEPA filters on the top (E4FVCOU926) (Charles River Laboratories, Massachusetts, USA)

II.10.4 Instrumentation

Analytic Balance (Explorer; Ohaus)
Laminar Flow Cabinets (NU-425-600E; Nuaire)
Microcentrifuges (Labofuge 200; Heraeus)
Wallthrough double door Autoclave (AMARO 2000; A. J. Costa)
37ºC Incubator (Model 600; Memmert)

II.10.5 Quality control

The described study requires important security measures that were strictly followed since the work performed involved infection of the animals with *M. tuberculosis*. In this manner, the state of the art biosafety level 3 conditions of the Unit of Mycobacteriology-P3-Safety Laboratory were maintained, by replacing the HEPA

filters (P3 Safety Laboratory Air Control Devices; Tecniclima, 1999) every 6 months by a certified company (Tecniclima, Portugal). Also, additional annual maintenance was conducted for the wall-through autoclave.

II.10.6 Method

II.10.6.1 Quarantine

Before toxicity assays were initiated the animals were kept in quarantine for 3 weeks. Mice were divided in groups of 5 animals each with water and food *ad libitum*. Every day the animals were observed for any alterations in behaviour, indicative of stress or disease. The illumination of the laboratory safety facility was daily checked. During the quarantine period none of the animals developed any visible scar or signal of disease. By this manner, all the animals were considered for toxicity testing.

II.10.6.2 Toxicity assays

Mice were divided in 6 groups of 5 animals each. From these, 5 groups were daily injected with 0.1 mL of a TZ solution in concentrations of 0.01, 0.02, 0.05, 0.10, 0.2 and 0.5 mg/day. These doses are equivalent to 25, 50, 100, 200, 400 and 1200 mg TZ/kg/day in the human. The remaining group (control) was submitted to the same protocol however, it was injected with a saline solution. Daily monitoring was performed recording all the alterations in behaviour or in weight.

II.10.6.3 Infection studies

All the animals were intraperitoneally (i.p.) infected with *M. tuberculosis* H37Rv ATCC27294 with 1×10^6 bacteria in 0.1 ml of sterile saline, strictly following all the safety and security measures recommended for experiments involving animals and handling of infectious agents (CDC, 1999; Hau and Van Hoosier, 2003; NIH, 1996). After the infection, mice were divided into groups of 5 animals each. After three days post-infection, treatment with TZ (Sigma-Aldrich Química SA, Madrid, Spain) was initiated, except for the control group. Each day, four groups of mice (to be treated) were injected i.p. with 0.1 mL of TZ in the concentrations of 0.05, 0.10, 0.2 and 0.5

mg/day, respectively. These doses are equivalent in the human to 100, 200, 400 and 1200 mg TZ/kg/day, considering an average weight of 70 Kg for an adult. The maximum dose of 0.5 mg/day is equivalent, on a kg basis, to approximately twice the maximum dose a chronically treated psychotic patient receives per day. The control group received no treatment however, in order to be submitted to the same stress than the animals of the other groups, was injected with a saline solution (PBS, Sigma-Aldrich Química SA, Madrid, Spain) with the same volume used to administrate the agent to the mice. The animals were observed daily in order to monitor alterations of behaviour and were weighed daily before the administration of TZ. At the end of 30 days, and thereafter at monthly intervals, mice from each group were sacrificed by cervical dislocation and their lungs, liver and spleen removed and weighed. One lung, a lobe of the liver and a portion of the spleen were kept for histological studies. The remaining portions of these organs were weighed, diced and transferred to homogenizing conical tubes containing 1 mL of 0.1N NaOH. The organs were manually homogenised and aliquots of 0.1 mL transferred in triplicate to 1.0 mL of saline (PBS, Sigma-Aldrich Química SA). From these, aliquots of 0.1 mL were serially transferred to 1.0 mL of saline and 0.1 mL of the resulting solution plated onto 7H11 agar plates (Mycobacteria 7H11 Agar; Difco, Madrid, Spain). The plates were incubated at 37ºC and after 3 to 4 weeks of incubation the CFU were counted and extrapolated to CFU/kg of tissue.

CHAPTER III.

Clinical Concentrations of Thioridazine Enhance the Killing of Intracellular Methicillin-resistant S*taphylococcus aureus*: an *In Vivo*, *Ex Vivo* and Electron Microscopy Study

This chapter contains data published in:

Martins, M., W. Bleiss, A. Marko, D. Ordway, M. Viveiros, C. Leandro, J. Molnar, J. E. Kristiansen, J. Wecke, and L. Amaral. 2004. Clinical concentrations of thioridazine enhance the killing of intracellular methicillin-resistant *Staphylococcus aureus*: an *in vivo*, *ex vivo* and electron microscopy study. In Vivo **18**:787–794.

To reduce the global burden of TB by 2015, the WHO in line with the Millennium Development Goals and the Stop TB Partnership, has defined 4 main objectives, namely: the achievement of universal access to high-quality diagnosis and patient-centred treatment; the reduction of the human suffering and socioeconomic burden associated with TB; the protection of poor and vulnerable populations from TB, TB/HIV and MDR-TB and finally to support the development of new tools and enable their timely and effective use. In order to accomplish these objectives the targets considered were endorsed by the Stop TB Partnership and by 2005 detected at least 70% of new sputum smear-positive TB cases and cure of at least 85% of these cases. By 2015, these targets are expected to reduce prevalence of and deaths due to TB by 50% relative to 1990 and, by 2050 eliminate TB as a public health problem (<1 case per million population). In order for this strategy to succeed, 6 main components were established, namely:

1) Pursue high-quality DOTS expansion and enhancement through the political commitment with increased and sustained financing; **the case detection through quality-assured bacteriology**; standardized treatment with supervision and patient support; an effective drug supply and management system and the monitoring and evaluation system, and impact measurement;

2) Address TB/HIV, MDR-TB and other challenges by implementing collaborative TB/HIV activities; prevent and control MDR-TB and address prisoners, refugees and other high-risk groups and special situations;

3) Contribute to health system strengthening by actively participate in efforts to improve system-wide policy, human resources, financing, management, service delivery, and information systems and share innovations that strengthen systems, including the Practical Approach to Lung Health, as well as to adapt innovations from other fields;

4) Engage all health care providers: Public-Public, and Public-Private Mix approaches and defining International Standards for TB Care;

5) Empower people with TB, and communities by advocacy, communication and social mobilization; community participation in TB care and patients' Charter for Tuberculosis Care;

6) **Enable and promote a Programme-based operational research to develop new diagnostics, drugs and vaccines.**

At the Unit of Mycobacteriology (IHMT/UNL) we have focused on objectives 1 and 6 which have, with the implementation of the TB Task Force (previously described in the introduction of this thesis) yielded reduction of new cases of MDR-TB from an excess of 28% to less than 8% (DGS, 2006) and developed **new drugs which have activity against XDR-TB/MDR-TB.**

The main objective of my thesis research is the development of new drugs that have activity against XDR-TB/MDR-TB. Because phenothiazines have been shown by our group to have activity against these antibiotic resistant strains of *M. tuberculosis*, I have selected thioridazine, a derivative of chlorpromazine but which produces far milder side effects, for assessment of its *in vitro, ex vivo* and *in vivo* activity against *M. tuberculosis*; have examined the mechanism by which it produces its anti-TB effects; and as a consequence have provided this information for the synthesis of derivatives of TZ derivatives by a colleague of our laboratory (Professor György Hajós, Hungarian Academy of Science, Budapest, Hungary). The results presented in the next series of sections will describe the studies conducted that define *in vitro, ex vivo* and *in vivo* activity of TZ derivatives against *M. tuberculosis* strains, the mechanism by which these agents produce their anti-TB effects as well as the methods that have been developed for the characterisation of a specific form of resistance which render bacteria resistance to two or more unrelated antibiotics. Each chapter will present an initial outline and the most important results obtained as well as a general discussion. Whenever relevant, the publication itself or aspects of that publication that resulted from this component of the thesis will be presented.

CHAPTER OUTLINE

This chapter is focused on the *in vitro* and *ex vivo* effects of TZ on Methicillin-resistant *S. aureus* (MRSA) strains. MRSA has been used as a model that evaluates the effects of the phenothiazine since these are essentially the same as those produced with strains of *M. tuberculosis* and are produced and evaluated within a day as opposed to three or more weeks when *M. tuberculosis* is used. The data obtained from the MRSA model is then used for the planning of experiments with *M. tuberculosis.* The minimum inhibitory and minimum bactericidal concentrations of CPZ and TZ against MRSA strains were determined and the intracellular killing activity of these phenothiazines on MRSA strains evaluated. Electron microscopy studies were also performed to determine the effects of TZ on the ultrastructure of MRSA strains *in vitro* and on MRSA that has been phagocytosed by macrophages. The results obtained with Methicillin-resistant *S. aureus* strains establish the parameters to be applied in subsequent work involving *M. tuberculosis* strains.

SUMMARY

Chlorpromazine (CPZ) is concentrated by human macrophages where it kills intracellular mycobacteria when the concentration outside the macrophage is sub-clinical. We have previously demonstrated that thioridazine (TZ), a much milder phenothiazine, has similar activity and kills intracellular methicillin-susceptible *S. aureus* at sub-clinical concentrations (Ordway *et al*., 2002a). We have extended this latter study to include methicillin-resistant *S. aureus* (MRSA) and show that TZ kills intracellular MRSA at clinically relevant concentrations. The ultrastructure of MRSA exposed to *in vitro* concentrations of TZ just below its MIC and that of MRSA phagocytosed by macrophages previously exposed to a clinically relevant concentration of TZ was also studied. TZ inhibits the replication of phagocytosed MRSA, affecting the structure of the cell envelope resulting in lysis of the bacterium 6 hours post-phagocytosis. These ultrastructural changes are identical to those produced *in vitro* by a TZ concentration that is near the MIC. Because macrophage intracellular MRSA is not killed by the macrophage and its intracellular location protects it from antibiotics that are unable to reach that site, recurrent infections which result may be successfully managed with the use of TZ.

The materials and methods employed for this study are described in Chapter II, (sections II.3 and II.7) as well as by publication #1 listed in the Appendix. Nevertheless, the legends of the tables and figures contain sufficient information relevant to the methods employed.

RESULTS

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of CPZ and TZ against MRSA strains. The *in vitro* activity of CPZ and TZ against MRSA strains is summarised in Table 1. Briefly, although CPZ and TZ have similar MICs against either strain of *S. aureus*, the activity of TZ is greater than that exhibited by CPZ. The MICs for CPZ and TZ against these *S. aureus* are consistent with those previously reported (Amaral *et al*., 1992; Ordway *et al.*, 2002a; Radhakrishnan *et al.*, 1999). The bactericidal activity of each of these compounds is expressed at a higher concentration of each compound and, again, the activity of TZ, as expressed by the MBC, is higher than that of CPZ although the difference is not statistically significant.

Table 1. MIC and MBC of CPZ and TZ against methicillin-resistant *S. aureus* (MRSA) strains[a].

		MIC and MBC of compounds (mg/L)		
		MRSA1	**MRSA2**	**MRSA3**
CPZ	**MIC**	40	40	40
	MBC	80	70	80
TZ	**MIC**	20	20	30
	MBC	60	40	50

[a]100,000 CFU were incubated at 37°C with increasing concentrations of each compound in 10 mL TSB for 18 hours. The MIC of each compound-strain was determined from visible examination of cultures that contained no evidence of growth. The MBC of each compound-strain was determined by plating the cultures beyond each MIC on TSB agar plates and counting CFUs. **CPZ** (chlorpromazine), **TZ** (thioridazine).

The intracellular killing activity of CPZ and TZ on MRSA strains. A minimal concentration of CPZ or TZ of 0.1 mg/L, as shown by Figure 1, after a slow initial effect (2 hours), consistently reduced the number of viable MRSA cells by the end of 4 hours after phagocytosis by human PBMDM. Enhanced intracellular killing activity continued to increase with time such that, by the end of 6 hours, killing was almost complete. Similar results were obtained with the use of the THP-1 cell line (data not shown). The lower concentration of 0.01 mg/L of either of these two phenothiazines had a marginal effect on the number of viable bacteria recovered 6 hours subsequent to phagocytosis. The concentration of 0.1 mg/L was confirmed in this study, and in previous studies, to be non-toxic to the macrophage and not to affect the phagocytic processes of this cell (Ordway *et al.*, 2002a).

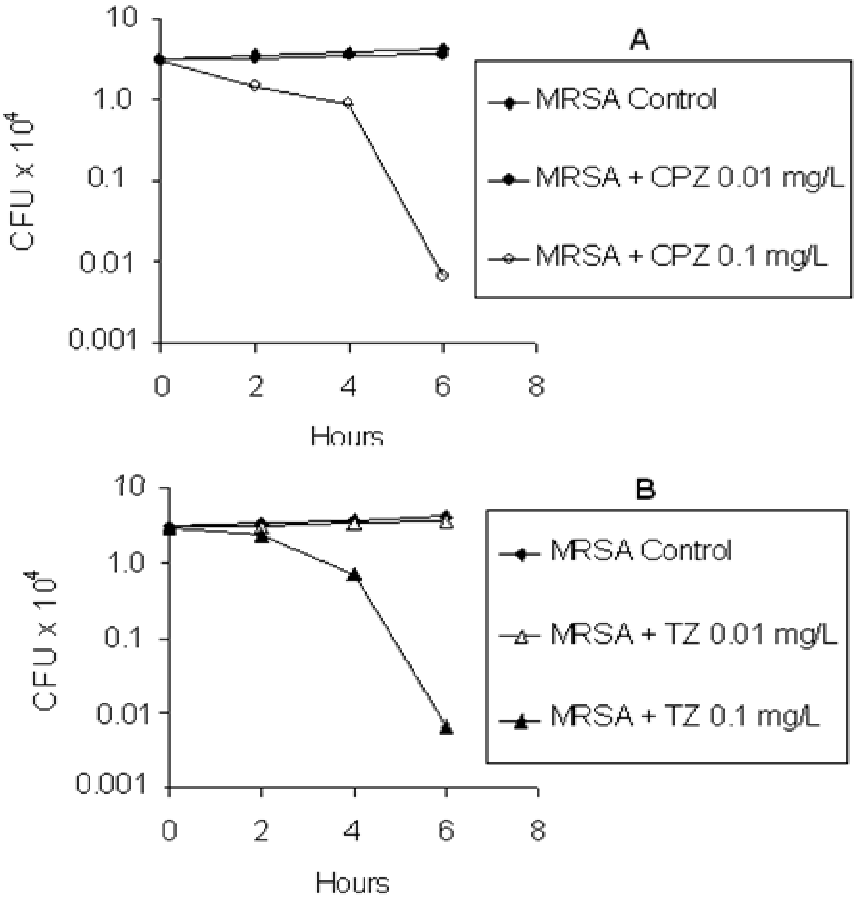


Figure 1. The average effect of chlorpromazine (CPZ) and thioridazine (TZ) on intracellular growth of the 3 MRSA strains tested. The THP-1 and human PBMDM were infected for 30 minutes with the methicillin-resistant clinical strains of *S. aureus* (MRSA), at concentrations of 10 CFU per macrophage (10:1). CPZ and TZ were then separately added to the cultures at concentrations of 0.01 and 0.1 mg/L. Control cultures received no drug. Results are expressed as the

mean CFU of the 3 MRSA strains tested in 3 independent experiments. Data presented was obtained with the use of human PBMDM and is similar to that obtained from the use of THP-1 cells.

The effects of TZ on the ultrastructure of MRSA strains *in vitro*. The *in vitro* presence of a concentration of 20 mg/L of TZ that is below that which totally eliminates the replication of MRSA produced the typical ultrastructure of MRSA is shown by Figure 2. Briefly, major alterations noted were: blebbing of the cell wall (a); thickening of the cell wall and cross-walls (b); partial separation of the internal portion of the cell wall (c); continued production of cross-walls without subsequent separation of the replicated cells (d); asymmetrical cross-wall formation (e); partial loss of cell wall (f) and lysis of the cell (g). Only (f) and (g) alterations are expected to ultimately, result in cell death, inasmuch as even the most severe changes (a-e) are reversible when the organism is transferred to TZ-free medium (data not shown). All of the alterations noted have been previously reported for methicillin-susceptible *S. aureus* exposed *in vitro* to sub-inhibitory concentrations of chlorpromazine (Kristiansen and Amaral, 1997).

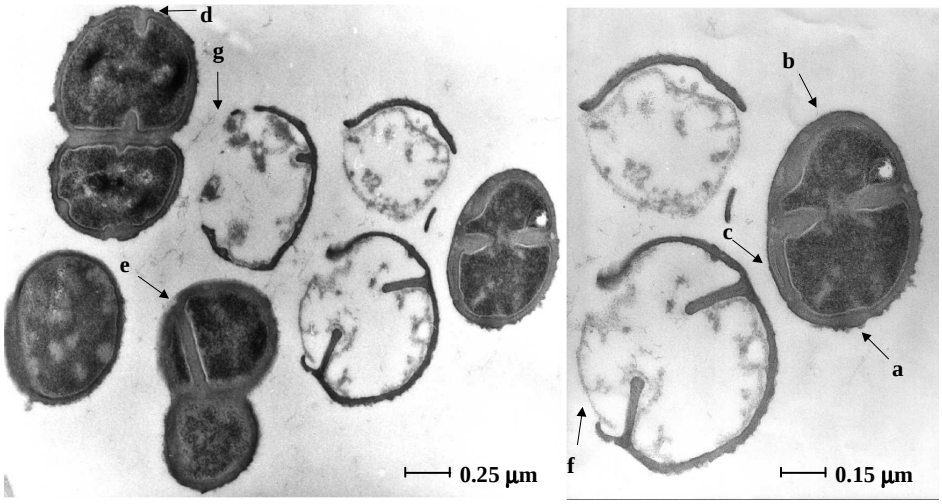


Figure 2. **The *in vitro* effect of TZ on the ultrastructure of MRSA after 18 hours of culture.** Blebbing of cell wall (a), thickened cell wall and cross-walls (b), partial partitioning of the internal portion of the cell wall (c), continued production of cross-walls without separation of the replicated cells (d), asymmetrical cross-wall formation (e), partial loss of cell wall (f), evidence of lysis (g). It is important to note that this concentration of TZ does not kill the majority of cells and that, with the exception of (f) and (g), the alterations noted are reversible. The strain selected for the electron microscopy studies was the MRSA3.

The ultrastructure of MRSA that has been phagocytosed by macrophages previously exposed to a concentration of TZ of 0.1 mg/L. The typical ultrastructure of phagocytosed MRSA of a 6-hour culture control is presented in Figure 3 and is quite normal and identical to that previously shown for *in vitro* controls (Kristiansen and Amaral, 1997). There is ample evidence that cell division is taking place within the phagosome, as noted by the presence of cross-walls in different degrees of completion and almost complete separation of the two replicated cells from each other. It should be noted that, because the thickness of the section was very small (80 nm), many of the cells present in Figure 3 show only portions of the cell that are distal to the cross-wall. In contrast to the ultrastructure of the 6-hour control population of intracellular MRSA, phagocytosis of the MRSA followed by exposure to 0.1 mg/L of TZ results in the alteration of the ultrastructure of MRSA as early as 4 hours post-phagocytosis (Figure 4). Evidence of replication was scarce as noted by the absence of cross-walls in the majority of phagocytosed MRSA and, when cross-walls are present, they are thickened (Figure 4a). The periphery of the cell wall appeared eroded (Figure 4b). Further alteration of the ultrastructure of *S. aureus* was evident after 6 hours (Figure 5). It should be noted that phagocytosed MRSA showed an incomplete cell wall and in some cases, an incomplete plasma membrane that allowed the spilling of the cytoplasm out of the bacterium. The presence of cross-walls in this population of intracellular MRSA was then quite rare suggesting that replication had been essentially inhibited.

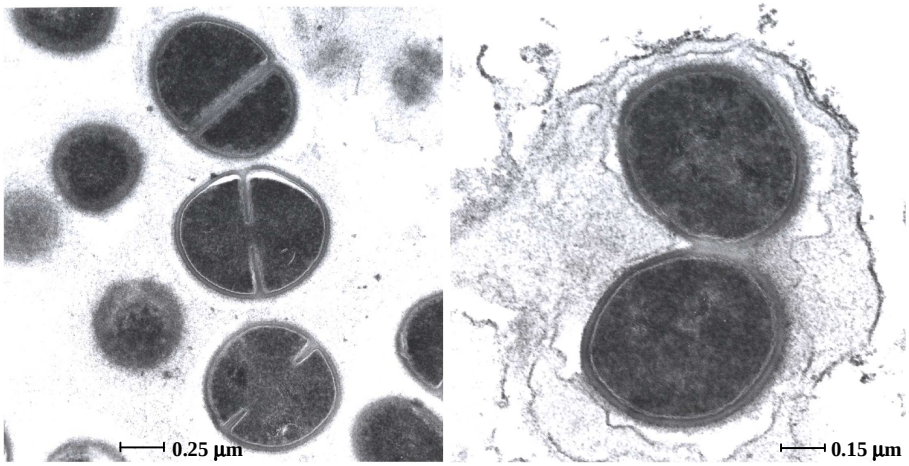


Figure 3. **The ultrastructure of MRSA 6 hours after it has been phagocytosed by the human macrophage (Controls).** Presence of normal morphology, evidence of cross-wall formation at different stages.

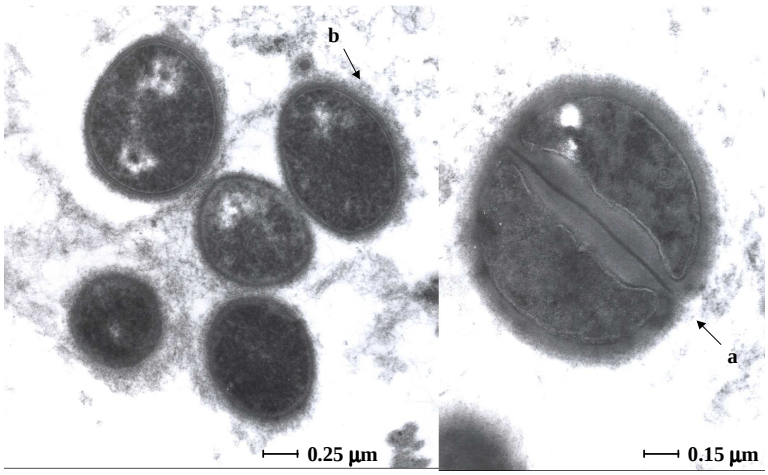


Figure 4. The ultrastructure of MRSA 4 hours after it has been phagocytosed by the human macrophage that had been subsequently exposed to a concentration of TZ of 0.1 mg/L. Cross-walls are scarce and when present are bizarre (a); periphery of cell wall is eroded (b).

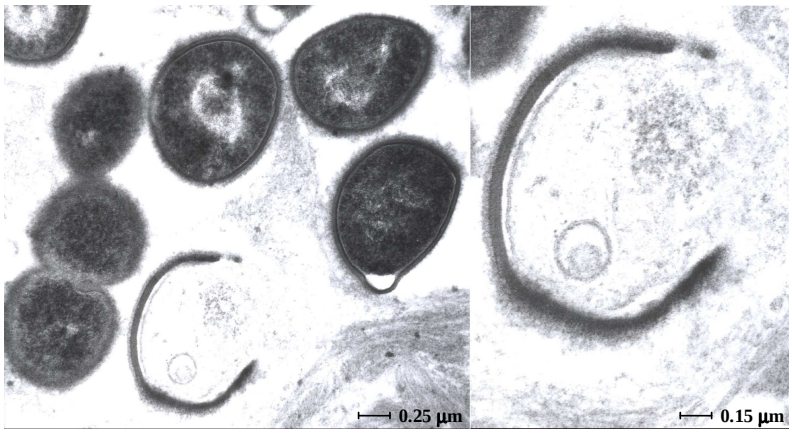


Figure 5. The ultrastructure of MRSA 6 hours after it has been phagocytosed by the human macrophage that had been subsequently exposed to a concentration of TZ of 0.1 mg/L. The same effect was observed in all TZ exposed cells. **Note:** Incomplete cell wall, incomplete plasma membrane, evidence of lysis. Compare with Figure 2.

DISCUSSION

The main objections raised for the use of chlorpromazine as an antibacterial agent are: (i) its known toxicity when chronically employed and, (ii) its *in vitro* antibacterial activity which takes place at concentrations which are well beyond those achievable in the human. The results obtained in this study suggest that, as previously shown (Amaral and Kristiansen, 2000; Amaral and Kristiansen, 2001; Amaral *et al*., 1996; Amaral *et al*., 2001a; Amaral *et al*., 2001b; Bettencourt *et al*., 2000; Ordway *et al*., 2002b; Viveiros and Amaral, 2001), thioridazine has *in vitro* antibacterial activity equal to that of chlorpromazine. Furthermore, the current study also showed that a concentration of either phenothiazine corresponding to one easily achieved in patient plasma kills intracellular staphylococci regardless of their antibiotic susceptibility. Because the *in vitro* concentration of either phenothiazine needed to kill MRSA is of the order of 60 mg/L, and since both of these compounds are known to be concentrated by macrophages/tissues rich in macrophages (Ordway *et al.*, 2002a), we hypothesise that the concentration of either drug needed to kill intracellular *Staphylococcus* is reached as a result of the macrophage's ability to concentrate these compounds. This belief is further supported by the demonstration that whereas a concentration of TZ near its MIC is needed to alter the morphology of MRSA *in vitro*, the altered morphology are reproduced in phagocytosed MRSA by a concentration of TZ in the cell culture medium that is 200 to 300 times lower. Phenothiazines intercalate between nucleic bases of DNA (Stolze and Mazon, 1991) and result in inhibition of all DNA-based processes (Hawtrey *et al*., 2002; Kruglova and Zinenko, 1993; Webb and Hass, 1984). The *in vitro* concentration needed for sufficient TZ to traverse the cell envelop of MRSA and reach DNA for its intercalation must be exceedingly greater than that present in the medium to which the macrophage-phagocytosed MRSA is exposed. We believe that the killing of intracellular staphylococci by TZ is the result of the macrophage achieving the sufficiently high concentrations of drug needed for the penetration of the drug and its subsequent intercalation into the bacterium DNA. However, because of the killing activity of the macrophage, at least with respect to methicillin-resistant *S. aureus*, it may well be that the initial process of killing might first involve the action of macrophage-lysosomal enzymes on the cell wall of the bacterium making it more permeable to the phenothiazine. Thus the concentrations achieved within the macrophage need not be equal to those corresponding to the *in vitro* MBC. Because the partial loss of the cell

wall and plasma membrane produced *in vitro* is identical to that present in MRSA that have been phagocytosed by the macrophage and subsequently treated with TZ, we conclude that the TZ-concentrating effect inside the macrophage is the primary cause for the killing of MRSA. MRSA strains account for a large number of deaths that result from nosocomial infections (Hiramatsu *et al*., 2001; Metlay, 2002; Noskin, 2001). The use of fluoroquinolones rapidly resulted in resistance of *S. aureus* (Appelbaum and Hunter, 2000). Furthermore, some of these quinolones were found to be toxic and subsequently removed from use (Appelbaum and Hunter, 2000). Vancomycin is the only really effective drug that remains and resistance to this compound is already taking place (Boyle-Vavra *et al*., 2001; Ward *et al*., 2001). We believe that TZ has potential for the therapy of persistent and recurrent MRSA infections that result from the inability of the antibiotics to reach the bacterium after it has been phagocytosed (Talon *et al*., 2002). Because TZ has been in safe use for many decades and its use as an anti-bacterial agent is anticipated to be, for a shorter period of time than that employed for the psychotic disorder therapy, safe and effective for the management of persistent and recurrent intracellular infections. Because the number of lethal bacterial targets affected by the phenothiazine is large, resistance to thioridazine is anticipated to be low, if not, improbable (Amaral *et al*., 2001a, b; Kristiansen and Amaral, 1997). Nevertheless, clinical trials are strongly recommended.

CHAPTER IV.

In vitro and *ex vivo* activity of TZ and its derivatives against *Mycobacterium tuberculosis* and *M. avium*

This chapter contains data published and accepted for publication in:

Ordway, D., M. Viveiros, C. Leandro, R. Bettencourt, J. Almeida, M. Martins, J. E. Kristiansen, J. Molnar, and L. Amaral. 2003. Clinical concentrations of thioridazine kill intracellular multidrug-resistant *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **47**:917–922; **Martins, M., Z. Schelz, A. Martins, J. Molnar, G. Hajös, Z. Riedl, M. Viveiros, I. Yalcin, and L. Amaral.** 2007. *In vitro* and *ex vivo* activity of thioridazine derivatives against *Mycobacterium tuberculosis*. Int. J. Antimicrob. Agents **29**:338–340; **Viveiros, M., M. Martins, I. Couto, J. E. Kristiansen, J. Molnár, and L. Amaral.** 2005. The *in vitro* activity of phenothiazines against *Mycobacterium avium*: potential of thioridazine for therapy of the co-infected AIDS patient. In Vivo **19**:733–736. **Martins, M., M. Viveiros, and L. Amaral.** 2008. Inhibitors of Ca^{2+} and K^{+} transport enhance intracellular killing of *M. tuberculosis* by non-killing macrophages. In Vivo **22**: *In press*.

CHAPTER OUTLINE

This chapter is focused on the *in vitro* and *ex vivo* activity of TZ against mycobacteria.

The main headings addressed in this chapter are:

- **The effect that clinical concentrations of TZ have on intracellular multidrug-resistant *Mycobacterium tuberculosis*.** *Ex vivo* studies were conducted on monocyte derived macrophages infected with multidrug-resistant *M. tuberculosis*. From the results obtained with the parental compound (TZ), parameters such as concentration, time of incubation, ratio of infection, *etc*., were selected;

- **The *in vitro* and *ex vivo* activity of TZ derivatives against *M. tuberculosis*.** The *in vitro* activity of twenty-two TZ derivatives was evaluated against *M. tuberculosis* strains and further extended to the infected monocyte derived macrophages;

- **The *in vitro* activity of TZ, as well as other phenothiazines against *M. avium*.** *M. avium* shows resistance to most of the compounds available to treat mycobacterial infections. However, in patients presenting with AIDS these infections are more difficult to treat and therefore, the need for new non-toxic compounds is obvious. Because phenothiazines, especially TZ, have significant activity against *M. tuberculosis*, the *in vitro* activity of CPZ, TZ, promazine, promethazine and desipramine against *M. avium* strains was evaluated.

IV.1 Clinical concentrations of thioridazine kill intracellular multidrug-resistant *Mycobacterium tuberculosis*

SUMMARY

The phenothiazines chlorpromazine (CPZ) and thioridazine (TZ) have equal *in vitro* activities against antibiotic-sensitive and -resistant *Mycobacterium tuberculosis*. These compounds have not been used as anti-*M. tuberculosis* agents because their *in vitro* activities take place at concentrations which are beyond those that are clinically achievable. In addition, chronic administration of CPZ produces frequent severe side effects. Because CPZ has been shown to enhance the killing of intracellular *M. tuberculosis* at concentrations in the medium that are clinically relevant, we have investigated whether TZ, a phenothiazine whose negative side effects are less frequent and serious than those associated with CPZ, kills *M. tuberculosis* organisms that have been phagocytosed by human macrophages, which have nominal killing activities against these bacteria. Both CPZ and TZ killed intracellular antibiotic-sensitive and -resistant *M. tuberculosis* organisms when they were used at concentrations in the medium well below those present in the plasma of patients treated with these agents. These concentrations *in vitro* were not toxic to the macrophage, nor did they affect *in vitro* cellular immune processes. TZ thus appears to be a serious candidate for the management of a freshly diagnosed infection of pulmonary tuberculosis or as an adjunct to conventional anti-tuberculosis therapy if the patient originates from an area known to have a high prevalence of multidrug-resistant *M. tuberculosis* isolates. Nevertheless, we must await the outcomes of clinical trials to determine whether TZ itself may be safely and effectively used as an anti-tuberculosis agent.

Materials and Methods employed in this study are described in publication #5 and in Chapter II (sections II.5 and II.8).

RESULTS

MICs and MBCs of CPZ and TZ for antibiotic-susceptible and MDR *M. tuberculosis* strains. The average MICs and MBCs of CPZ and TZ for three antibiotic-susceptible strains (including strain ATCC27294) and three antibiotic-resistant *M. tuberculosis* strains are summarized in Table 1. Briefly, the MBCs of CPZ and TZ were significantly greater than the respective MICs for antibiotic-susceptible and -resistant strains of *M. tuberculosis*. The activities of TZ against each of the strains were greater than those of CPZ. Lastly, MDR *M. tuberculosis* strains were significantly more resistant to the actions of CPZ and TZ than the antibiotic-susceptible strains were. It is noteworthy that because the MICs and MBCs were not determined by the broth dilution method and, indeed, were the precise concentrations used against a constant standardized inoculum, the differences noted above are deemed significant.

Table 1. MICs and MBCs of CPZ and TZ for antibiotic-sensitive and MDR strains of *M. tuberculosis*[a]

Drug	Susceptible strains		Resistant strains					
	ATCC27294 / A / B		C		D		E	
	MIC (mg/L)	MBC (mg/L)	MIC (mg/L)	MBC (mg/L)	MIC (mg/L)	MBC (mg/L)	MIC (mg/L)	MBC (mg/L)
CPZ	10	20	30	40	20	30	20	30
TZ	15	30	30	50	20	30	20	30

[a] The individual MICs of RIF, INH, STR, EMB, CPZ and TZ, were determined by the BACTEC 460-TB method, as described previously (Amaral and Kristiansen, 2000; Bettencourt *et al*., 2000; Viveiros *et al*., 2002). Stock solutions containing various amounts of each compound were freshly prepared in BACTEC 460 medium, and 0.1-mL aliquots of each solution were added to BACTEC 460 vials. The final concentrations of CPZ and TZ in the BACTEC 460 vials were 0, 1, 5, 10, 15, 20, 30, 40, 50, and 60 mg/L. A total of 0.1 mL of BACTEC 460 medium containing an adjusted concentration of mycobacteria corresponding to approximately 10^5 to 10^6 CFU was added to each vial (Viveiros *et al*., 2002). The MBCs of CPZ and TZ were determined by extending the MIC curves well beyond each MIC and 10 μL aliquots of the 10 mL cultures at zero time and those after 30 days that showed no evidence of growth in the BACTEC 12B vials were subjected to counting of the numbers of CFU (Viveiros *et al*., 2002). The MIC and MBC determinations were repeated three times, and the values obtained did not differ significantly. Strains ATCC27294, A, and B are susceptible to INH, RIF, STR, and EMB. Strain C is resistant to all four drugs. Strains D and E, besides being resistant to INH and RIF, are also resistant to STR.

Toxicities of CPZ and TZ against THP-1 cells, PBMDMs, and subsets of the Ficoll preparation. The potential toxicities of CPZ or TZ against THP-1 cells and PBMDMs were evaluated by three distinct methods in a previous study, and 50% of the full toxicity was shown to take place at concentrations that exceeded 3.0 mg/L (Ordway *et al*., 2002a). Similar results were obtained in this study (data not shown). The toxicities of the phenothiazines at concentrations below those anticipated to be present in the plasma of a patient treated with 600 mg of either phenothiazine per day (Amaral *et al*., 2001a) against subsets of the Ficoll monocyte preparation were examined in the present study. Briefly, as shown in Figure 1, the concentrations of CPZ and TZ that correspond to those readily evident during initial therapy of a patient, *i.e.*, 0.1 mg/L, had a nominal effect on annexin V binding, determination of which is the method of choice for the demonstration of apoptosis (Ordway *et al*., 2002a). The percentage of annexin V binding indicative of early apoptosis is 10% for alpha-beta and gamma-delta T cells and $CD14^+$ macrophages (Ordway *et al*., 2002a). The data presented in Figure 1 also show that the presence of live mycobacteria during a culture period of 3 days did not increase the percent annexin V binding by the various monocyte subsets evaluated.

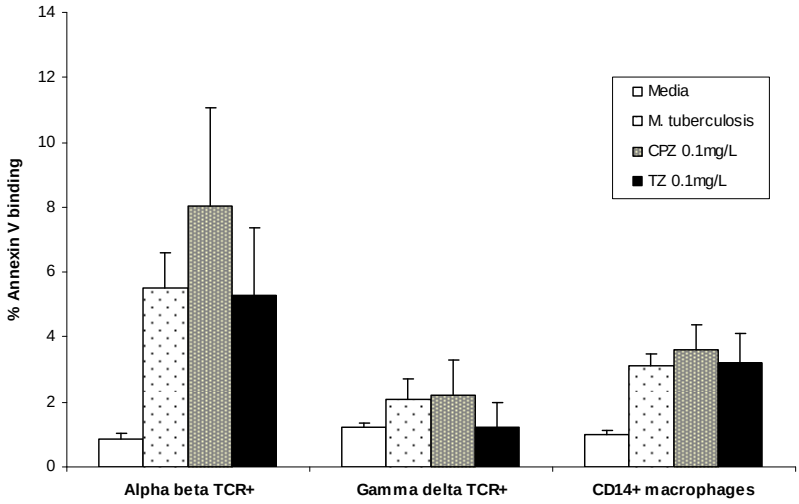


Figure 1. Effects of CPZ and TZ on percent annexin V binding by monocyte subsets of Ficoll preparations of PBMCs. Subpopulations of the Ficoll preparation containing PBMCs from healthy donors were separately stimulated for 3 days with *M. tuberculosis* (H37Rv ATCC27294) or with concentrations of CPZ and TZ that ranged from 0 to 0.5 mg/L. After 3 days the cells were harvested and stained with fluorescent antibodies against alpha-beta TCR^+, gamma-delta TCR^+, and $CD14^+$ macrophages in combination with annexin V. Cells were analyzed with an Ortho absolute flow cytometer. Results are expressed as the mean ± standard deviation percent annexin V binding in lymphocytes. Annexin V binding in excess of 10% is an indication of early apoptosis (Ordway *et al*., 2002a).

Effects of CPZ and TZ on killing of *M. tuberculosis* phagocytosed by PBMDMs or THP-1 macrophages. THP-1 macrophages and PBMDMs were selected for use in the assays of the phagocytosis of *M. tuberculosis* on the basis of the fact that these cells, unlike neutrophils, possess slight killing activities against bacteria (Hoidal *et al*., 1981; Ordway *et al*., 2002a). This provides an understanding of the killing activities of agents against intracellular bacteria. As shown in Figure 2, the phagocytosis of approximately 10^5 mycobacterial cells by an equal number of PBMDMs does not result in any effective killing for 3 days post-infection, regardless of the organism's susceptibility to INH, RIF, STR, and EMB (including strain H37Rv ATCC27294) or resistance to INH and RIF (MDR *M. tuberculosis*). In contrast to this lack of killing, when CPZ or TZ was added to the infected macrophage cultures at 0.1 mg/L, the killing of strain ATCC27294 and the antibiotic-susceptible and MDR *M. tuberculosis* strains was enhanced within 1 day post-infection (Figure 3A and B). Complete killing took place by the end of the third day post-infection (Figure 3A and B). Lower concentrations of these phenothiazines were ineffective, and the rates of survival of all intracellular mycobacterial strains were indistinguishable from those for their controls (Figure 3A and 3B). Although the results presented above were obtained by use of PBMDMs, similar results were obtained by use of the THP-1 macrophage cell line (data not shown).

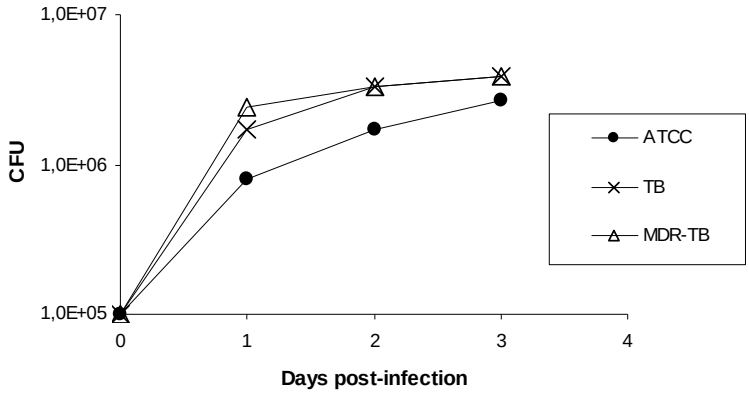


Figure 2. Average killing activities of human PBMDMs against *M. tuberculosis* ATCC 27294 (ATCC), two antibiotic-susceptible strains (strains A and B [TB]), and three MDR *M. tuberculosis* strains (strains C, D, and E; resistant at least to RIF and INH [MDR-TB]). The killing activities against the strains used were not significantly different.

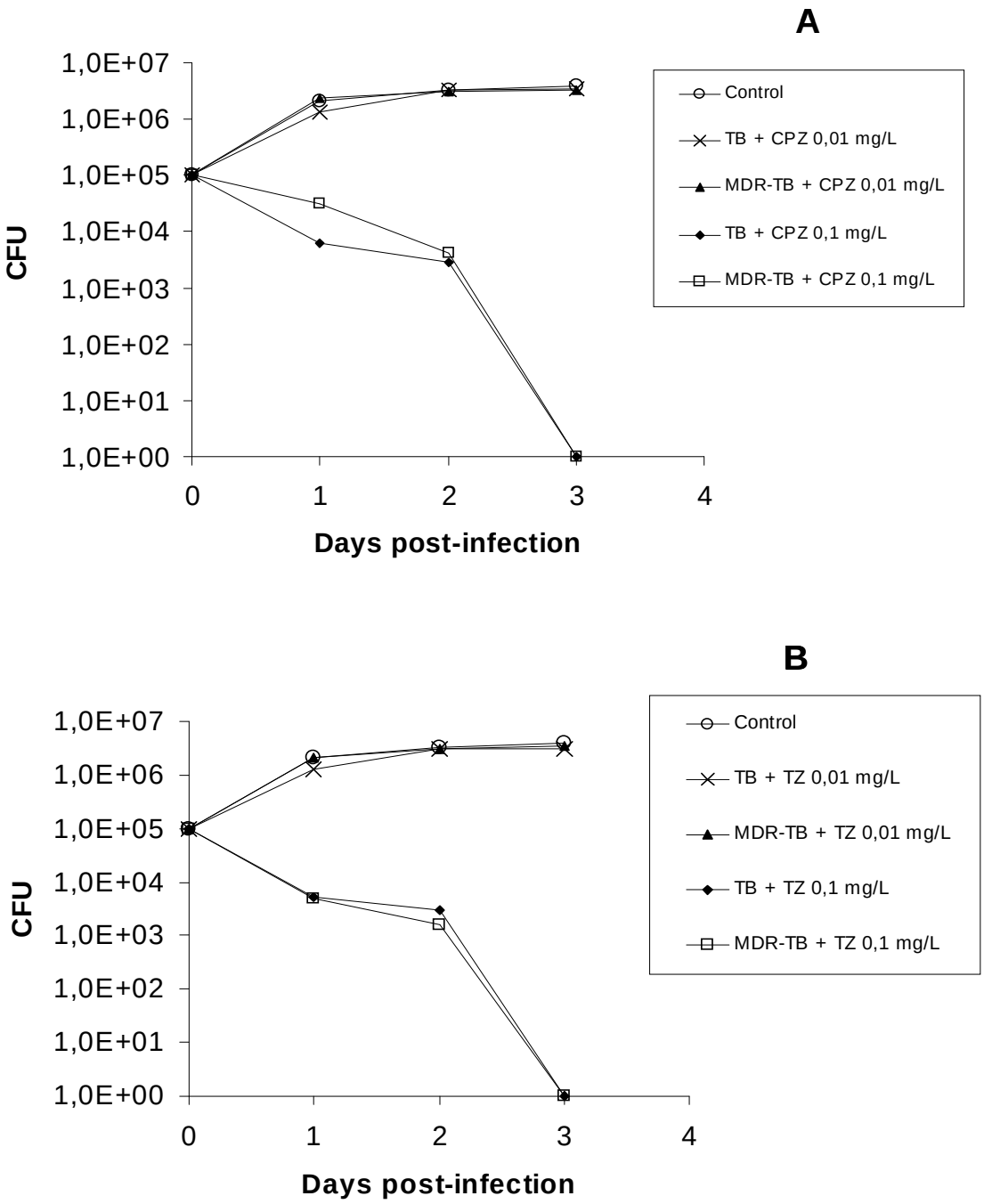


Figure 3. Average killing activities of human PBMDMs against three MDR *M. tuberculosis* strains (resistant to RIF and INH) (MDR-TB) and three antibiotic-susceptible *M. tuberculosis* strains (TB) including strain ATCC27294 when either CPZ (A) or TZ (B) was present in the medium at concentrations of 0.01 and 0.1 mg/L. The control value is the average numbers of CFU for cultures of all six strains to which no phenothiazine was added, inasmuch as the CFU did not differ appreciably between strains.

DISCUSSION

The toxicity associated with the chronic use of CPZ is well known (Amaral *et al*., 2001a; Kaplowitz *et al*., 1986; Kodovanti *et al*., 1990). In addition, CPZ has been shown to affect the activities of a variety of T lymphocytes *in vitro* (Ghezzi, 1991; Grabski *et al*., 2001; Hieronymus *et al*., 2000), although requiring concentrations of CPZ which are beyond those clinically relevant (*i.e.*, in excess of 1 mg/L) or fairly close to the highest concentration of CPZ in plasma that might result from its aggressive use (≥0.5 mg/L). Our previous study (Ordway *et al*., 2002a) and the present study show that an *in vitro* concentration of 0.1 mg/L of either CPZ or TZ, which is equivalent to that anticipated to be present in the plasma of a patient treated with either phenothiazine, has no significant toxicity against macrophages or alpha-beta TCR^+, gamma-delta TCR^+, or $CD14^+$ macrophages. Concentrations of CPZ or TZ that are well below their range for toxicity against human macrophages, THP-1 macrophages, or subsets of T lymphocytes *in vitro*, as shown in this and other studies (Ordway *et al*., 2002a), were shown in the present study to enhance the killing of *M. tuberculosis* strains that are phagocytosed by either PBMDMs or THP-1 cells. Complete killing is evident by the end of 3 days post-infection at a minimum concentration of 0.1 mg/L, regardless of the antibiotic susceptibility of the strain. When similar *M. tuberculosis* strains are exposed to concentrations of CPZ or TZ *in vitro*, the minimum concentration of either CPZ or TZ required to completely inhibiting growth ranges from 8 to over 30 mg/L (Amaral *et al*., 1996; Bettencourt *et al*., 2000; Crowle *et al*., 1992; Viveiros and Amaral, 2001). The minimum *in vitro* concentration of either phenothiazine required for complete killing (MBC), as shown in our study, ranges from 20 to 50 mg/L and is similar to that reported previously (Amaral *et al*., 1996). Because these phenothiazines are concentrated by macrophages (Amaral and Kristiansen, 2001), cells that have little killing actions of their own (Hoidal *et al*., 1981; Ordway *et al*., 2002a), it is likely that the concentration of either phenothiazine required to kill *M. tuberculosis* cells *in vitro* is achieved because the macrophage is able not only to concentrate the compounds but also to make these compounds available in an active form to the cytoplasmic structure of the macrophage that houses the entrapped phagocytosed bacterium (Amaral and Kristiansen, 2001). Nevertheless, it is also possible that the lytic action of the macrophage lysosomal apparatus, which is apparently weak, might be sufficiently effective to affect the integrity of the bacterium's cell wall. This weakened cell wall might be more permeable

for the penetration of these compounds, which eventually intercalate between the nucleic acid base pairs of the bacterium's DNA, where DNA-dependent functions may be affected (de Mol *et al*., 1983). Regardless of the cause of killing, *in vitro* resistance of MDR *M. tuberculosis* to either CPZ or TZ, as opposed to that of the antibiotic susceptible strains, is not evident after the MDR strain is phagocytosed by macrophages that have been treated with CPZ or TZ. The demonstration that clinically relevant concentrations of either CPZ or TZ that are neither toxic *in vitro* to the macrophage nor inhibitory of T-cell activity *in vitro* kill phagocytosed mycobacteria supports their use in the management of intracellular *M. tuberculosis* infections. TZ has significantly fewer serious side effects than CPZ (Amaral *et al*., 2001a), making the use of TZ as an anti-TB agent far more attractive than the use of CPZ. However, its use is not without some risk, inasmuch as almost twice as many sudden deaths due to cardiac failure take place in patients managed with TZ compared to the number of deaths from such causes in the general population (Glassman and Bigger, 2001). Although TZ may induce episodes of torsade de pointes that result in sudden death, there are only 10 to 15 such events in 10,000 person-years of observation (Glassman and Bigger, 2001). Regardless, careful monitoring of cardiac function during TZ management is strongly recommended. Admittedly, the use of TZ for the management of an active pulmonary TB infection caused by antibiotic-susceptible *M. tuberculosis* strains provides no advantage over the use of present therapies. Moreover, because the concentration of TZ needed to kill or even inhibit mycobacterial replication when the bacterium is outside the macrophage is far beyond that which can be achieved in the patient, it cannot be used for the management of a cavitary pulmonary *M. tuberculosis* infection of moderate to severe status. Nevertheless, even for patients with cavitary infections of moderate status whose origins are associated with a high prevalence of MDR *M. tuberculosis*, especially when there is a lack or absence of appropriate, effective clinical laboratory support, TZ could serve as an adjunct to conventional therapy during initial management of the patient, at least until the susceptibility data for the causative organism are made available (Viveiros and Amaral, 2001). Because the length of such management is anticipated to be weeks, side effects associated with chronic TZ therapy are not anticipated. Normally, such a recommendation would not be made until the effectiveness of TZ therapy was defined subsequent to clinical trials. However, given the severity of MDR *M. tuberculosis* in parts of the world where effective therapy is not possible, the use of TZ deserves consideration.

IV.2 *In vitro* and *ex vivo* activity of thioridazine derivatives against *Mycobacterium tuberculosis*

SUMMARY

Thioridazine (TZ) has previously been shown by us to have *in vitro* and *ex vivo* activity against antibiotic-susceptible and multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB). Because current therapy of MDR-TB is highly problematic even when all five 'first line of defence' drugs are employed, there is a need for effective antituberculosis drugs. New derivatives of TZ were synthesised and their *in vitro* activity against a reference strain of *M. tuberculosis* was evaluated with the aid of the BACTEC 460 system. Derivatives that presented significant activity were evaluated by *ex vivo* studies and were shown to enhance the killing of intracellular *M. tuberculosis*.

Materials and Methods employed in this study are described in publication #3 and in Chapter II (sections II.5 and II.8).

RESULTS AND DISCUSSION

The results obtained in this study show that from the twenty-two TZ derivatives tested against *M. tuberculosis* H37Rv ATCC27294, in the BACTEC 460 system, six (#1867; #1875; #1926; #1470; # 1870; #1876) have higher activity (MIC=5-10 mg/L) than TZ (MIC=15 mg/L) (Table 1).

Table 1. Minimum inbibitory concentration of TZ and its derivatives against *Mycobacterium tuberculosis*

	Phenothiazines	**MIC (mg/L)**
Thioridazine	**TZ**	**15**
TZ derivatives	**#1867; #1875; #1926**	**5**
	#1470; # 1870; #1876	**10**
	#1687; #1688; #1689; #1868; #1532-2; #1686; #1872	20
	#1550; #1819; #1820; #1821; #1869; #1871; #1873; #1874; #1929	>20

The evaluation of TZ and active derivatives for potential toxicity was performed on lymphocytes cultured for up to 3 days and is summarised by Table 2. Briefly, TZ was cytotoxic at concentrations that exceed 0.5 mg/L (Ordway *et al*., 2002b; Ordway *et al*., 2003b). However, at this same concentration no significant toxicity was detected for the TZ derivatives (trypan blue exclusion by over 90% of lymphocytes).

Table 2. Percent toxicity of TZ and TZ derivatives on human lymphocytes

	Toxicity (%)		
Phenothiazines	**0.1 mg/L**	**3 mg/L**	**10 mg/L**
TZ	0	50	100
#1867; #1875; #1926; #1470; #1870; #1876	0	1.6	5.3

If a compound is to be useful as an antituberculosis agent it must have activity against intracellular *M. tuberculosis*. Because our previous studies demonstrated that TZ at a clinically relevant concentration of 0.1 mg/L enhanced the killing of phagocytosed *M. tuberculosis* (Ordway *et al*., 2003b), derivatives that had the greatest *in vitro* activity and were devoid of significant toxicity were selected for evaluation of similar killing activity. As shown by Figure 1, macrophages derived from human blood did not appreciably kill phagocytosed *M. tuberculosis* during a period of 3 days' incubation. These results are consistent with those previously presented (Martins *et al*., 2007b; Ordway *et al*., 2003b). In contrast, cultures containing the phenothiazine TZ (used as a positive control) at a concentration of 0.1 mg/L yielded only a few live mycobacteria after 3 days of culture. When tested *ex vivo* for the enhancement of the macrophage killing activity, the non-toxic TZ derivatives (#1867, #1875, #1926, #1470, #1870, #1876), also demonstrated *ex vivo* activity against *M. tuberculosis* (see Figure 1). Derivatives #1875, #1926 and #1876 enhanced the killing of mycobacteria at a concentration of 0.1 mg/L. When compared with the parental molecule (TZ), derivative #1875 was especially active and more effective than TZ, since after 2 days post-infection no colony forming units (CFU) were retrieved from the lysed cultures.

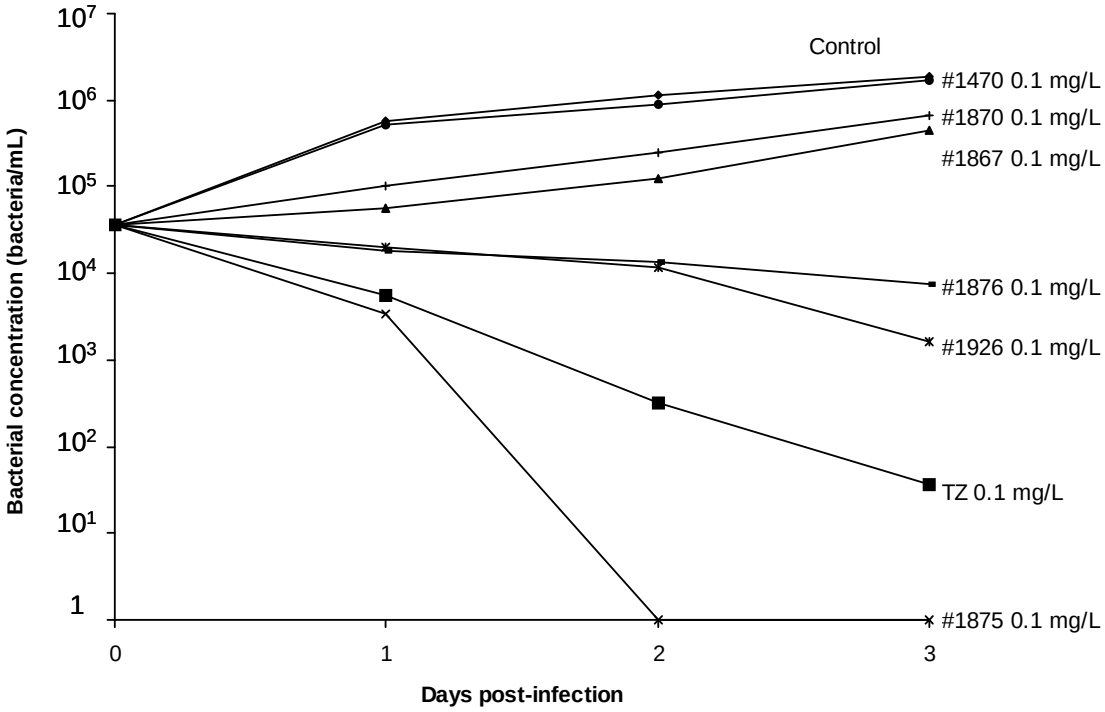


Figure 1. Effect of TZ and its derivatives in the killing activity of macrophages infected with *M. tuberculosis* H37Rv ATCC27294. Human macrophages (1×10^5 cells/mL) were infected with *M. tuberculosis* at a ratio of 1:10 (1 macrophage:10 bacteria). After 1 hour of phagocytosis, cells were washed to remove non-phagocytosed bacteria. Aliquots of the tested compounds were added to the corresponding wells and cells incubated for 0, 1, 2 and 3 days. After incubation, supernatants of the cells were removed and plated in order to determine whether the addition of the agents cause the lysis of the macrophage and subsequent release of the bacterium. Adhered cells were lysed with SDS 0.01% in order to release all the intracellular bacteria and aliquots of the lysed cells were plated in 7H11 plates and incubated at 37ºC. After 3 to 4 weeks, CFU were counted and bacterial concentrations calculated. The data presented are averages from three independent experiments each of which was conducted in triplicate.

The results obtained in this study demonstrate the *in vitro* and *ex vivo* potential of new non-toxic derivatives of TZ against *M. tuberculosis*. However, further evaluation of these new derivatives for *in vivo* toxicity and effectiveness in rendering the *M. tuberculosis* infected mouse free of this infection are absolutely necessary before these compounds can be seriously considered for the therapy of a pulmonary TB infection.

IV.3 The *in vitro* activity of phenothiazines against *Mycobacterium avium*: potential of thioridazine for therapy of the co-infected AIDS patient

SUMMARY

Patients presenting with Acquired Immune Deficiency Syndrome (AIDS) are predisposed to co-infection with *Mycobacterium avium*. The management of such patients is problematic due to underlying immuno-incompetence and the high resistance of *M. avium* to most non-toxic compounds. Therefore, the need for effective agents is obvious. Because phenothiazines, especially the relatively mild thioridazine, have significant activity against *Mycobacterium tuberculosis*, we investigated the *in vitro* activity of chlorpromazine, thioridazine, promazine, promethazine and desipramine against a reference and clinical strains of *M. avium*. The results obtained show that whereas all of the phenothiazines employed in this study had an minimum inhibitory concentration (MIC) against the strains studied that ranged from *ca*. 10 to >50 mg/L, as was previously shown for *M. tuberculosis*, thioridazine was the most active of the group against *M. avium*.

Materials and Methods employed in this study are described in publication #4 and in Chapter II (section II.5).

RESULTS

The *in vitro* activity of the phenothiazines CPZ, TZ, PMZ, PMTZ and DSP are presented in Figure 1. Briefly, the ascending order of activity, as defined by the MIC (lowest concentration that produces 100% inhibition of growth assessed by the inhibition of the $^{14}CO_2$ generated) in mg/L, was as follows: TZ (10), CPZ (25), PMZ (25-50), PMTZ (25-50) and DSP (>50). Because it has generally been assumed that the resistance of *M. avium* to drugs employed for the management of pulmonary tuberculosis produced by *M. tuberculosis* is due to the cell wall of the former, it was surprising to discover that *M. avium* is more sensitive to TZ than is *M. tuberculosis*, the latter being completly inhibited in its growth by concentrations of TZ that exceed 25 mg/L. However, with respect to CPZ, PMZ, PMTZ and DSP, the sensitivity of *M. avium* to these agents is practically identical to that produced against *M. tuberculosis*

(Bettencourt *et al*., 2000). Because the assays were conducted in triplicate and the experiments repeated at least twice, the difference between the MICs of TZ for *M. avium versus M. tuberculosis* were considered to be significant.

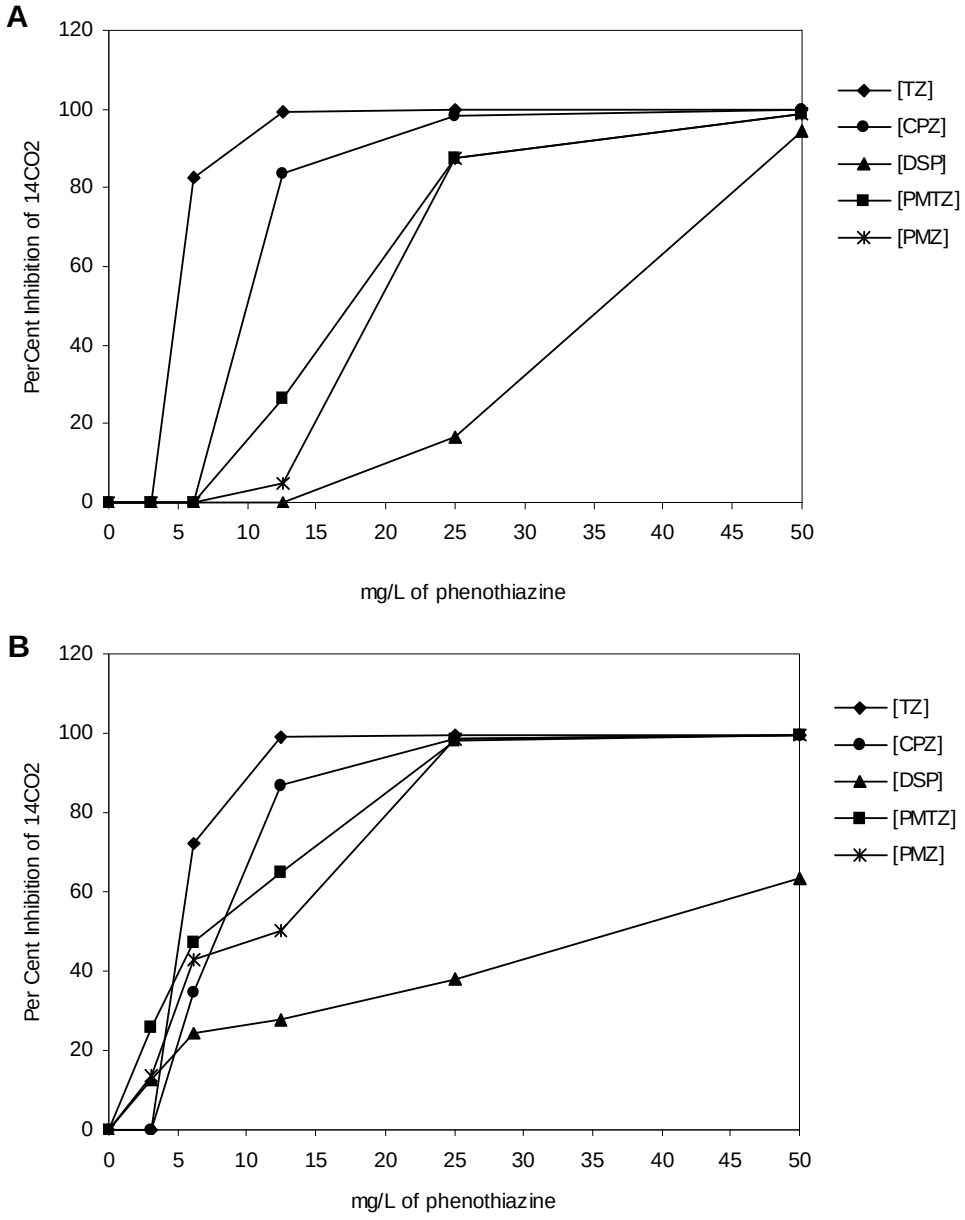


Figure 1. The *in vitro* activity of chlorpromazine (CPZ), thioridazine (TZ), promazine (PMZ) and desipramine (DSP) against *Mycobacterium avium*. *Mycobacterium avium* strain CIP 14 031 002-S4 (Pasteur Institute, Paris, France) (A) and a *Mycobacterium avium* strain isolated from an AIDS patient (B) were incubated in BACTEC 12B vials that contained concentrations of each phenothiazine that ranged from 0.0 to 50 mg/L. The experiment was repeated twice and essentially the same quantitative data was obtained. The results represent the data obtained from one such experiment and describe the activity against both strains.

DISCUSSION

Patients presenting with AIDS are at risk of acquiring a variety of opportunistic infections (Bellamy *et al*., 2004). Although each of these infections may cause death to any given AIDS patient, pulmonary infections caused by *M. avium* produce the highest mortality (Bellamy *et al*., 2004; Hoffmann and Brunner, 2004) since effective drugs are few and relatively ineffective against this organism (Hoffmann and Brunner, 2004). Currently, azithromycin and clarithromycin are the two most effective agents for the therapy of *M. avium* pulmonary non-tubercular infections (Hoffmann and Brunner, 2004). However, there is now evidence that these agents produce ototoxicity (Uzun *et al*., 2001) and arrhythmia (Ohtani *et al*., 2000), respectively. The need for effective drugs for the management of this infection is obvious. The results obtained in our current study indicate that each of the phenothiazines studied had *in vitro* activity against the ATCC and clinical strains. The response of the reference and clinical strains was quantitatively very similar, thereby suggesting that other clinical strains will show similar responses. The order of increasing effectiveness against these strains was TZ, CPZ, PMZ, PMTZ and DSP. TZ was by far the most effective of the phenothiazines evaluated, presenting with an MIC of *ca*. 10 mg/L. Surprisingly, *M. avium* is far more sensitive to the phenothiazine TZ than are strains of *M. tuberculosis* (Amaral *et al*., 2001a, 2001b). Nevertheless, the MIC of 10 mg/L for TZ is not clinically achievable. However, TZ does enhance the killing of intracellular *M. tuberculosis* (Ordway *et al*., 2003b) when the concentration of this agent in the medium is of the order of 0.1 mg/L and, hence, within clinical range and below any associated with toxicity (Ordway *et al*., 2002a, b; Ordway *et al*., 2003b). Killing is considered to be the result of the agent being concentrated by the macrophage to levels equivalent to those that produced *in vitro* killing (Ordway *et al*., 2002a, b; Ordway *et al*., 2003b) and, because phenothiazines are concentrated by lysosomes (Daniel *et al*., 2001; Wojcikowski and Daniel, 2002), the killing of the bacterium by macrophages that have little killing activity of their own (Ordway *et al*., 2003b) is probably due to the fusion of the phagosome with the lysosome containing the highly concentrated phenothiazine (Ordway *et al*., 2002a, 2002b; Ordway *et al*., 2003b). The greater sensitivity of *M. avium* to TZ as opposed to that produced against *M. tuberculosis* is interesting. Although at this time we cannot provide an adequate explanation for this difference, we can confidently conclude that the cell wall of *M. avium*, generally considered being the cause for its resistance to anti-

TB agents, does not lead to greater resistance to TZ. The results of the current study are not sufficient to recommend that TZ be used for the management of AIDS patients co-infected with *M. avium*. Furthermore, even when the study that evaluated the killing activity of TZ against phagocytosed *M. avium* showed that the concentration required for such killing was comparable to that expected to be present in the plasma of patients chronically treated with this agent, the use of the mild TZ is not without risk, as shown by recent studies demonstrating that, for a very small number of patients, TZ is associated with serious arrhythmia such as “torsade de pointes” (Vieweg and Wood, 2004). However, the relationship is now in question since the number of risk factors presented by the patient may be more significant for the arrhythmia than that produced by the agent itself (Titier *et al*., 2005).

In the future, studies evaluating the ability of TZ, its derivatives and other phenothiazines to enhance the killing activity of the infected macrophage will be performed to determine if these compounds also demonstrate activity against phagocytosed *M. avium*.

CHAPTER V.

The Curative Activity of Thioridazine on Mice Infected With *Mycobacterium tuberculosis*

This chapter contains data published in:

Martins, M., M. Viveiros, J. E. Kristiansen, J. Molnar, and L, Amaral. 2007. The curative activity of thioridazine on mice infected with *Mycobacterium tuberculosis*. In Vivo **21**:771–776.

CHAPTER OUTLINE

This chapter describes all the studies conducted on Balb/C mice infected with *M. tuberculosis*. The effectiveness of TZ was evaluated. Infected animals were treated with different doses of TZ and the results are presented by CFU counts obtained from the infected organs.

SUMMARY

The aim of the study was to evaluate the effectiveness of thioridazine (TZ) at different dose levels on mice that had been infected intraperitoneally (i.p.) with a high dose of the *M. tuberculosis* ATCC H37Rv strain. Groups of five female Balb/C mice were infected i.p. with 10^6 CFU/mL. After thirty days, treatment with TZ was initiated, except for the control group. Mice were treated with TZ at equivalent concentrations to that used in the humans (1200 mg/day), ranging from 0.05 to 0.5 mg/day. The results demonstrated that a daily dose of 0.5 mg/day of TZ reduced the number of colony forming units retrieved from the lungs of infected mice within one month. By the end of 300 days of therapy, although mycobacteria were still retained, their presence, in comparison to that of the control was 8 orders of magnitude lower.

Materials and Methods employed in this study are described in publication #5 and in Chapter II (section II.10).

RESULTS

The average absolute number of CFU retrieved from the lungs of mice after infection with approximately 10^6 *M. tuberculosis* ATCC H37Rv is shown in Figure 1A. Daily doses of TZ below 0.5 mg did not produce any significant change in the bacterial load as compared to the untreated control mouse group during the entire period of the study (data not shown). Thirty days after the mice were treated daily with 0.5 mg/day of TZ there was an excess of five log reduction in the CFU/kg retrieved from the lung as compared to the control group (Figure 1B). The effects of 0.5 mg/day on the bacterial load recovered from the lung of the animals becomes more prominent with each passing 30 day interval, such that by the end of 300 days of treatment the difference between the

treated group and its control untreated group is approximately 8 orders of magnitude (Figure 1B). The bacterial load recovered from the liver or the spleen, unlike that recovered from the lung, continued to increase regardless of therapy with TZ and was similar to that recovered from the untreated infected control mouse group (data not shown).

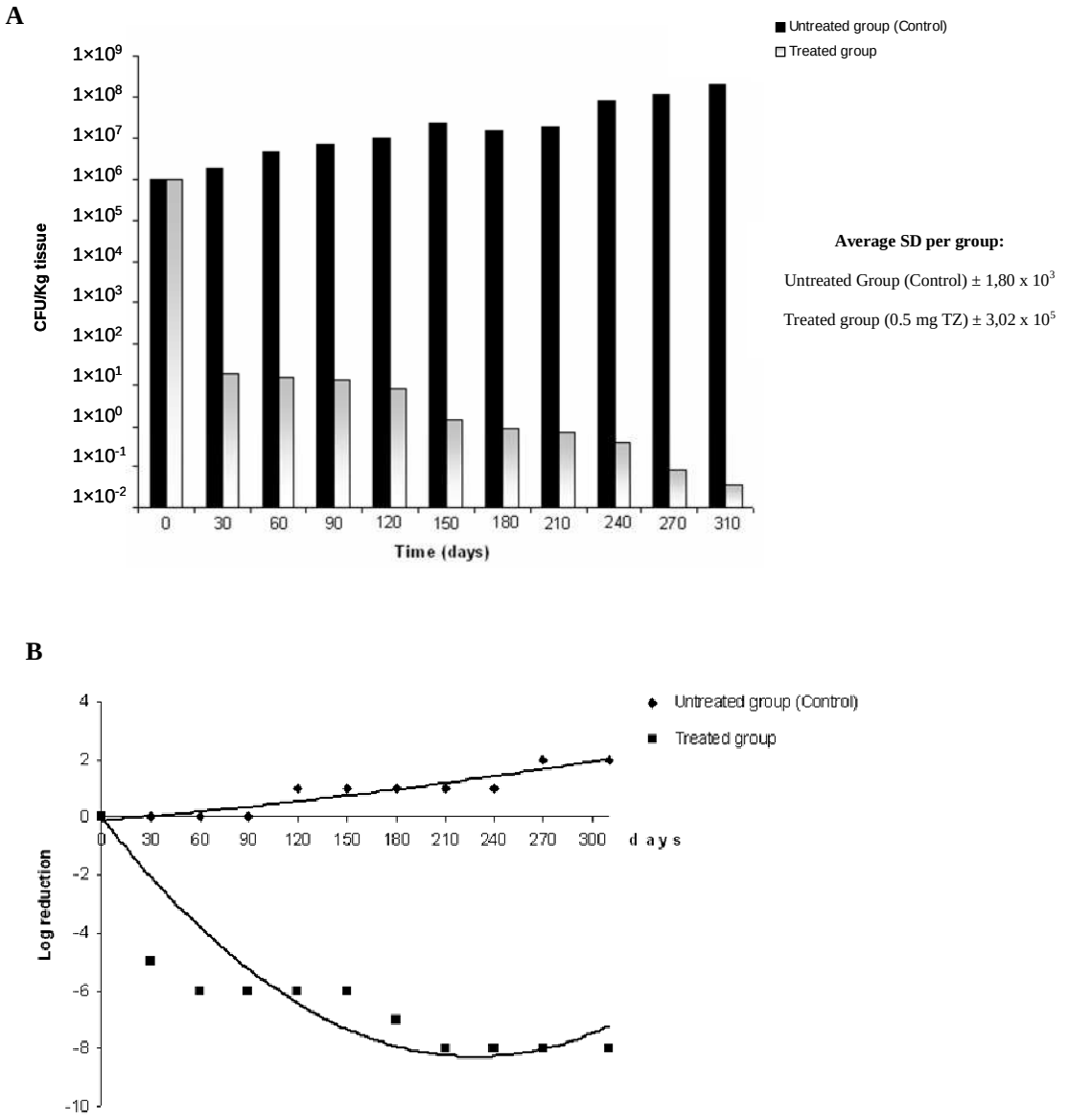


Figure 1. Effect of daily TZ treatment of mice infected with *M. tuberculosis*. Mice were infected i.p. with approximately 10^6 *M. tuberculosis* ATCC H37Rv. After thirty days of infection, mice were treated daily with a dose equivalent in the human to 1200 mg of TZ (0.5 mg/day). A, Average absolute number of colony forming units (CFU) per kg of mouse lung retrieved from the lungs of treated and non-treated (control) infected mice. B, Log reduction of *M. tuberculosis* per kg of mouse lung retrieved from the lung of the treated mice as compared to the control group (no treatment).

DISCUSSION

Although TZ therapy appears to be very effective, after 300 days a few CFU per mg of lung tissue could still be recovered, hence the infection persisted, albeit at a very low level. The question of why mycobacteria could still be recovered 300 days after daily therapy, whereas experimental data obtained from human macrophages that had been infected *ex vivo* could be completely rid of *M. tuberculosis* subsequent to exposure to as little as 0.1 mg/L of TZ (Ordway *et al*., 2003b) can be answered from the data obtained from spleens and livers of both treated and untreated mice. Daily therapy with TZ did not reduce the number of CFU of mycobacteria retrieved from the liver or the spleen (data not shown) during the time of the experiment as opposed to the decrease of mycobacteria that was evident in the lungs. The single dose of mycobacteria (10^6 CFU) introduced i.p. is massive compared to that to which a human is exposed and subsequently infected with. This massive dose was necessary inasmuch as mice, even though the strain used in this study is relatively more readily infected with mycobacteria than other strains (Ordway *et al*., 2005b), are relatively resistant to infection by *M. tuberculosis*. Taking this massive dose into account, as well as the possibility that the spleen and liver were sources of mycobacteria, it was surprising to note the effectiveness of TZ therapy. The experimental protocol employed in this study does not shed any light as to whether TZ is effective against mycobacteria that are trapped in the liver and spleen given the massive i.p. dose administered. Recent studies have provided evidence that phenothiazine derivatives can clear the lungs of mice that have been infected with only 200 CFU of *M. tuberculosis* (Weinstein *et al*., 2005; Yano *et al*., 2006). In light of these latter studies, the significance of TZ therapy becomes even greater given its effectiveness against an infection that was 5,000 times greater. Nevertheless, we must remember that although TZ is effective for the therapy of pulmonary TB of the mouse, other than anecdotal evidence and laboratory studies that support the use of TZ (Amaral *et al*., 2004), its effectiveness for therapy of human pulmonary TB remains to be determined.

CHAPTER VI.

Effect of *Carpobrotus edulis*, reserpine, ouabain and verapamil on the growth of phagocytosed multidrug-resistant *Mycobacterium tuberculosis* and methicillin-resistant *Staphylococcus aureus*

This chapter contains data published and accepted for publication in:

Martins, M., D. Ordway, M. Kristiansen, M. Viveiros, C. Leandro, J. Molnar, and L. Amaral. 2005. Inhibition of the *Carpobrotus edulis* methanol extract on the growth of phagocytosed multidrug-resistant *Mycobacterium tuberculosis* and methicillin-resistant *Staphylococcus aureus*. Fitoterapia **76**:96–99; **Martins, M., M. Viveiros, D. Ordway, J. E. Kristiansen, J. Molnar, and L. Amaral.** 2006. Reserpine, ouabain and the calcium channel blocker verapamil, cause intracellular killing of *Staphylococcus aureus*. Research J. Microbiology **1**:203–209; **Martins, M., M. Viveiros, and L. Amaral.** 2008. Inhibitors of Ca^{2+} and K^{+} transport enhance intracellular killing of *M. tuberculosis* by non-killing macrophages. In Vivo **22**: *In press*.

CHAPTER OUTLINE

From all the data obtained several questions were posed relative to the mechanism of action of TZ as well as other phenothiazines, namely:

- Is the effect of the enhancement of the macrophage killing activity due to a concentration of the compound inside the macrophage?

- Or is this effect due to the inhibition of the Ca^{2+} and K^{+} pumps present in the membrane of the macrophage since the phenothiazines are known to inhibit efflux systems of bacteria (Amaral *et al*., 2001b; Amaral *et al*., 2007c; Martins *et al*., 2008)?

In order to understand these questions, several studies were conducted. Inhibitors of efflux pumps systems were selected, namely, the methanol extract of *C. edulis*, ouabain, reserpine and verapamil. Plants extracts are a good source of compounds that inhibit the efflux pumps of bacteria as well as eukaryotic cells. Some alkaloids present in the plant extracts have been identified as inhibitors of bacteria, such as *S. aureus*, and also of efflux pumps of eukaryotic cells. Its mode of action is mainly based on the inhibition of K^{+} flux. Others inhibitors of the K^{+} transport such as ouabain, reserpine and verapamil (Contreras *et al*., 2004; Orlov *et al*., 2004), exert their action by direct or indirect inhibition of K^{+} transport by limiting access of Ca^{2+} to Ca^{2+}-dependent ATPases (Gao *et al*., 2004).

This chapter presents all the data obtained from the studies conducted with these inhibitors in macrophages infected with MDR *M. tuberculosis* and MRSA.

VI.1 Inhibition of the *Carpobrotus edulis* methanol extract on the growth of phagocytosed multidrug-resistant *Mycobacterium tuberculosis* and methicillin-resistant *Staphylococcus aureus*

SUMMARY

The *Carpobrotus edulis* methanol extract, inactive *in vitro* against the methicillin-resistant *Staphylococcus aureus* or the multidrug-resistant *Mycobacterium tuberculosis*, does inhibits the growth of these two bacteria once they are phagocytosed by monocyte derived human macrophages.

Material and methods are described in chapter II (sections II.7 and II.8), as well as in publication #6 listed in the Appendix.

RESULTS

Concentrations of methanol extract that are five times higher than that which produce toxicity against monocyte derived macrophages do not inhibit the *in vitro* growth of methicillin-resistant *S. aureus* or multidrug-resistant *M. tuberculosis* (data not shown).

In contrast to these results, the fivefold diluted methanol extract that is well below the level that is toxic to the macrophage effectively inhibits the growth of phagocytosed Methicillin resistant *S. aureus* within 6 h of culture (Table 1), and that of multidrug-resistant *M. tuberculosis* within 3 days of culture (Table 2).

Table 1. Effects of *C. edulis* leaves methanolic extract (CELME) on phagocytosed *S. aureus*[a]

Bacteria	CFU[b]×10[5] (hours)		
	1	**2**	**6**
S. aureus ATCC 25923	2.0	3.2	3.6
S. aureus ATCC 25923+CELME	1.9	3	2.5
S. aureus MRSA[c]	3.0	3.5	3.7
S. aureus MRSA[c]+CELME	0.3	0.5	0.3

[a] Approximately 0.5 to 1.0×10^5 CFU could be retrieved from 100,000 macrophages after the period employed for phagocytosis of approximately 10^5 CFU presented to the macrophages (zero time). CFU presented are calculated from colonies resulting from the plating of serial dilutions of 0.1 ml aliquots of cultures after the lysing of the macrophages. About 20 µl of undiluted aqueous methanol extract was added to 180 µl of medium and this total volume was added directly to the cultures containing the macrophage-phagocytosed MRSA (20 µl of water served as the blank added to controls). Final volume of the culture was 250 µl. [b] CFU: Colony Forming Units of bacteria. [c] Results are expressed as the mean of the two strains tested.

Table 2. Effects of *C. edulis* leaves methanolic extract (CELME) on phagocytosed *M. tuberculosis*[a]

Bacteria	CFU[b]×10[6] (days)		
	1	**2**	**3**
M. tuberculosis H37Rv ATCC 27294	0.8	2	1.9
M. tuberculosis H37Rv ATCC 27294+CELME	0.5	1	0.6
M. tuberculosis MDR-TB[c]	2.1	3	3.9
M. tuberculosis MDR-TB[c] +CELME	1.1	1.3	1.2

[a] Approximately 0.5×10^6 CFU could be retrieved from 100,000 macrophages after the period employed for phagocytosis of approximately 10^6 CFU presented to the macrophages (zero time). CFU presented are calculated from colonies resulting from the plating of serial dilutions of 0.1 ml aliquots of cultures after the lysing of the macrophages. Concentrations of extract, final volumes, *etc*., are identical to that described in legend of Table 1. [b] CFU: Colony Forming Units of bacteria. [c] Results are expressed as the mean of the two strains tested.

VI.2 Reserpine, ouabain and the calcium channel blocker verapamil, cause intracellular killing of *Staphylococcus aureus*

SUMMARY

Killing of bacteria by neutrophils is dependent upon the availability of potassium. Although macrophages derived from human peripheral blood monocytes have little killing activity of their own, they can be transformed into effective killers of *Staphylococcus aureus* and *Mycobacterium tuberculosis* by *in vitro* exposure of the macrophage to clinically relevant concentrations of phenothiazines, namely, thioridazine or chlorpromazine. Because transport mechanisms dependent upon the availability of calcium are inhibited by these agents, the possibility that other agents which have similar activity also have the ability to enhance the killing of bacteria by the macrophage derived from peripheral blood monocytes was investigated. In this study we show that the presence of increasing concentrations of ouabain, reserpine or verapamil in the medium enhances the killing of *Staphylococcus aureus*. Because these concentrations have no activity on the replication or killing of the bacterium, killing is deemed to be due to the macrophage itself. A model is presented which describes the mechanism by which these agents and phenothiazines indirectly activate lysosomal enzymes as a result of the inhibition of potassium efflux pumps that would normally pump the ion from the phagocytic vacuole to the cytoplasm of the macrophage.

Materials and Methods employed in this study are described in publication #7 and in Chapter II (section II.7).

RESULTS

Toxicity of reserpine, ouabain and verapamil on Monocyte Derived Macrophages and their *in vitro* activity against *S. aureus*

The toxicity of reserpine, ouabain and verapamil on human monocyte derived macrophages (MDM) was determined, along the same guidelines and approaches followed for CPZ and TZ on Chapter IV (section IV.1) (Ordway *et al*., 2002a; Ordway *et al*., 2003b). Briefly, concentrations as high as 100 mg/L of reserpine, ouabain or verapamil did not produce any toxicity against MDM (data not shown).

In vitro activity of reserpine, ouabain and verapamil against *S. aureus*

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for ouabain, reserpine and verapamil were determined by the broth microdilution method. These compounds at the highest concentrations employed for the determination of toxicity had no effect on the growth rate or final growth of *S. aureus* (MIC and MBC>100 mg/L).

Effect of reserpine, ouabain and verapamil on the killing of *S. aureus* that have been phagocytosed by human monocyte derived macrophages

The effects of ouabain, reserpine and verapamil on *S. aureus* that have been phagocytosed by human monocyte derived macrophages are summarised by in Figure 1. Reserpine, ouabain and verapamil enhanced the macrophage killing activity when added to the culture media (Figura 1A). The effects were similar to those obtained with CPZ and TZ although these latter agents produced an enhancement of the macrophage killing activity at concentrations in the medium that were far lower than the ones employed for reserpine, ouabain and verapamil (Fig. 1B).

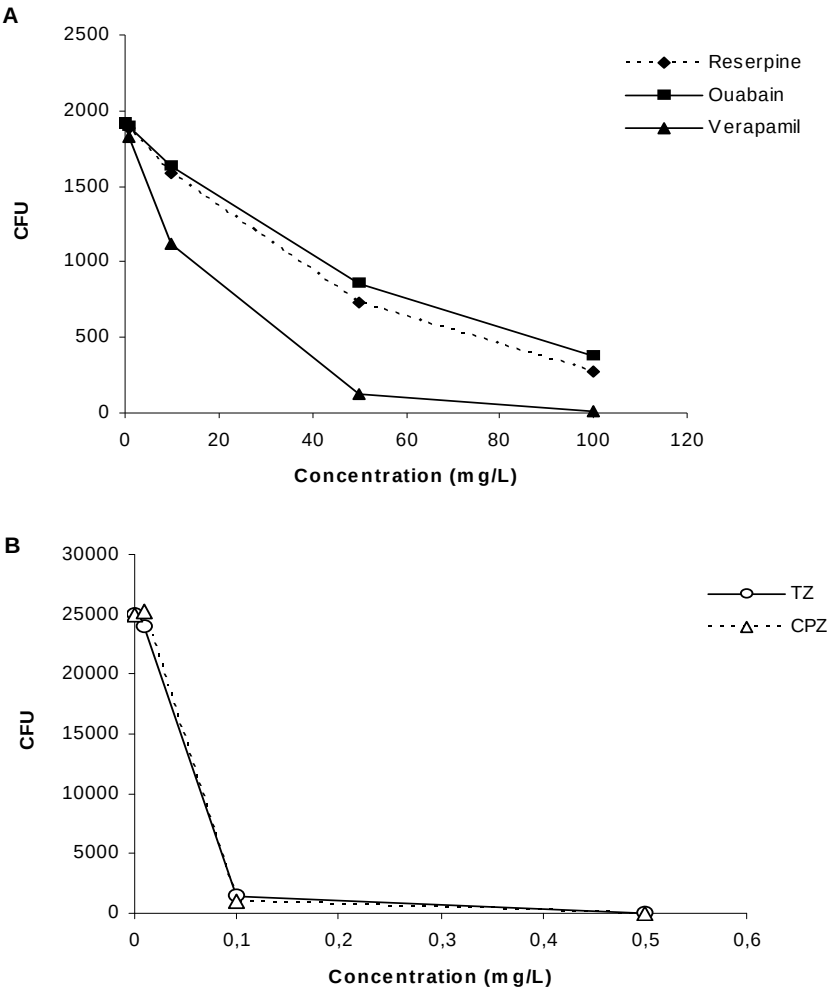


Figure 1. The effect of reserpine, ouabain, verapamil (A), CPZ and TZ (B) on *S. aureus* that have been phagocytosed by human monocyte derived macrophages. Macrophages derived from peripheral blood monocytes (1×10^6 cells/mL) were suspended in 1 mL RPMI medium and distributed to the wells of a 24 microwell plate. After 6 days of incubation, wells were washed with RPMI medium to remove non-adherent cells (lymphocytes). The adherent monocyte derived macrophages (MDM) were counted and the yield per well was approximately 10^5 with less than 5% variation of each well from this number. 10^6 bacteria were added for 30 minutes and after this period the wells were washed to remove any non-phagocytosed bacteria and fresh RPMI medium (1 mL) added to each well. One hundred microliter of each compound was added to yield the concentration that ranged from 0.0 to the maxima identified in the Figure. The microplates were incubated for 0 to 6 h and the MDM lysed with SDS thereby releasing the bacteria which were then counted by the colony forming unit (CFU) method (Ordway *et al*., 2002a, 2003b). The data in Figure 1A was obtained from a number of repeat experiments involving different donations of derived MDM and due to the biological variation resulting from differences of donors, the data was normalised and the curves presented generated by regression analysis. The data presented by 1B is actual data obtained from a representative experiment. Figure 1A. The addition of each of these agents to the medium after the phagocytosis of the bacterium produced significant enhancement in the killing of *S. aureus* in a concentration dependent manner with a maximum effect on the killing of intracellular staphylococci at the highest concentration employed.

DISCUSSION

The results obtained in our current study show, for the first time that inhibitors of potassium transport have the capacity to enhance the killing of bacteria that have been phagocytosed by monocyte derived macrophages that have little killing action of their own. Our previous studies (Ordway *et al*., 2002a, 2003b) suggested that the killing activity of phenothiazines was due to the ability of the macrophage to concentrate the agent to levels comparable to those *in vitro* that had bactericidal activity. However, the results of the current study suggest that agents that have similar activity as phenothiazines with respect to effects on calcium binding also enhance the killing of intracellular bacteria. Because the agents reserpine, ouabain and verapamil, unlike the phenothiazines (Amaral *et al*., 2004), are shown in the current study to be devoid of any *in vitro* activity against *S. aureus*, we attribute their intracellular activity to their effects on the transport of potassium in as much as these agents inhibit this transport (Kanoh, 1999; Galli, 1988). The results obtained in our current study coupled to those results recently obtained by others (Ahluwalia *et al*., 2004; Reeves *et al*., 2002), suggest the mechanisms presented by Figure 2 as those involved in the killing of intracellular bacteria. Firstly, the transport of potassium into the macrophage is dependent upon calcium dependent type pumps present in the plasma membrane of the macrophage. The process of phagocytosis involves the invagination of the plasma membrane to which the bacteria is first bound (Figure 2A). The relative position of the potassium pump would now be reversed in the phagosome and the transport of potassium would effectively be from the inside of the phagocytic vacuole to the cytoplasm of the macrophage (Figure 2B). Normally, the activity of these pumps would be expected to reduce the potassium concentration of the phagosome and hence the latent hydrolases in the lysosome that now fuses with the phagosome remain inactive. These pumps would be subjected to being inhibited by agents that inhibit calcium dependent ATPase activity and hence the efflux of K^+ from the phagosome-lysosome complex would be inhibited (Figures 2C to E). The availability of this ion for the activation of hydrolases present in the lysosome would be insured-hence killing would take place (Figure 2E). With respect to the activity of phenothiazines on intracellular bacteria, we should also consider that when bacteria are trapped in the phagosome-lysosome, there is also leakage of K^+ from the bacteria that is promoted by the agent (Kristiansen *et al*., 1982). The killing activity of macrophages that is enhanced by inhibitors of the potassium pump that is present in the

membrane of the phagosome is a subject that is now under investigation and involves the isolation of the phagocytic vacuole and the evaluation of potassium efflux activity.

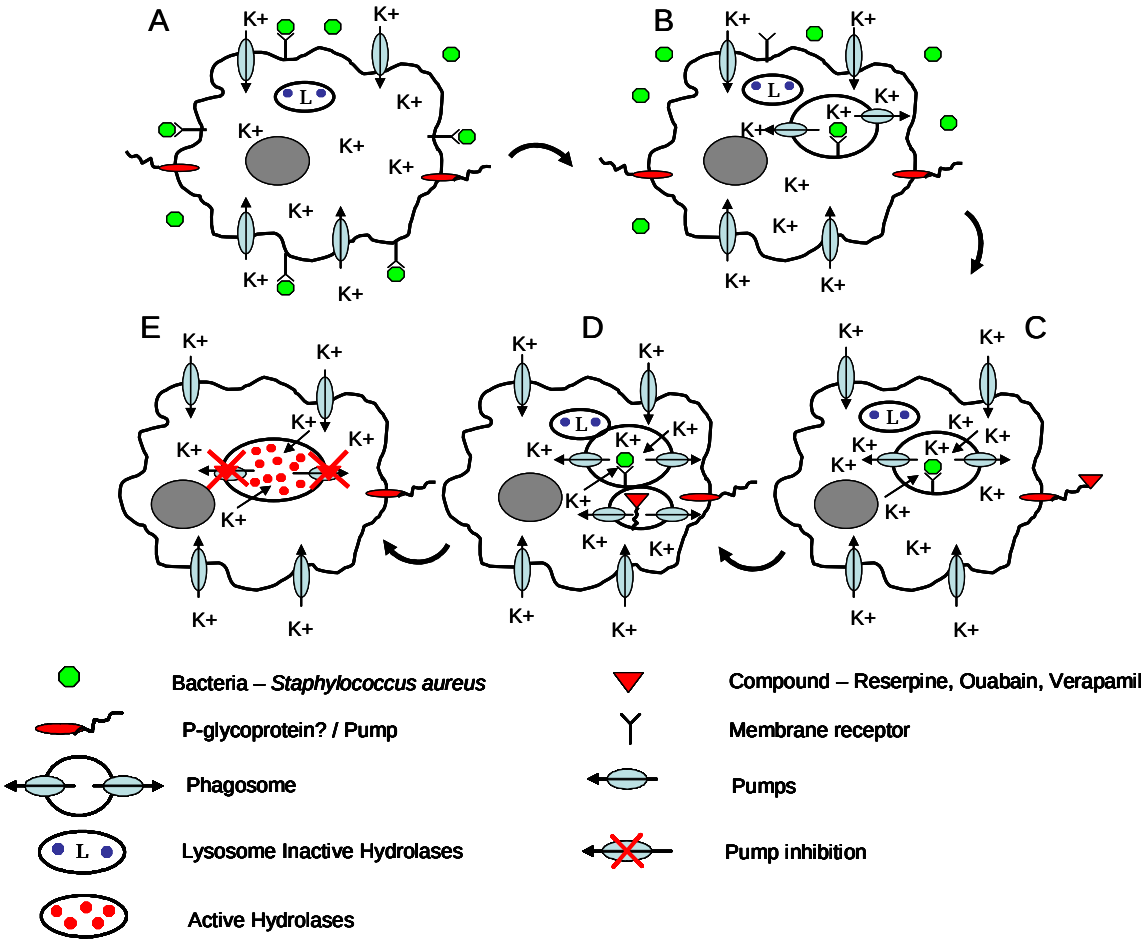


Figure 2. Model suggested for the killing of intracellular *Staphylococcus aureus* by ouabain, reserpine and verapamil. (A) Internalization of the bacteria by the macrophage, (B) Invagination of the macrophage membrane containing the bacteria, (C) Phagosome trapping with the bacteria, (D) Fusion of the phagosome with the lysosome and (E) Inhibition of the pumps by the compounds reserpine, ouabain and verapamil with the activation of the hydrolases and subsequent bacterial killing.

VI.3 Inhibitors of Ca^{2+} and K^{+} Transport Enhance Intracellular Killing of *M. tuberculosis* by Non-Killing Macrophages

SUMMARY

Human monocyte-derived macrophages that have little killing activity of their own kill intracellular *Staphylococcus aureus* when cultured in the presence of inhibitors of calcium and potassium efflux pumps. The aim of this study was to evaluate the effect of inhibitors such as ouabain, reserpine and verapamil in the killing activity of macrophages infected with *Mycobacterium tuberculosis*. Macrophages obtained from peripheral blood were infected with *M. tuberculosis* ATCC27294 H37Rv and treated with reserpine, ouabain and verapamil. After three days post-infection, macrophages treated with the inhibitors demonstrated an enhancement of the killing activity destroying the internalized bacteria. Whereas drugs that target the bacterium are predicted to lose effectiveness due to mutation of the bacterial target, drugs that enhance killing by macrophages that normally do not kill mycobacteria may yield a more effective form of therapy of infections caused by multidrug resistant *M. tuberculosis.*

Materials and Methods employed in this study are described in publication #8 and in Chapter II (sections II.5 and II.8).

RESULTS

The evaluation of ouabain, reserpine and verapamil for direct *in vitro* activity against *M. tuberculosis* H37Rv ATCC27294, by the BACTEC 460-TB method, that identifies the minimum inhibitory concentration (MIC) for each agent, is summarised by Table 1. Briefly, whereas TZ (positive control) has an MIC of 15 mg/L, a concentration of ouabain, reserpine or verapamil as high as 80 mg/L had no significant effect on the replication of the mycobacterium.

Table 1. Minimum inhibitory concentration of TZ and other efflux pumps inhibitors against *M. tuberculosis* H37Rv ATCC27294

	MIC (mg/L)
Phenothiazine (positive control)	
TZ	15
Other efflux pump inhibitors	
Reserpine	100
Ouabain	>200
Verapamil	128

The evaluation of toxicity of ouabain, reserpine and verapamil on lymphocytes cultured for up to 3 days is summarised by Table 2. Briefly, whereas TZ was cytotoxic at concentrations that exceed 0.5 mg/L, concentrations of ouabain, reserpine and verapamil as high as 100 mg/L did not produce significant toxicity, *i.e.*, the percentage of lymphocytes that exhibited trypan blue staining of their cytoplasm did not exceed 10%.

Table 2. Percent toxicity of reserpine, ouabain and verapamil on human lymphocytes

	Toxicity (%)		
Phenothiazine (positive control)	**0.1 mg/L**	**3 mg/L**	**10 mg/L**
TZ	0	50	100
Other efflux pump inhibitors	**2 mg/L**	**50 mg/L**	**100 mg/L**
Reserpine	0	0.54	1.1
Ouabain	0	5.5	10[a]
Verapamil	0	2.4	4.8

[a] Concentration of 100 mg/L of ouabain produces marginal toxicity.

In order to determine whether the enhanced killing obtained with TZ and its derivatives (results presented in Chapter IV.2) could be due to the inhibition of transport of Ca^{2+} and K^+, we examined whether common inhibitors of Ca^{2+} and K^+ transport can enhance the killing of intracellular bacteria at concentrations of agents which are non-toxic. As can be seen in Figure 1, these efflux pump inhibitors enhanced the killing activity of

infected macrophages, however, at concentrations much higher (80 mg/L) than those used for TZ (0.1 mg/L). Verapamil demonstrated a greater activity than TZ. The maximum concentration of the K^+ inhibitors examined for ability to enhance the killing of intracellular *M. tuberculosis* was 80 mg/L inasmuch as this concentration was devoid of any significant toxicity against human lymphocytes (see Table 2).

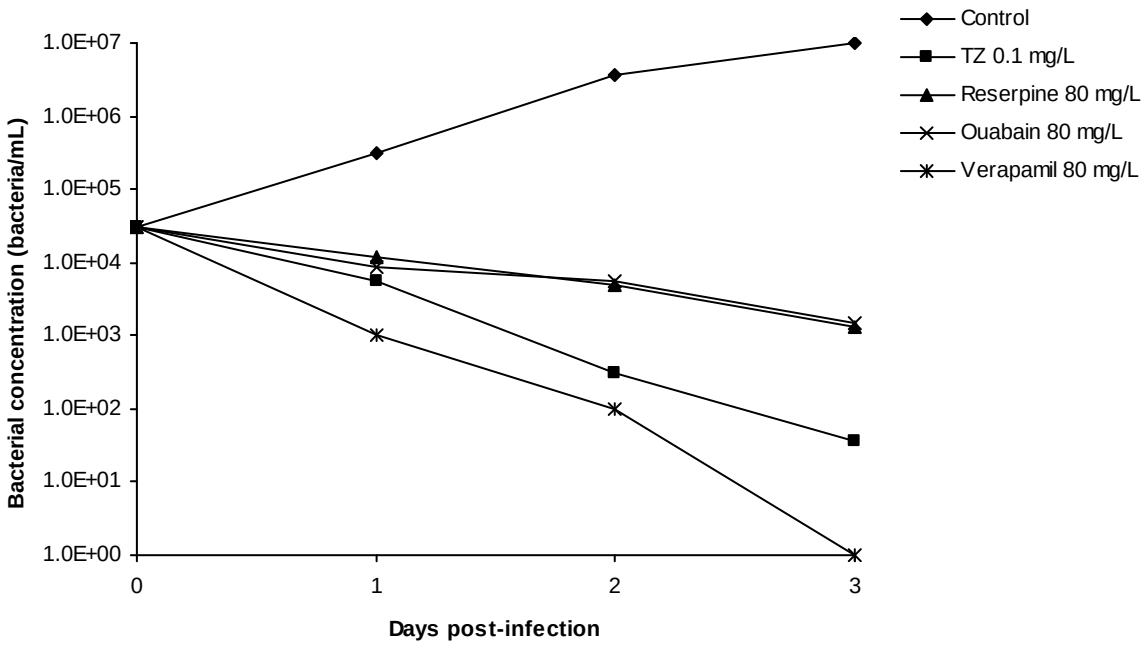


Figure 1. Effect of TZ and other efflux pumps in the killing activity of macrophages infected with *M. tuberculosis*. Cultures of isolated human macrophages (1×10^5 cells/mL) were infected with *M. tuberculosis* at a ratio of 1:10 (1 macrophage:10 bacteria). After 1 hour of phagocytosis, cells were washed to remove non-phagocytosed bacteria. Aliquots of the tested compounds were added to the wells and cells incubated for 0, 1, 2 and 3 days. After incubation, supernatants of the wells were removed and kept for CFU study in order to determine whether the addition of the agents cause the lysis of the macrophage and subsequent release of the bacterium. Adhered cells were lysed with SDS 0.01% in order to release all the intracellular bacteria and aliquots of the lysed cells were plated on 7H11 medium. Plates were incubated at 37ºC for 3 to 4 weeks. After the incubation period, CFU were counted and bacterial concentrations calculated. The data presented are averages from three independent experiments each of which was conducted in triplicate.

DISCUSSION

Phenothiazines inhibit the replication of *S. aureus* (Kristiansen *et al*., 2006; Martins *et al.*, 2006b; Ordway *et al*., 2002b), and *M. tuberculosis* (Bettencourt *et al*., 2000; Martins *et al.*, 2007b;Ordway *et al*., 2003b) and are bactericidal at concentrations that are multiples of their MIC (Amaral and Kristiansen, 2000). Because phenothiazines are concentrated as much as 100-fold by cells that are rich in lysosomes (Amaral and Kristiansen, 2000; Amaral *et al*., 2001a, 2006, 2007b; Daniel and Wojcikowski, 1997a, 1999b), the enhanced intracellular killing produced by TZ and its derivatives could be the result of concentrations that are comparable to those that are bactericidal (Bettencourt *et al*., 2000; Ordway *et al*., 2003b;). Because previous studies have shown that concentrations of verapamil and reserpine that are considerably higher than those employed in our study do not significantly affect the growth of bacteria (Viveiros *et al*., 2002; Kristiansen *et al*., 2006), enhanced killing is probably not due to any antimicrobial activity that results from their being concentrated by the macrophage.

Recent studies have shown that the killing activity of the neutrophils is dependent upon the transport of Ca^{2+} (Ahluwalia *et al*., 2004), K^+ and hydrolysis of ATP (Reeves *et al*., 2002). Because these processes are inhibited by phenothiazines (Amaral *et al*., 2004, 2007b; Ordway *et al*., 2002b) enhanced killing may be due to the effect that the phenothiazine has on the transport of Ca^{2+} and K^+. This raises the question, of how these inhibitors of Ca^{2+} and K^+ transport enhance the killing of intracellular mycobacteria. After phagocytosis, the bacterium is contained in a phagosome that was formed from the invagination of the plasma membrane of the macrophage. The plasma membrane is rich in pumps that transport Ca^{2+} and K^+ into the cell, and due to invagination, would now pump these ions to the cytoplasm of the cell. When fusion of the phagosome with a lysosome takes place, the absence or low concentration of Ca^{2+} and K^+ prevents the acidification of the phagolysosome and hence the bacterium is not killed. The unification of a vacuole that contains the inhibitor of Ca^{2+} and K^+ transport with the phagolysosome inhibits the transport of these ions to the cytoplasm and they begin to accumulate within the phagolysosome. This would soon be followed by the activation of the V-ATPase system, which is probably ouabain insensitive (probably also phenothiazine insensitive); K^+ is extruded and hydrogen ions flood into the phagolysosome resulting in the acidification of the phagolysosome and activation of

hydrolytic enzymes. The killing of the bacterium subsequently takes place. However, the possibility that a direct action of the agent on the phagosome-trapped bacterium due to the agent being concentrated to levels equivalent to those that kill the bacterium *in vitro* cannot be ruled out.

The question of whether the results obtained in this study will further contribute to the therapy of MDR-TB is intriguing, because:

Firstly, therapy of mice infected with *M. tuberculosis* with phenothiazines derivatives has proven successful (Amaral *et al*., 2007b; Weinstein *et al*., 2005; Yano *et al*., 2006). Secondly, these results are consistent with the predictions provided by *ex vivo* studies (Amaral *et al*., 2007b; Martins *et al*., 2006b, 2007b; Ordway *et al*., 2002b, 2003b), demonstrating the relevance of testing these compounds on infected macrophages.

The results of the current study suggest an entirely different approach for the therapy of TB, and especially, MDR-TB. This approach involves the design of agents which target the macrophage and transform it into an effective bacterial killer. Whereas agents which target the bacterium are predicted to become ineffective due to mutations of the bacterial target, agents that enhance the killing activity of the macrophage will not suffer from this limitation. Moreover, this approach is also viable for the therapy of other intracellular infections such as malaria, leishmaniasis, trypanosomiasis inasmuch as phenothiazines have already been shown to be effective (Amaral *et al*., 2001b, 2006; Grácio *et al*., 2003; Happi *et al*., 2006; Wainwright and Amaral, 2005). Perhaps the search for effective agents for the therapy of intracellular infections should be extended to include all agents that are known to inhibit Ca^{2+} and K^{+} transport processes.

CHAPTER VII.

The effect of efflux pumps inhibitors, such as SILA compounds, on macrophages infected with MDR *M. tuberculosis*. New methods for the screening of efflux pump activity and efflux pump inhibitors

This chapter contains data published and submitted for publication in:

Martins, M., M. Viveiros, J. Ramos, I. Couto, J. Molnar, and L. Amaral. 2008. SILA compound 421, an inhibitor of efflux pumps of cancer cells, enhances the killing of intracellular MDR-TB. *Submitted to J. Antimicrob. Chemother.*; **Martins, M., B. Santos, A. Martins, M. Viveiros, I. Couto, A. Cruz, J. M. Pagès, J. Molnar, S. Fanning, L. Amaral, and Management Committee Members of COST B16; European Commission/European Science Foundation.** 2006. An instrument-free method for the demonstration of efflux pump activity of bacteria. In Vivo **20**:657-664.

CHAPTER OUTLINE

This chapter is focused on the effect of SILA compounds and infected macrophages as a special example relationship between efflux pumps inhibitors and the effect on infected macrophages. Human macrophages infected with MDR/XDR *M. tuberculosis* were treated with SILA compounds and the effect on the enhancement of the macrophage killing activity was assessed by colony forming units. These new and enthusiastic results provide evidence for a new compound that presents *in vitro* activity against XDR-TB and also enhance the macrophage killing activity. The fact that efflux inhibitors not only have activity against the bacteria itself but also *ex vivo*, lead us to develop methods for the rapid screening of new active compounds.

The screening methods to be described, allow the rapid detection of efflux pumps inhibitors using an agar-based approach and a broth microplate system. These methods were developed at the Unit of Mycobacteriology (IHMT/UNL) and allow the rapid screening of efflux pump activity in large collection of clinical isolates, as well as efflux pump inhibitors (that reverse resistance or reduce antibiotic resistance) identification. These methods will be eventually adapted for the identification and screening of efflux activity of clinical isolates of *M. tuberculosis* and *M. avium* during my post-graduate work.

VII.1 SILA compound 421, an inhibitor of efflux pumps of cancer cells, enhances the killing of intracellular MDR-TB

SUMMARY

Multi-Drug Resistant Tuberculosis (MDR-TB) and Extensively-Drug Resistant Tuberculosis (XDR-TB) infections are problematic to manage, especially when the patient presents with AIDS. There is a dire need for effective anti-TB agents and none as of now have been made available. We have previously shown that agents which have the ability to inhibit efflux pumps of multi-drug resistant (MDR) bacteria and cancer cells also enhance the killing of intracellular mycobacteria possibly by increasing the availability of K^+ and Ca^{2+} needed for the activation of lysosomal enzymes of the phagolysosomal unit. In the herein study to be described, the newly synthesized and recently patented SILA compound 421, were tested for *in vitro* and *ex vivo* activity against an XDR-TB strain. Minimum inhibitory concentration of each agent was determined by the BACTEC 460 method. The effect of each compound on the killing activity of human monocyte-derived macrophages infected with the XDR-TB strain was determined by exposing the macrophage that had phagocytosed the bacterium to varying concentrations of each agent and the killing activity assessed by colony forming units counting. SILA compound 421 was shown to have *in vitro* activity against XDR-TB (MIC < 3.5 mg/L) and transforms non-killing macrophages into effective killers of phagocytosed XDR-TB. Because this agent is devoid of any cytotoxic activity it has good potential as an anti-XDR-TB/MDR-TB drug.

Materials and Methods employed in this study are described in publication #9 and in Chapter II (sections II.5 and II.8).

RESULTS

The *in vitro* activity of TZ and SILA compounds 409 and 421 is summarized by Table 1. Briefly, the MIC of TZ, SILA compounds 409 and 421 are 15, 12.5, and 3.125 mg/L, and 20, 12.5, and 3.125 mg/L, for *M. tuberculosis* ATCC H37RV and XDR-TB strains, respectively. These compounds were evaluated for cytotoxicity against human lymphocytes via the trypan blue exclusion method (Ordway *et al*., 2003b) and found not

to increase the retention of trypan blue at concentrations as high 100 mg/L as compared that of the drug-free controls (data not shown). These results are compatible with those of others who demonstrated that the compounds do not produce toxicity to the mouse (Fusi *et al*., 2008).

Table 1. The *in vitro* activity of TZ and SILA compounds 409 and 421 against *M. tuberculosis* strains.

***M. tuberculosis* strains**	**MIC (mg/L)**		
	TZ	**SILA 409**	**SILA 421**
H37Rv ATCC	15	12.5	3.125
XDR-TB	20	12.5	3.125

The activity of TZ (positive control) and of SILA compounds 409 and 421 against phagocytosed XDR-TB was evaluated. As shown by Figure 1, whereas the lowest concentration of TZ and SILA compound 421 that enhances the killing of intracellular XDR-TB is 0.1 mg/L, at this concentration SILA compound 409 does not enhance killing.

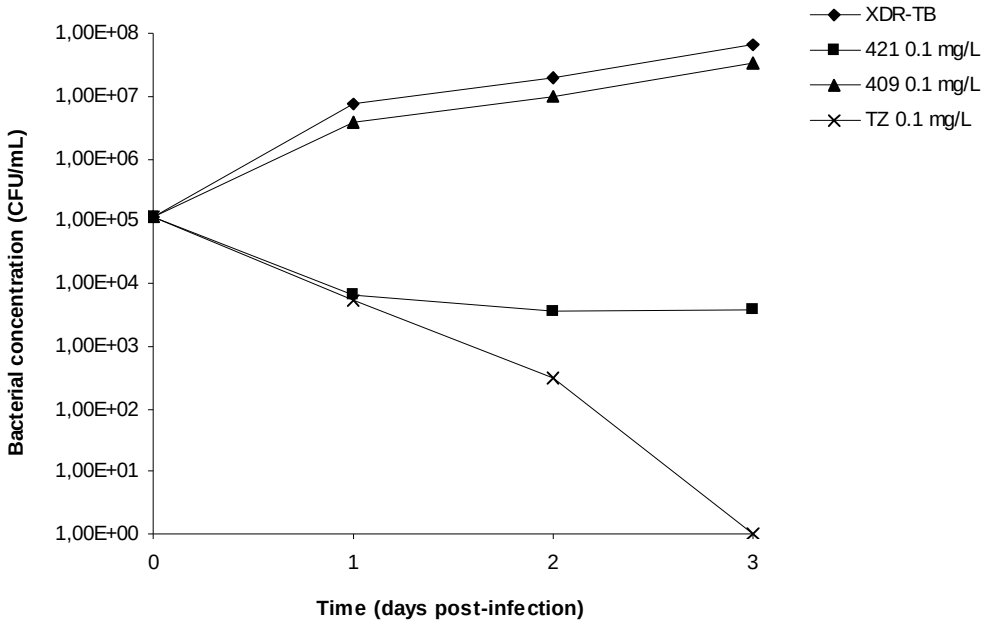


Figure 1. *Ex vivo* effect of SILA compounds 421 and 409 in human macrophages infected with XDR-TB. Average killing activities of human monocyte-derived macrophages against XDR-TB (filled losanges), when either TZ (cross), SILA compounds 421 (filled squares) and 409 (filled triangles) were present in the medium at concentrations of 0.1 mg/L. The results are the average numbers of CFU for cultures obtained from three independent experiments.

DISCUSSION

Global infections of antibiotic resistant *M. tuberculosis* continue to escalate especially in areas of the world that are poverty stricken and are laden with economic strife (Dorman and Chaisson, 2007). Although the problem of antibiotic resistance is not nearly as acute in Western European countries, there are some urban centres of Western Europe where the rates of new cases of MDR-TB and now, XDR-TB, are high (Migliori *et al*., 2007; WHO/IUATLD, 2004; Zignol *et al*., 2006;). Lisbon and Porto, the two major cities of Portugal are examples where the rates of MDR-TB are exceedingly high (DGS, 2006) although with respect to Lisbon, there has been a dramatic reduction of new cases of MDR-TB due to the implementation of the Faster TB Track Programme of the Institute of Hygiene and Tropical Medicine and the ability of its Mycobacteriology laboratory to identify within one day of receiving a positive acid fast stained sputum specimen, the patient that is infected with MDR-TB (Viveiros *et al*., 2005b). With respect to Porto, that as of this writing is not using the systems and programmes of the Institute of Hygiene and Tropical Medicine of Lisbon, the rates of new cases of MDR-TB continue to escalate (DGS, 2006). With respect to XDR-TB, retrospective evaluation of MDR-TB strains isolated from patients during the past 8 years, clearly, a significant number are of an XDR-TB nature. The XDR-TB strain employed in the current study was isolated 3 years ago and is an example of this assumption. Undoubtedly, the pulmonary TB laboratory plays a major role in the control of TB (Bergmann and Woods, 1996; Ridderhof *et al*., 2007). However, until the pulmonary TB is completely eliminated, effective therapy is an absolute requirement if the disease is to be controlled (WHO, 2007b). Although antibiotic susceptible TB infections are not difficult to manage effectively under conditions that optimize therapy (Ducati *et al*., 2006), inexperience of the physician or non-compliance of the patient contribute to the selection of antibiotic resistant strains of *M. tuberculosis* (Drobniewski and Balabanova, 2002; Rao, 1998;). When the patient is infected with MDR-TB, therapy is problematic and cure rates, although they can be as high as 85% in certain locales (Raviglione, 2003), are very low wherever the frequency of MDR-TB is high (Vanacore *et al*., 2004). If the patient is co-infected with HIV or presents with AIDS, mortality is complete within one year of diagnosis regardless of therapy (Kawai *et al*., 2006). Now with the advent of XDR-TB, therapeutic success is highly improbable with currently available drugs, and this includes the increasing number of agents that make up the “second line

of defence" drugs (Kim HR *et al*., 2007). And as of the time of this writing, no new drugs are on the horizon; and only two clinical trials of new agents are active with little success in evidence (Zhang, 2007). There is an obvious dire need for effective anti-MDR-TB/XDR-TB compounds, and, this is where our studies have been directed during the past 8 years (Amaral and Kristiansen, 2000; Amaral *et al*., 1996, 2001a, 2001b, 2004, 2006, 2007c; Bettencourt *et al*., 2000; Kristiansen and Amaral, 1997; Martins *et al*., 2005, 2007a, 2007b; Ordway *et al*., 2002b; Viveiros and Amaral, 2001; Wainwright and Amaral, 2005).

We have recommended in our rprevious published studies (Amaral and Kristiansen, 2000; Amaral *et al*., 2001a, 2001b, 2006, 2007b; Martins *et al*., 2008) that until effective anti-MDR-TB agents are made available, TZ be considered for the therapy of MDR-TB (Amaral and Kristiansen, 2000; Amaral *et al*., 2001b, 2006). Because TZ does cause prolongation of QTc when used at a considerably high dose (Amaral *et al*., 2007b), there has been resistance to use this compound for therapy of MDR-TB even for compassionate reasons, *i.e*., after everything else has failed and the mortality is certain (Amaral *et al*., 2006, 2007b). Perhaps the derivatives of TZ which have been shown to be even more effective than the parent compound will receive serious attention (Martins *et al*., 2007b). In the meanwhile, SILA compound 421, a cancer cells efflux pump inhibitor, that has been studied and reported herein, has significant *in vitro* activity at a concentration acceptable for a potential anti-MDR-TB/XDR-TB drug (the MIC is quite low, namely, less than 3.5 mg/L). Although this concentration is free of any cytotoxicity, what is of far greater significance is that the enhancement of killing of intracellular XDR-TB by non-killing human macrophages takes place when the concentration of the agent in the medium is 0.1 mg/L. One would expect that a similar concentration of the agent in the interstitial fluids of the XDR-TB infected patient would also be effective and enhance the killing of XDR-TB at the site where this organism resides-namely, the pulmonary macrophage (Amaral *et al*., 2007b; Martins *et al*., 2008). Hence, barring any unforeseen negative aspects, SILA compound 421 is a very exciting anti-MDR-TB/XDR-TB agent and for this it deserves immediate attention from the pharmaceutical concern.

VII.2 An instrument-free method for the demonstration of efflux pump activity of bacteria

SUMMARY

The aim of the study was to develop a simple, inexpensive, reproducible ethidium bromide (EB)-agar based method that is independent of any specialized instrumentation, for the demonstration of efflux pump activity, which is responsible for antibiotic resistance of bacteria and therefore, can be the basis for the identification of new antimicrobial agents. A series of agar plates containing varying concentrations of EB were swabbed with strains of *Escherichia coli* or *Staphylococcus aureus*, which differed with respect to efflux pump activity. The plates were incubated at different temperatures and time periods and the measurements of fluorescence were used to evaluate the efflux activity of each culture. This simple assay allowed us to identify the efflux of EB in different bacteria following an overnight incubation. The minimal concentration of EB that produced fluorescence was significantly greater at 37ºC than at 4ºC, suggesting the presence of an energy-dependent pump. The method was shown to simultaneously identify strains of a mixed culture that differed from each other with respect to the activity of their efflux pumps. The method, in conjunction with the use of antibiotic-containing disks, provides an additional advantage for the easy identification and selection of colonies that differ with respect to antibiotic susceptibility and degree of efflux pump activity. Because the method is very reproducible it may form the basis for inter-laboratory standardization of efflux pump activity of multi-drug resistant (MDR) clinical isolates, as well as the screening of compounds for the ability to inhibit efflux pumps of bacteria.

Materials and Methods employed in this study are described in publication #9 and in Chapter II (section II.4).

RESULTS

The use of a system that is to contain an agent for the demonstration of an efflux pump or its activity must first be preceded by studies that determine the effect of that agent on the growth of the bacteria under study (Viveiros *et al*., 2002). As evident from Table 1,

EB, a common bacterial efflux pump substrate, has differential activity against the bacteria listed. This efflux pump substrate was chosen because of its fluorescent properties that make it easily trackable at low concentrations.

Table 1. The minimum inhibitory concentration (MIC) of EB against bacteria.

Bacterium	MIC for EB (μg/ml)
E. coli K-12 AG100 (*acrAB*)	150
E. coli K-12 AG100 (*acrAB* TET)	>200
E. coli K-12 AG100A (*ΔacrAB*)	5
Enterobacter aerogenes EA27	>150
S. aureus ATCC25923	5
MRSA COL	10
MRSA HPV107	>30

The MIC for EB was determined by the use of the broth dilution method previously described (Viveiros *et al*., 2005a).

Therefore, the concentrations of EB present in the agar that is to be streaked or swabbed with the bacterial inoculum must be well below those that have an effect on growth. Furthermore, because EB will fluoresce under UV light, the selection of the maximum concentration of EB in the agar must be one that is below the resolution of the UV detector employed. With these considerations in mind, the maximum concentration of EB in agar was below 7.5 μg/ml. Examples of the growth at 37ºC of streaked *S. aureus* ATCC25923 strain in agar containing concentrations of EB that ranged from 0.0 to 4.0 μg/ml demonstrate that at a concentration of 0.4 μg/ml EB bacteria begin to exhibit fluorescence, whereas the agar-EB itself does not exhibit any detectable fluorescence at a concentration as high as 4.0 μg/ml of EB (Figure 1).

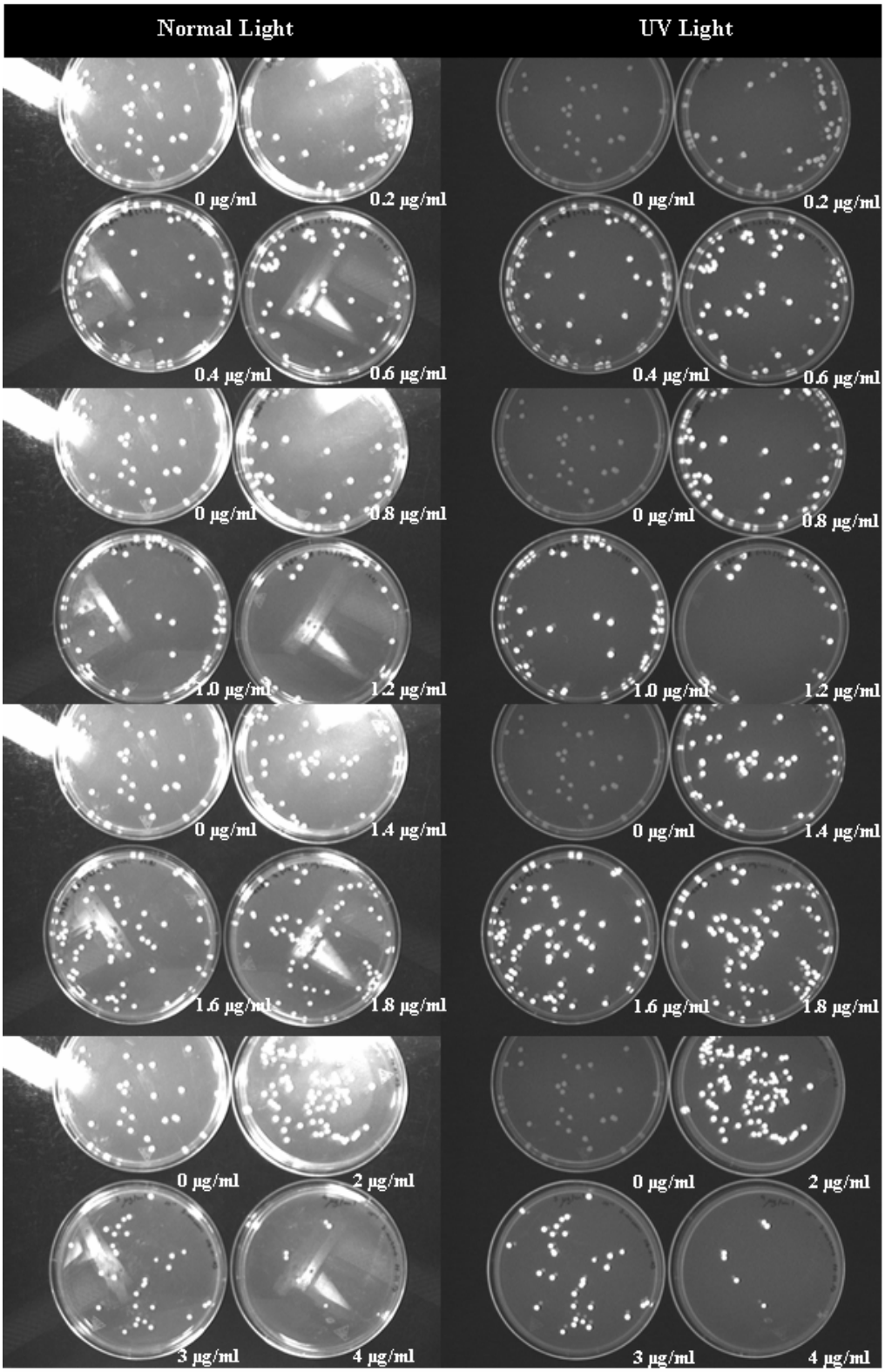


Figure 1. **Fluorescence associated with isolated *S. aureus* colonies grown on agar containing increasing concentrations of EB.** *S. aureus* ATCC25923 grown overnight in TSB was diluted with saline to yield approximately 100,000 cells per ml. This dilution was further diluted 100-fold and from this a small loopful streaked onto TSB agar containing concentrations of EB ranging from 0.0 to 4.0 µg/ml. Plates were incubated overnight and examined under UV illumination. Photographs

were obtained with the aid of the Eagle Eye (Stratagene, USA) instrument. Fluorescence of the colonies is associated with the minimal concentration of EB-agar of 0.4 µg/ml. Also note that concentrations of EB as high as 4.0 µg/ml do not produce any fluorescence from the agar itself.

The use of swabbing affords certain advantages over the streaking method such as providing a more representative and perhaps uniform result if the strain employed is homogeneous with respect to the presentation of fluorescence at a given concentration of EB. As shown by Figure 2, swabbing of *S. aureus* ATCC25923 strain yields the same result — namely that the minimal concentration of EB that produces significant fluorescence is identical to that yielded by the streaking method (0.4 µg/ml). As the concentration of EB is increased, more fluorescence is associated with the confluent mass. Figure 2 also demonstrates that the minimal concentration of EB that produces fluorescence of the ATCC strain is markedly lower than that of two MRSA strains COL and HPV107, the latter of which has an MDR phenotype.

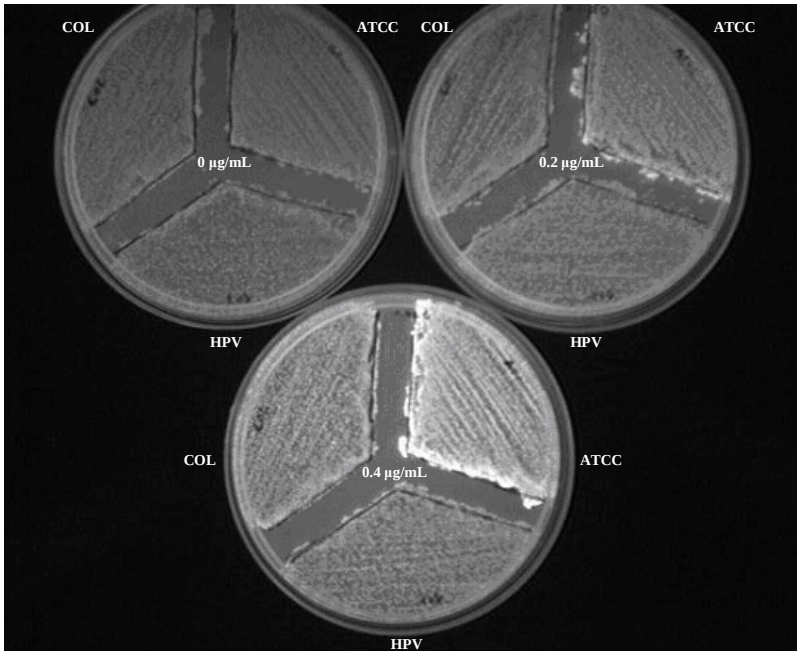


Figure 2. **The minimal concentration of EB that produces fluorescence of *S. aureus* ATCC, MRSA COL and MRSA HPV107 MDR phenotype.** Strains of *S. aureus* were grown overnight in TSB and 10 µl aliquots were diluted in 10 ml of saline. With the aid of a sterile swab, individuals sectors of each agar plate, which contained various concentrations of EB was swabbed with the ATCC, MRSA COL and MRSA HPV107 strains, respectively. The minimum concentration of EB producing fluorescence of the ATCC strain was 0.4 µg/ml whereas the MRSA COL and HPV107 strains did not fluoresce until a concentration above 1.8 µg/ml of EB was used.

The effect of temperature on the retention of EB is shown by Figure 3. The transfer of the EB plates that had been incubated at 37ºC to 4ºC resulted in fluorescence of the cultures at a concentration that produced no fluorescence at 37ºC. The set of duplicate plates returned to 37ºC maintained the same minimal concentration of EB that produced fluorescence after the first 24 hours at 37ºC (data not shown).

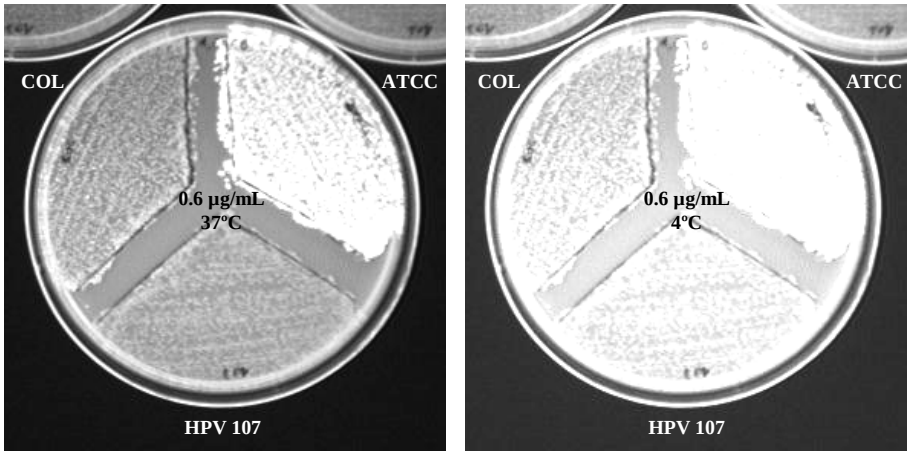


Figure 3. The effect of temperature on the minimal concentration of EB that produced fluorescence of *S. aureus* ATCC, MRSA COL and MRSA HPV107. Agar plates containing EB were examined for concentrations that did not produced fluorescence of MRSA COL and HPV107 strains but that did produce ample fluorescence of the ATCC strain. These plates were transferred to 4ºC and examined after 24 h. The minimal concentration of EB that produced fluorescence was reduced from about 1.8 to < 0.5 µg/ml (example shown is for 0.6 µg/ml and shows highly evident fluorescence).

The minimal concentration of EB that produced fluorescence by different bacteria cultured on EB-containing agar and which have different degrees of efflux pump activity is summarized by Table 2.

Table 2. Lowest concentration of EB that produced fluorescence associated with bacterial cell mass after 24 hours at 37ºC, after 48 hours at 37ºC and after transfer from 24 hours at 37ºC to 4ºC.

Bacterium	Lowest EB concentration producing fluorescence (µg/ml)		
	After 24h at 37ºC	Transfer to: 4ºC	After 48h at 37ºC
E. coli K-12 AG100 (*acrAB*)	0.4	0.1	0.4
E. coli K-12 AG100 (*acrAB* TET)	2.0	0.1	2.0
E. coli K-12 AG100A (Δ*acrAB*)	0.1	<0.1	0.1
Enterobacter aerogenes EA27	1.9	0.1	1.9
S. aureus ATCC25923	0.4	0.05	0.4
MRSA COL	1.6	0.5	1.6
MRSA HPV107	2.2	0.5	2.2

From overnight cultures in LB or TSB broth, 100 µl were transferred to 10 ml of saline, mixed, and one sterile cotton swab dipped into the mixture, dabbed onto the inside of the tube, and replicate LB or TSB agar plates containing concentrations of EB ranging from 0.0 to 2.2 µg/ml were extensively swabbed to yield a uniform distribution of cells. The plates were incubated for 24 h at 37ºC and then illuminated briefly by a fluorescent lamp and the lowest concentration of EB associated with uniform fluorescence of the colonies recorded. One set of replicate plates were returned to 37ºC and the other replicate transferred to 4ºC. The plates were examined periodically and the lowest concentration of EB that produced uniform fluorescence of the colonies present recorded.

The demonstration of fluorescence associated with the bacterial mass takes place at different concentrations of EB for each of the bacteria studied - The transfer of these plates to 4ºC reduced the minimal concentration of EB that produced fluorescence (Table 2). These assays have been conducted on at least six separate occasions, each time in duplicate, and the data obtained has been consistent. Colonies from the 4ºC or 37ºC plates that showed fluorescence at a minimal concentration of EB associated with the cell mass when transferred to the appropriate broth (TSB or LB) were as viable as their respective initial controls (data not shown). Therefore, the presence of EB associated with the bacterial cell mass does not result in any measurable lethality.

To determine whether the method allows the distinction between strains of one species that differ significantly from each other by the activity of efflux pumps, *E. coli* K-12 AG100A (Δ*acrAB*) and the AcrAB over-expressed strain (AG100 TET) were separately cultured in LB broth overnight. The AcrAB-TolC system in *E. coli* has been identified as the predominant drug efflux pump of this organism and is the major described MDR mechanism (Elkins and Nikaido, 2002). We have used an *E. coli* K-12 strain whose genes that code for this main efflux pump (AcrAB) have been deleted (*E. coli* AG100A) and also a strain that contains an over-expressed intact *acr*AB operon. This last strain (*E. coli* AG100TET) has a high level of resistance to TET due to its slow and gradual culture in media containing this antibiotic (Viveiros *et al*., 2005a). Since *E. coli* K-12 AG100A (Δ*acrAB*) and *E. coli* K-12 AG100 (*acrAB* intact) strains are resistant to kanamycin (KAN) and TET, respectively, separate disks containing these antibiotics were placed on each EB-agar plate. As shown in Figure 4, the colonies of the KAN-resistant strain, AG100A (Δ*acrAB*), that grow up to the KAN disk exhibit heavy fluorescence, whereas those that grow around the TET disk, the AG100 (*acrAB* intact), strain do not fluoresce. These results show that whereas the strain which has the main efflux pump deleted (Δ*acrAB*), as expected, cannot extrude the EB at concentrations above 0.4 μg/ml, the strain that has the intact acrAB efflux pump readily extrudes EB.

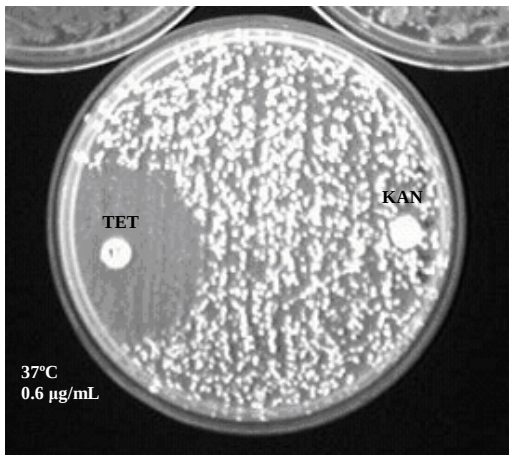


Figure 4. The use of the EB-agar method for the demonstration of different strains of *E. coli* that differ with respect to the absence and over-expressed AcrAB efflux pump. The EB-agar plate was swabbed with a mixture of acrAB deleted *E. coli* AG100A, resistant to KAN, and acrAB *E. coli* AG100, which has high level resistance to TET (Viveiros *et al*., 2005a). The plate was incubated at 37ºC, examined and photographed. Colonies around the TET disk that do not fluoresce are those that contain an over-expressed intact *acrAB* operon which is the cause of high level

resistance to this antibiotic whereas those that surround the KAN disk have the *acrAB* operon deleted and these fluoresce at a concentration of 0.6 µg/ml of EB.

The results presented in Figure 5 show that the colonies of MRSA HPV107 growing right up to the disks containing the OXA, ERY, and CIP begin to show evidence of fluorescence at 1.8 µg/ml of EB, whereas those present in the areas that are far from these disks begin to show fluorescence at 0.4 µg/ml. Removal of single white colonies from the areas around the antibiotic-containing disks and fluorescent colonies from the EB plates containing 0.4 µg/ml of EB with subsequent culture and testing for susceptibility confirmed that the white colonies were of the HPV107 MDR strain and those that fluoresced were the ATCC strain. Transfer of these plates to 4ºC reduced the minimal concentration of EB needed to produce fluorescence of the colonies surrounding the disks (data not shown).

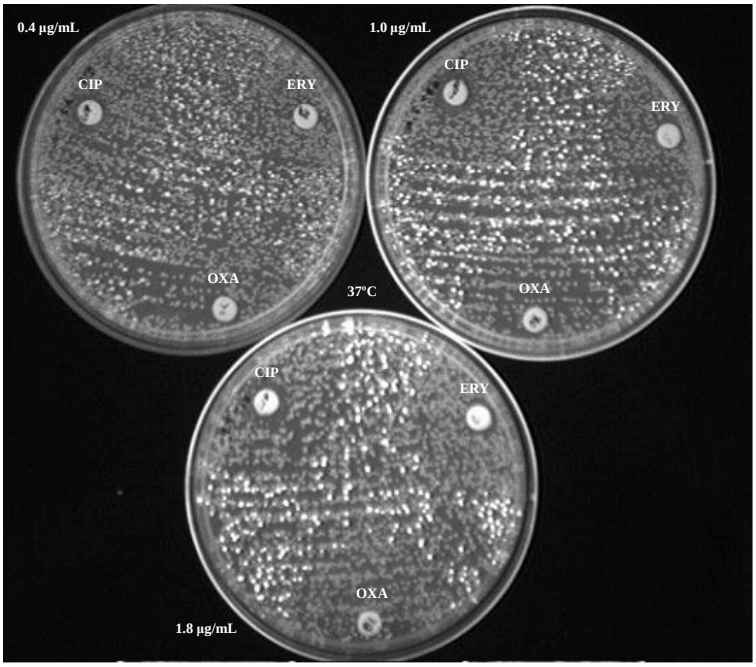


Figure 5. The ability of the method to distinguish the antibiotic-susceptible *S. aureus* ATCC25923 strain from the antibiotic-resistant MRSA HPV107 strain. Aliquots of 10 µl of *S. aureus* ATCC25923 and 40 µl of MRSA HPV107 strains from overnight cultures were added to 10 ml of saline and from this 10 µl were diluted 100-fold with saline. The mixture was swabbed onto EB-containing plates and antibiotic susceptibility disks containing CIP, ERY and OXA applied, and the plates incubated overnight at 37ºC. Colonies growing right up to the disks of CIP begin to show evidence of fluorescence at 1.8 µg/ml. The zone of inhibition noted is devoid of colonies that

fluoresce. Isolation of non-fluorescent colonies and fluorescent colonies were later shown by susceptibility assay to be MRSA HPV107 and *S. aureus* ATCC strains, respectively.

DISCUSSION

The extrusion or retention of the common efflux pump substrate, EB, has been the primary means by which the activity of bacterial efflux pumps has been assessed by most studies (Beyer *et al*., 2000; Bolhuis *et al*., 1996; Giraud *et al*., 2000; Kaatz *et al*., 2002, 2003; Kern *et al*., 2006; Paulsen *et al*., 1996). Because efflux pumps are temperature dependent one could predict that the extrusion of EB would be reduced by decreasing the temperature in which a stationary culture is maintained. The results obtained in this study show exactly this – namely, that the fluorescence manifested by this agent, associated with a colony or confluent bacterial mass maintained at 37ºC, is evident at a given concentration of EB. The simple transfer to 4ºC will markedly reduce the concentration of EB that produces similar fluorescence. The manifestation of fluorescence at a lower concentration of EB produced at 4ºC must be the result of retention of this compound as opposed to an increase in the diffusion into the bacterial cell, since the rate of diffusion at 4ºC would be much lower than that taking place at 37ºC. The viability of cells cultured on agar containing concentrations of EB associated with the onset of fluorescence has been evaluated with the aid of colony forming units (CFU) when colonies showing fluorescence at minimal concentrations of EB were transferred to saline, diluted and aliquots then cultured in TSB or LB, depending on the species of bacteria under study, and evaluated at intervals by the use of the CFU method, they were found to have growth rates indistinguishable from those of controls pre-grown in EB-free broth (data not shown).

The results presented in this study demonstrate that the degree of efflux pump activity between strains of bacteria, as well as species, can easily be assessed by the use of the EB-agar method requiring no specialized instrumentation. The method affords a level of precision that may, after extensive study, provide the means by which efflux pump activity can be evaluated by a consortium of clinical laboratories. If the method progresses to that point, it may find its way into the clinical laboratory and provide the clinician with an additional laboratory tool that identifies the source of MDR of a given bacterial clinical isolate.

The method may prove extremely useful when introducing plasmids that contain individual genes that code for distinct components of efflux pumps into bacteria. It would be expected that when these genes are expressed, the colonies on the agar surface would exhibit fluorescence at significantly higher concentrations of EB than those colonies not having the plasmid-carrying gene (uninfected colonies). If the resolution of this discrimination is sufficiently high, then one may expect that the method may allow the use of plasmids that do not contain antibiotic-resistant genes, required by other methods for the selection of plasmid-carrying efflux pump colonies. The ability to use plasmids that are free from antibiotic resistant marker genes means that larger genes may be introduced without having to increase the size of the plasmid. Moreover, the smaller the plasmid used, the greater its retention by the bacterium, hence, the higher the efficiency of recovery.

The advantages provided by the method transcend its simplicity. Firstly, an agar culture containing a mixture of colonies that differ with respect to the degree of efflux pumps expressed can be replicate plated to agars containing increasing amounts of EB. This affords easy identification, quantification and isolation of the phenotypes exhibited. Secondly, the replicate cultures themselves can then be transferred to agar containing a more limited range of EB concentrations in combination with concentrations of an agent that is being examined for the inhibition or activation of efflux pump(s). The applications of this method can be further extended to the rapid characterization of bacteria harboring plasmids containing genes that code for distinct components of specific efflux pumps, their regulators and sensors. Variations of the replicate plating can also be tested, in order to evaluate the possible automation of the method, adapting it to microwell-plate technology. The method is currently being used by members of the Unit of Mycobacteriology for the assessment of over-expressed efflux pump systems of mycobacteria as well as those of *S. aureus* that have been made extremely resistant to EB (MIC increased from 5 to over 100 mg/L of EB) (Couto *et al*., 2008, submitted).

Lastly, this method can be easily adapted for hightroughput screening of compounds with inhibitory activity againt efflux pumps of bacteria. These can later be tested for the enhancement of the killing activity of the infected macrophage, along the same lines previously described, in order to detect new antimicrobial agents.

CHAPTER VIII.

The future of chemotherapy

This chapter contains data published in:

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CHAPTER OUTLINE

This review chapter is divided into two parts:

The first part of this chapter is focused on the discovery of new anti-microbial/mycobacterial agents that are known efflux pump inhibitors, and that can be used in the therapy of MDR bacteria, namely, MDR-TB. A new approach that uses these agents in conjunction with the classical antibiotics is analysed and described. These now termed “helper compounds” could provide the basis for a novel strategy implemented in the management and control of MDR and XDR-TB. This chapter also summarises much of my published work that has been jointly conducted with colleagues from Ireland, Denmark, Germany, Hungary, France, India and the USA, all of whom are identified in the Acknowledgement section of this Thesis.

The second part of this chapter provides: a) The rationale for my thesis and future work; b) Results from my research that are relevant to the therapy of XDR-TB and MDR-TB and, c) alternative therapy supported by my studies.

VIII.1 The future of chemotherapy: From where will new antimicrobial agents come from?

SUMMARY

Multidrug resistance in Gram-negative bacteria is now known to be primarily caused by overexpression of efflux pumps that extrude unrelated antibiotics from the periplasm or cytoplasm of the bacterium prior to their reaching their intended target. This review chapter focuses on a variety of agents that have been shown to be efflux pump inhibitors (EPIs) and which, if used as 'helper compounds' in combination with antibiotics to which the organism is initially resistant, may produce the required cure. Although not all of the EPIs may serve a helper role owing to their toxicity, they may nevertheless serve as lead compounds.

Antibiotics or Non-antibiotics

An antibiotic as defined by Webster's dictionary is a substance produced by a microorganism that destroys or inhibits the growth of another microorganism. In contrast to an antibiotic, there are medicinal compounds that are used for the therapy of non-infectious pathology and that have antimicrobial properties (Haug *et al*., 2007). For lack of a better name, these latter compounds are termed 'non-antibiotics' (Nishimura, 1986) and given their potential for the therapy of some problematic infections (Amaral *et al*., 2006; Kristiansen and Amaral, 1997), they may eventually achieve antimicrobial status. The group of 'non-antibiotics' consists of two subgroups, each with activities that are distinctly different. Those non-antibiotics that have direct antimicrobial activity make up the first group (antimicrobial non-antibiotics) (Kristiansen and Amaral, 1997). The second group consists of two subclasses, one of which alters the permeability of the microorganism to a given antibiotic (Amaral and Lorian, 1991; Dutta *et al*., 2007; Viveiros and Amaral, 2001), termed 'helper compounds', whilst the second subgroup enhances the killing activity of macrophages that have phagocytosed the microorganism (Amaral *et al*., 2007b) and may be termed 'macrophage modulators'. The purpose of this review of 'non-antibiotics as helper compounds' is to discuss the mechanisms by which some non-antibiotics have direct antibacterial activity, some assist the penetration of antibiotics by their ability to inhibit efflux of the antibiotic prior to it reaching its

intended target, others directly transform non-killer cells into effective killers of bacteria which cause infections that are problematic to manage, and lastly some of which have the ability to cure bacteria of plasmids and hence may have potential in the area of animal husbandry.

History of 'non-antibiotics'

Prior to end of the 19th century, the work of Paul Ehrlich demonstrated the antimicrobial activity of methylene blue, a dye that is the 'poster child' for a group of heterocyclic compounds, the phenothiazines. Methylene blue was also shown to obviate the mobility of microorganisms and this finding quickly spurred studies by others to see whether the dye would also affect the mobility of a mammal and indeed it did, inasmuch as intravenous (i.v.) administration of this dye produced lethargy in humans (Amaral and Kristiansen, 2001). Because the dye turned the patient blue, it could not be used for therapy of neurological disease that required restraining the patient from overactive activity. Nevertheless, interest in the dye as a potential neuroleptic was maintained for the next 50 years, ultimately resulting in the first neuroleptic non-coloured phenothiazine, CPZ (Lopez-Munoz *et al*., 2005). Interest in the antimicrobial properties of methylene blue was not sufficient to motivate its exploitation as an antimicrobial agent. However, with global use of CPZ, anecdotal reports demonstrated that patients receiving CPZ therapy could be cured of bacterial infections (Amaral and Kristiansen, 2000). Nevertheless, because the appearance of these reports took place during the 'golden age of antibiotics' (1950s to 1970s), there was little need for the use of CPZ as another antibiotic, especially since the serious side effects produced by this agent were rather frequent (Amaral *et al*., 2004). However, because antibiotic resistance, especially multidrug resistance, has become common (WHO/IUATLD, 2004), a number of investigators turned their interest to CPZ and other derived phenothiazines as potential agents against MDR organisms.

Antimicrobial activity of non-antibiotics

The *in vitro* antimicrobial activities of non-antibiotics have been widely described. However, with few exceptions the concentrations of these agents needed to inhibit *in vitro* growths are well beyond those that can be achieved clinically (Amaral and Kristiansen, 2000). Nevertheless, phenothiazines such as trimethoprim and

trimeprazine, at concentrations that are tolerated by the mouse, protect the animal from developing *Salmonella* infections (Guha *et al*., 2000). Similar protection of the mouse against infection by highly virulent *Salmonella* strains is afforded by diclofenac sodium (Dutta *et al*., 2004, 2007). Non-antibiotic phenothiazines and their derivatives have been shown to protect mice against *Escherichia coli* infection (Komatsu *et al*., 1997). However, although the above non-antibiotics protect the mouse against a Gram-negative infection, they have not yet been shown to cure a *Salmonella*-infected mouse. Nevertheless, children who present with recurrent pyelonephritis due to *E. coli* can be cured with CPZ and gentamicin, whereas therapy with the antibiotic alone results in failure (Gunics *et al*., 2000; Molnar *et al*., 1990).

Although the use of phenothiazines for therapy of Gram-negative infections is still very far from being seriously considered, phenothiazines appear to have great potential for the therapy of MDR-TB (Amaral and Kristiansen, 2000; Amaral *et al*., 2006, 2007b; Kristiansen and Amaral, 1997; Viveiros and Amaral, 2001). As is the case for *in vitro* activity against Gram-negative bacteria, a large number of phenothiazines have been shown to have *in vitro* activity against *Mycobacterium tuberculosis* (Amaral *et al*., 1996; Bate *et al*., 2007; Bettencourt *et al*., 2000; Chakrabarty *et al*., 1993; Gadre *et al*., 1998, 1999; Madrid *et al*., 2007; Ratnakar and Murthy, 1992, 1993; Ratnakar *et al*., 1995; Reddy *et al*., 1996; Yano *et al*., 2006). However, the concentrations of phenothiazines that inhibit *in vitro* growth cannot be achieved clinically (Amaral *et al*., 2004). Nevertheless, because phenothiazines are concentrated as much as 100-fold by macrophages (Daniel and Wojcikowski, 1999b; Ordway *et al*., 2003b) and *M. tuberculosis* is an intracellular infection of the human macrophage (Saunders and Britton, 2007); phenothiazines have been studied for activity against intracellular mycobacteria. These studies have shown that killing of phagocytosed *M. tuberculosis* by non-killing macrophages is enhanced by concentrations of the phenothiazine that are well below those employed for the therapy of psychoses (Crowle *et al*., 1992; Martins *et al*., 2007b; Ordway *et al*., 2003b; Reddy *et al*., 1996) (discussed on **Chapter IV**). It is important to note that whereas many compounds have been shown to have in vitro activity, few of these penetrate the macrophage and retain activity against intracellular mycobacteria. With respect to the phenothiazines, because these compounds are concentrated many-fold by the macrophage, it is probable that any phenothiazine will have intracellular antimycobacterial activity. In line with the demonstration that

phenothiazines promote the killing of intracellular mycobacteria, mice infected with *M. tuberculosis* have been cured of this infection when treated with thioridazine (Martins *et al*., 2007a) (**Chapter V**) and with analogues of CPZ (Weinstein *et al*., 2005). Because a given phenothiazine has the same activity against *M. tuberculosis* regardless of its resistance to one, two or more antibiotics (Amaral *et al*., 1996), phenothiazines should be seriously considered for the therapy of MDR-TB (Amaral and Kristiansen, 2000; Amaral *et al*., 2006, 2007b) and extensively drug-resistant (XDR)-TB (Amaral *et al*., 2007c). However, because some phenothiazines such as CPZ produce a plethora of serious side effects, only those phenothiazines that are relatively free of serious effects are to be considered as candidates for the therapy of MDR-TB (Amaral and Kristiansen, 2000; Amaral *et al*., 1996, 2006, 2007b; Kristiansen and Amaral, 1997).

Mechanism of action by which phenothiazines express *in vitro* and *ex vivo* activity

In vitro

Phenothiazines inhibit transport of calcium (Ca^{2+}) by preventing its binding to Ca^{2+}-binding proteins such as calmodulin (CaM) (Weiss *et al*., 1980). This means that enzyme systems which are dependent upon Ca^{2+}, such as those involved in generating cellular energy from hydrolysis of ATP, are inhibited (Francis *et al*., 2002; Garcia *et al*., 1995). Among these phenothiazine-sensitive systems are transporters that extrude from the cell a variety of noxious agents and hence protect the cell from these agents. All bacteria studied to date contain a variety of transporters that intrinsically recognise noxious agents, such as antimicrobial agents and detergents, and extrude these agents from the periplasmic space of the cell envelope (Piddock, 2006b). When these transporters are overexpressed, the bacterium becomes resistant to a variety of unrelated antibiotics, hence their MDR phenotypes. Phenothiazines have been shown to reverse MDR phenotypes of bacteria and therefore render these bacteria susceptible to antibiotics to which they were initially resistant (Amaral *et al*., 2000; Kawase and Motohashi, 2003; Kristiansen *et al*., 2003, 2006; Kristiansen *et al*., 2007; Michalak *et al*., 2006). Because multidrug resistance is due to the overexpression of efflux pumps that are the cause of MDR bacteria (Piddock, 2006a) and these efflux systems are driven by energy provided by the proton-motive force which is dependent upon Ca^{2+}-dependent enzyme systems (Molnar *et al*., 1997; Plishker, 1984), the inhibition of Ca^{2+} binding to Ca^{2+}-dependent enzymes will render the bacterium susceptible to that which

they were initially resistant (Bhatnagar and Singh, 2003). Therefore, it is not surprising to note that phenothiazines reverse MDR phenotypes of bacteria. Inhibition of intrinsic efflux of a noxious agent such as a phenothiazine is believed to result in greater permeability to other noxious agents, including the phenothiazine itself. Increased permeability ensures that phenothiazine molecules eventually reach the DNA, intercalate between the bases (Rohs and Sklenar, 2004), inhibit all DNA-based processes (Dastidar *et al*., 2000; Ratnakar and Murthy, 1993) and hence inhibit replication. The *in vitro* concentrations of the phenothiazine that inhibit replication of the bacterium are generally many hundred-fold greater than that which can be achieved in the patient (Mengozzi *et al*., 1994).

Ex vivo

In contrast to the *in vitro* activity, the enhancement of killing of intracellular bacteria promoted by a phenothiazine has generally been considered to be due to concentration of the phenothiazine to a level that is compatible with the *in vitro* concentration that inhibits replication of the bacterium (Amaral *et al*., 2007b; Crowle *et al*., 1992; Ordway *et al*., 2003b). However, because phenothiazines can inhibit any transport process, including those for K^+ and Ca^{2+} (Chukhlova *et al*., 1984; Kim and Kim, 2005), and these ions are essential for acidification of the phagolysosome and subsequent activation of its hydrolases (Pillay *et al*., 2002), the possibility that enhanced killing is not due to a concentration effect but is related to the inhibition of K^+ and Ca^{2+} transport processes has been considered and investigated (Wittekindt *et al*., 2006) (**Chapter VI**). Although the concentrating effect cannot be ruled out, recent experiments have shown that common inhibitors of K^+ and Ca^{2+} transport enhance the killing of intracellular bacteria and support the contention that phenothiazines enhance killing by the same mechanism (Ahluwalia *et al*., 2004).

Efflux pump inhibitors (EPIs) of MDR bacteria

Antibiotic-sensitive bacteria that are gradually exposed to increasing concentrations of a given antibiotic develop increasing resistance to that antibiotic (Martins A *et al*., 2007; Viveiros *et al*., 2005a). Accompanying this induced resistance are increases in resistance to other unrelated antibiotics (Martins A *et al*., 2007; Viveiros *et al*., 2007). This induced resistance can be reversed with the transfer of the bacterium to a drug-free

medium or by exposure of the bacterium to an EPI such as phenylalanine arginyl β-naphthylamide (PAβN), phenothiazines such as thioridazine and CPZ, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and reserpine (Guillier *et al*., 2006; Kristiansen *et al*., 2006). These studies suggest that exposure to an antibiotic at concentrations that do not completely inhibit replication of the bacterium serve to induce the bacterium to withstand the action of the antibiotic even though its concentration has been increased (Bamberger *et al*., 1997). Development of a MDR phenotype suggests that the development of multidrug resistance in Gram-negative bacteria in patients treated with subinhibitory doses of the antibiotic occurs via the same mechanism.

Nevertheless, the above studies only demonstrate an association of induced MDR phenotypes with the development of an overexpressed putative efflux pump system. Recent studies (Viveiros *et al*., 2007) have demonstrated that exposure of *E. coli* to increasing concentrations of tetracycline (TET) results in significantly increased activity of genes that regulate genes coding for transporters of the resistance-nodulation-cell division (RND) superfamily efflux pumps (Elkins and Nikaido, 2003). The stress genes *soxS* and *rob* are increased when the organism is first exposed to TET. This is followed by increased activity of *marA*, *marB* and *marR*, which remain increased in activity during prolonged exposure to increasing concentrations of TET, whereas those of *soxS* and *rob* decrease to levels that approximate to that of unexposed controls. Together with the increased activity of regulator genes, there are increases in the activity of genes that code for the two main transporters, AcrB and YhiV, which remain at their highest level while the organism remains cultured in the presence of high concentrations of TET. Interestingly, the response to an antibiotic is not limited to the activation of efflux pump genes. *micF*, a gene that downregulates the post-transcription of porins (Guillier *et al*., 2006), is increased, as well as *ompX*, a gene that is considered to be a downregulator of porins by interfering with their assembly (Viveiros *et al*., 2007). Although inducement of TET resistance does not significantly alter the activity of *ompF* and *ompC* genes, the amounts of the outer membrane proteins OmpF and OmpC are decreased, possibly due to the increased activity of genes that code for proteases which degrade these outer membrane proteins prior to their assembly into the tri-barrel porin (Viveiros *et al*., 2005a). However, we cannot at this time rule out the possibility that downregulation of type F porin is due to the increased activity of the *micF* gene as noted (Viveiros *et al*., 2007). This gene produces a short anti-sense mRNA that binds to the tail end of the

OmpF mRNA, thereby preventing translation of OmpF mRNA. Hence, increase of *micF* activity results in less OmpF protein (Chen *et al*., 2004). It may be concluded that MDR phenotypic resistance of a Gram-negative bacterium results from treatment of a patient with subinhibitory doses of an antibiotic and that this MDR phenotype involves overexpression of efflux pumps and downregulation of porins, both systems working together to reduce the permeability of the organism to that antibiotic as well as to other unrelated antibiotics. Because the MDR efflux pump can be inhibited by a variety of agents, the use of EPIs as helper compounds to antibiotics to which the bacterium is initially resistant has become a focus of investigation.

The search for EPIs

Enterobacter aerogenes and *Klebsiella pneumoniae* are among the commonest Gram-negative bacteria involved in nosocomial respiratory and urinary tract infections. These bacteria exhibit a marked decrease in antibiotic susceptibility. In various clinical isolates, the MDR phenotype is strongly associated with a marked decrease in the synthesis of non-specific porins and the overproduction of active drug efflux systems. These modifications of envelope permeability contribute to a high level of resistance for structurally unrelated molecules such as β-lactams, quinolones, macrolides, tetracyclines and chloramphenicol (Piddock, 2006a; Poole, 2005).

Today, one challenge is to synthesise and characterise new molecules that are capable of circumventing efflux activity and restoring the internal concentration of common antibiotics that are substrates of efflux pumps. In addition, these compounds must be devoid of any intrinsic antibacterial activity at the concentration used (Viveiros *et al*., 2007). *Enterobacter aerogenes* and *K. pneumoniae* clinical isolates that exhibit a MDR phenotype and active efflux mechanisms have been used to test several quinoline derivative molecules as chemosensitisers or EPIs. This group of molecules has been selected due to their structural homology with the quinolones and with attention to previous results obtained with resistant microbes (Mahamoud *et al*., 2006). The respective minimum inhibitory concentrations (MICs) of these quinoline compounds are quite similar to those obtained with the commercially available EPI PAβN (MC-207, 110), which is the first inhibitor previously developed against *Pseudomonas aeruginosa* (Lomovskaya and Bostian, 2006). PAβN is currently used to detect efflux pump

activities in various Gram-negative bacteria, including resistant *E. aerogenes* and *K. pneumoniae* strains (Lomovskaya and Bostian, 2006; Pagès *et al*., 2005). Of the quinoline derivatives that have been tested for their capability to decrease resistance, certain of them (belonging to alkoxyquinoline, alkylaminoquinoline, thioalkoxyquinoline and chloroquinoline subclasses) are efficient chemosensitisers of chloramphenicol activity, with an 8- and 32-fold decrease in the chloramphenicol MIC in resistant strains. In addition, they significantly increase the intracellular accumulation of chloramphenicol or norfloxacin in resistant bacteria that exhibit an overproduction of efflux pumps, especially the AcrB pump in *E. aerogenes* and *K. pneumoniae*. The subsequent increase of intracellular drug concentration reached a level similar to that obtained with an energy poison which disrupts the inner membrane proton gradient required for the activity of the efflux pump, indicating a clear effect on the efflux mechanism (Pagès *et al*., 2005). Moreover, some of these compounds were also able to restore partially norfloxacin and tetracycline susceptibility of resistant clinical isolates and of a resistant variant strain (Mahamoud *et al*., 2006, 2007).

The variation obtained in the level of restoration of susceptibility conferred by the quinoline derivatives to antibiotic classes could be attributed to the differences in the pump sites involved in drug transport. It is possible that EPIs may have specific effects on drug transport mechanisms depending on their own affinity for specific residues located inside the pump cavity (affinity site). There are two possibilities that generate a collapse in drug transport. First, interactions resulting in the pump–inhibitor complex may saturate all the drug affinity sites if the antibiotic and the inhibitor have equal affinity for the same sites. Alternatively, inhibitor–pump interactions may induce steric hindrance if the respective binding sites for antibiotic and inhibitor on the pump are in close proximity (Mahamoud *et al*., 2007). It has been hypothesised previously that quinoline molecules may act as competitive inhibitors of the antibiotic flux that takes place inside the transporter, e.g. AcrB pump (Pagès *et al*., 2005). This proposal has been supported by some preliminary results showing a relationship between the dose of quinoline compound and the level of intracellular drug concentration reached during the incubation time. However, at the moment no direct and clear assay is available to measure and characterise the extrusion of intracellular antibiotic through transporter channels present in resistant isolates. Owing to the presence of active efflux mechanisms in various bacteria and the increase of resistant phenotypes, it is necessary

to develop an efficient assay to decipher the molecular parameters of transporters (Pagès *et al*., 2005) (**Chapter VII**). This assay will be very useful for the determination of specificity, kinetic constants for various drugs and efficiency of EPIs. In conjunction with molecular modelling using crystallisation data (Higgins, 2007; Lomovskaya *et al*., 2007), these kinetic data will yield great improvement in the design of future efflux inhibitors. With the recent determination of several three-dimensional (3D) structures of efflux transporters (Higgins, 2007; Murakami *et al*., 2006; Seeger *et al*., 2006), various models proposed for efflux activity and results obtained with current inhibitors, it seems reasonable to propose that this approach regarding 'pump–antibiotic–inhibitor' interactions (Mahamoud *et al*., 2007) will be used to improve the design of the new generation of EPIs.

Anti-plasmid activities of non-antibiotics

Antibiotic resistance is based on a complex of multifactorial processes in which the transfer of mobile genetic elements encoding resistance further aggravates the spread of resistant bacterial strains. Moreover, the use of antimicrobial drugs in clinical and veterinary medicine is a recognised driving force for the selection of resistant bacteria. This selective pressure is a major contributor to the emergence and evolution of MDR phenotypic bacteria. Bacterial plasmids play a major role in these processes with their ability to be replicated independently from the chromosome and be transferred by conjugation from a host cell to a recipient bacterium. Bacterial resistance constitutes a considerable therapeutic and economic burden, requiring new therapeutic approaches to overcome. Plasmid-mediated bacterial resistance may be tackled by curing of these resistance-carrying genetic elements (Spengler *et al*., 2003).

For plasmid curing, replication should be inhibited at three different levels simultaneously: the intracellular replication of plasmid DNA; partition; and intercellular transconjugal transfer. Various methods involving chemical and physical agents have been tested for this purpose and have revealed that super-optimal temperature, DNA intercalators (ethidium bromide, acridine orange, acriflavine or surface-active compounds, e.g. sodium dodecyl sulphate) are potent plasmid eliminators (Mándi *et al*., 1976). Based on these findings, Molnar and colleagues carried out a systematic assay to determine the structure–activity relationship among tricyclic antipsychotic drugs that

interfere with the genetic elements encoding resistance (Mándi *et al*., 1976; Molnár *et al*., 1975, 1980, 2003). Several members of this drug group and their derivatives, synthesised according to computer-aided drug design, exerted anti-plasmid activity. The antidepressants possessing a secondary amine side chain are more effective in inhibiting bacterial growth and in eliminating plasmids than are the drugs with tertiary amine side chains, whereas the plasmid-eliminating potency of quaternary amines is weaker as they are probably unable to penetrate the cell membrane. Inhibition of plasmid replication resulted in a single nick outside the replication origin of the superhelical structure. The process leads to further relaxation of the plasmid DNA. Intercalation of the compounds was proved by the increase in the melting point of DNA and by circular dichroism measurements (Barabas and Molnar, 1980; Nikaido, 2001). When the native plasmid DNA and its promethazine complex were analysed by agarose gel electrophoresis, the superhelical form was missing from the promethazine-treated plasmid DNA. The ratio of open circular and linear forms of plasmid DNA increased in promethazine-treated samples.

It has been proposed that the HOMO orbital energy, the conjugated π-electron system of the tricyclic skeleton, the symmetric π-electron distribution to the L-molecular region, and the superdelocalisability of the π-electron system on 10, 12, 13 atoms have special importance in anti-plasmid activity. In a computer-aided structure–activity relationship study, some correlations were found between anti-plasmid effect and the symmetry of the HOMO orbitals of phenothiazines and related compounds. The correlation coefficient was as high as 0.97 when the electrophilic superdelocalisability of the heavy atoms of the tricyclics on atoms C8, C9b and N10 were taken into consideration. In this way, the anti-plasmid and carcinogenic molecular orbitals were clearly differentiated (Amaral *et al*., 2004; Molnar *et al*., 1992b; Motohashi *et al*., 1992, 1999; Spengler *et al*., 2006; Tanaka *et al*., 1997). A possible mechanism of action is a complex formation of tricyclics with the guanine–cytosine-rich regions of the plasmid DNA, which are necessary for normal plasmid replication in an uncomplexed form (Miskolci *et al*., 2000). The use of phenothiazine drugs as resistance modifiers is restricted owing to their toxicity, because the concentrations required for anti-plasmid effects are beyond that which may be clinically achievable; therefore, only limited data are available regarding their resistance-modifying activity *in vivo*. Promethazine was studied in

children with frequently recurring pyelonephritis, in combination with gentamicin. Results were positive; the combination reduced the number of recurrences of urinary tract infections compared with the control group (Molnar *et al*., 1990). In another *in vivo* study, some patients recovered from urogenital infections despite the fact that plasmid elimination in the urine did not occur and the causative agents were resistant to gentamicin. These findings suggest that promethazine may affect the specific pili-mediated and plasmid-encoded adhesion or multiplication of bacteria on epithelial cells (Kásler *et al*., 1982). Promethazine and imipramine were investigated in the inhibition of adhesion of nephropathogenic *E. coli* strains in tissue culture using scanning electron microscopy. It was found that in addition to the direct antibacterial effect, it can be presumed that a low concentration of promethazine and imipramine can inhibit the reversible and irreversible attachment of bacteria to epithelial cells, since both drugs interfere with the function of the microfilaments of cells and bacteria via membrane effects (Hirota, 1960; Molnár *et al*., 1983, 1992b).

VIII.2 MDR- and XDR-TB; Rationale for alternative therapy; Recommended therapy based on the results obtained from Thesis Research

Global rates of pulmonary tuberculosis (TB) continue to increase. Moreover, resistance of the causative organism *Mycobacterium tuberculosis* to the two most effective anti-TB medications continue to rise. Now, multi-drug resistant TB (MDR-TB) has progressed to extensively drug resistant TB (XDR-TB) - a *M. tuberculosis* organism that is resistant the most effective second line drugs, and most commonly resistant to all of the antibiotics available. This work provides detailed, significant evidence that supports the use of an old neuroleptic compound, thioridazine (TZ), for the management of MDR-TB and XDR-TB infections (**Chapters IV, V and VI**). The argument has been previously presented but no one seems to be listening - and the disease continues unabated when there is a very good probability that the suggested drug will prove to be effective. When the prognosis is poor, available therapy predictably ineffective and death is inevitable, compassionate therapy with TZ should be contemplated. The risks are small and the rewards great.

Tuberculosis causes of morbidity and mortality worldwide and was declared by the World Health Organization a global emergency: over 2 billion infections worldwide, 9

million new cases and over 2 million TB deaths in 2004 (WHO, 2007a). Multidrug resistant TB (MDR-TB) is caused by *Mycobacterium tuberculosis* resistant to the two most potent first-line drugs; Rifampin (RIF) and Isoniazid (INH). MDR-TB not detected or managed properly can develop extensively drug-resistant TB (XDR-TB-resistant to RIF, INH, any fluoroquinolone and 1 of 3 injectable second line drugs (capreomycin, kanamycin and amikacin) (WHO, 2006b, 2007a). The danger from MDR and XDR-TB cannot be overstressed. XDR-TB represents a major threat to public health worldwide, as many of these strains may become virtually untreatable by any drug available. For that reason the WHO Global Task Force on XDR-TB stressed the need for research in the development of new anti-TB drugs and therapeutic strategies (WHO, 2006b).

Phenothiazines

Phenothiazines are heterocyclic compounds from which over 95% of all medicinal compounds ever used have been derived (Mosnaim *et al*., 2006). With respect to their antimicrobial properties, phenothiazines have been shown to have direct and indirect activities against a large gamut of bacteria (Amaral *et al*., 2006; Kristiansen and Amaral, 1997). Direct activity has been shown to be due to their reaching a large number of targets (Amaral and Kristiansen, 2001). Phenothiazines such as thioridazine (TZ) inhibit the binding of calcium to calcium binding proteins, and therefore indirectly inhibit calcium dependent enzymes that are involved in the hydrolysis of ATP (Amaral *et al*., 2007a). Phenothiazines also inhibit efflux pumps of multi-drug resistant (MDR) bacteria that extrude diverse and unrelated antibiotics prior to their reaching their targets (Viveiros *et al*., 2005a). However, because these *in vitro* activities take place with concentrations of the agent that are clinically irrelevant - that is, at concentrations that are many fold higher than those that can be achieved clinically, the use of these agents for the therapy of bacterial infections is not recommended. However, with respect to TB, the situation is quite different.

Tuberculosis - the disease

Pulmonary TB is an intracellular infection caused by the steadfast human bacterial pathogen *Mycobacterium tuberculosis.* Because infection is almost always silent, that is,

if symptoms are present they are sub-clinical, the infection goes unnoticed. Nevertheless, only about 5 to 10 % of all infections progress to active disease - that is, the organism has broken free of its intracellular prison (the alveolar macrophage) and can now be retrieved in the sputum of the patient. Interestingly, the distinction of infection from active disease is not always understood by physicians and hence there is some confusion between infection and active disease. Active disease is the infectious phase of TB and it is the phase of the disease which is recognised symptomatically. It is recognised symptomatically because a good part of the world's people are immunised with the Bacilli of Calmétte-Guérin (BCG) and therefore the diagnostic power of the simple purified protein derivative (PPD) test (also known as Mantoux test) that can readily, in the absence of BCG vaccination, detect a *M. tuberculosis* infection is obviated by the immune response due to vaccination alone. The quantitative Mantoux test that was once thought to distinguish by the size of induration a new *M. tuberculosis* infection from the immune response due to BCG vaccination has been shown to be unreliable and significantly affected by underlying pathology (Nagelkerke *et al*., 2001; Ponce de León *et al*., 2005).

Therapy of Active Disease

The therapy of antibiotic susceptible active disease with the two most effective anti-TB antibiotics isoniazid (INH) and rifampin (RIF) is highly effective when therapy is conducted at the correct dose, interval and duration. However, when the guidelines for therapy are not followed or when the patient is non-compliant, that is, fails to take the medication as instructed, the opportunity for the selection of a spontaneous mutation that renders one of the antibiotics totally ineffective, takes place. This patient can, as a consequence of the infectious nature of active TB disease, infect others. In turn, and by chance, if the newly infected patient progresses to active disease, is treated ineffectively with INH and RIF, or is non-compliant, the opportunity for the selection of a second mutation against the other antibiotic arises. The strain of *M. tuberculosis* is now considered to be MDR and it is known as MDR-TB. But the story does not end here since the resistance of MDR-TB has progressed to 5 or more antibiotics, and some of these strains are so highly virulent, extensively drug resistant TB (XDR-TB), that they have caused a panic to the extent that when such an XDR-TB infection is recognised, therapy with second line of defence drugs has been recommended world-wide (WHO,

2006b). Unfortunately, the frequency of resistance to each of these second lines of defence drugs is many folds higher than that for INH and RIF (Balabanova *et al*., 2005). Given the fact that when a patient that is infected with MDR-TB and co-infected with HIV and has progressed to advanced HIV infection or presents with full-blown AIDS, mortality within one year of diagnosis is certain regardless of therapy (Amaral *et al*., 2007c), some consideration for an alternative form of therapy should be made, if only along the lines of compassionate therapy (Amaral *et al*., 2004).

The Basis for Recommended Alternative Therapy of XDR-TB/MDR-TB

The activity of phenothiazines against antibiotic susceptible *M. tuberculosis* was first noticed soon after the first neuroleptic chlorpromazine (CPZ) was employed for the therapy of psychosis and patients infected with this organism were cured of the infection (Amaral *et al*., 2001b). However, because these anecdotal reports took place during the Golden Age of antibiotics, and because the therapy of active TB had been so successful that many believed the infection would eventually be eliminated, much as was believed to be the case for polio, there was no interest in developing CPZ for anti-TB use. Moreover, the global use of CPZ demonstrated that this compound produced very serious and frequent side effects (Amaral *et al*., 2001a). However, with the advent of the 1990's, a resurgence of pulmonary TB was noticed, at first quite slowly as was the case for New York City in 1992 when it was discovered that the rates of new cases of pulmonary TB had more than doubled during the previous decade (Munsiff *et al*., 2006) and of greater significance and importance, the large majority of these cases were resistant to one or more antibiotics (Munsiff *et al*., 2003). However late, this terrible news promoted the United States Centres for Disease Control and Prevention (CDC) in conjunction with the American Thoracic Society to establish a set of therapeutic guidelines, which when fully implemented and monitored by the City and State of New York, quickly reduced the rate of new cases to an all-time low (Munsiff *et al*., 2002). The TB and MDR-TB situation for most of the globe, however, continued to escalate although there are indications that the problem has begun to level off (WHO, 2007a). The once thought effective armamentarium of anti-TB drugs was and is inadequate for the management of XDR-TB/MDR-TB. Moreover, because much of TB and antibiotic resistant infections take place in disadvantaged countries that cannot even afford conventional drugs, there is little incentive for the development of new and effective

anti-TB compounds (Millet, 2006). As of this time, there are no new effective anti-TB drugs. And this will remain for the foreseeable future given the almost total absence of clinical trials with new agents. And all of this is understandable given the fact that the cost of delivering an anti-TB drug from the bench to the bedside exceeds half a billion US dollars (Wishart, 2007). Although, as previously stated, CPZ was not considered for the development of anti-TB drugs, interest in this compound remained. CPZ was shown to have *in vitro* activity against antibiotic susceptible *M. tuberculosis* (Amaral *et al*., 1996; Bettencourt *et al*., 2000; Kristiansen and Vergmann, 1986; Molnar *et al*., 1977; Ratnakar *et al*., 1995; Viveiros and Amaral, 2001) and against antibiotic mono and poly resistant strains (Amaral *et al*., 1996; Bettencourt *et al*., 2000; Viveiros and Amaral, 2001), against MDR-TB and XDR-TB (Amaral *et al*., 1996; Viveiros and Amaral, 2001). Because CPZ has many serious and frequent side effects, other phenothiazines were studied for activity against antibiotic susceptible (Amaral *et al*., 1996; Bettencourt *et al*., 2000; Viveiros and Amaral, 2001) and antibiotic resistant strains, including MDR-TB (Viveiros and Amaral, 2001). Among these phenothiazines, thioridazine (TZ), a derivative of CPZ, was shown to be as effective as CPZ *in vitro* (Amaral *et al*., 1996; Bettencourt *et al*., 2000; Viveiros and Amaral, 2001) and because TZ had fewer serious side effects than CPZ, TZ was intensively studied for its potential as an anti-XDR-TB/MDR-TB agent.

Pulmonary TB is an intracellular infection. This means that if a drug is to be effective against this bacterium it must have activity where the bacterium resides-namely, the macrophage component of the alveolus of the lung. Hundreds of compounds have been shown to have *in vitro* activity against *M. tuberculosis* but only a precious few are active against intracellular *M. tuberculosis*. Among the most active compounds is CPZ, first shown to have activity against intracellular *M. tuberculosis* by Crowle and his group in 1992 (Crowle *et al*., 1992). The significance of this finding was that the intracellular concentration of the medium that was required to promote complete killing was within clinical reach. This finding was confirmed 10 years later by Amaral and his international group (Ordway *et al*., 2002b) with the demonstration that a concentration of CPZ that is lower than that clinically employed for chronic therapy of psychosis promoted complete killing of *M. tuberculosis* and MDR-TB within 3 days of culture (**Chapter IV**). Moreover, TZ, the much milder drug (Ordway *et al*., 2003b) as well as three of its derivatives (Martins *et al*., 2007b), had intracellular activities that were equal

to or even greater than that promoted by CPZ (**Chapter IV**). Finally, TZ was shown to cure the mouse of a TB infection caused by the intraperitoneal introduction of over 1 million *M. tuberculosis* colony forming units (Martins *et al*., 2007a) - a massive dose compared to one that causes infection in man (**Chapter V**).

Mechanism of action of TZ enhanced intracellular killing of *M. tuberculosis* by non-killing human macrophages

Phenothiazines are concentrated by tissues rich in macrophages that contain lysosomes (Amaral *et al*., 2004, 2007c; Viveiros *et al*., 2005c). At first, the ability of the macrophage to concentrate CPZ or TZ to levels compatible with the *in vitro* inhibition of replication of *M. tuberculosis* was considered to be the mechanism by which enhanced killing of intracellular *M. tuberculosis* by non-killing macrophages took place (Ordway *et al*., 2003b). This assumption was further strengthened with electron microscopy studies that showed that the *in vitro* effects of TZ on the ultrastructure of *S. aureus* could be reproduced when the organism had been phagocytosed by non-killing human macrophages (Martins *et al*., 2004) (**Chapter III**). However, i) killing of intracellular bacteria has been shown to be dependent upon the availability of K^+ needed for the acidification of the phagolysosome and subsequent activation of hydrolases that degrade the bacterium and cause its death (Ahluwalia *et al*., 2004; Reeves *et al*., 2002); ii) TZ is an inhibitor of Ca^{2+} and K^+ transport (Eilam, 1983; Landmark *et al*., 1972) and hence enhanced killing could be due to the ability of TZ to insure adequate levels of K^+, within the phagolysosome, required for the killing. Because common inhibitors of K^+ transport that do not have *in vitro* activity against *M. tuberculosis* have been shown to enhance the killing of intracellular *M. tuberculosis* (Martins *et al*., 2008), it seems highly plausible that enhanced killing by TZ takes place by the same K^+ dependent mechanism (Amaral *et al*., 2007b) (**Chapter VI**). Further studies are needed for complete elucidation of the role of TZ and K^+ in enhanced killing of intracellular *M. tuberculosis*.

The Fly in the ointment - TZ cause arrhythmia/increase of QTc interval

Neuroleptics, typical or atypical, have been shown to cause QTc prolongation in humans (Pacher and Kecskemeti, 2004) that is augmented with metabolic inhibitors

(Harrigan *et al*., 2004). However, due to the association of sudden death-torsade de pointes with chronic administration of TZ, caution has been recommended, and in the United Kingdom, special legislation governing the use of TZ has been implemented. Given this information, it is quite natural to resist the use of TZ even for the therapy of psychosis. However, by far, TZ has been for the past 40 years the most used neuroleptic, and the most serious side effect of sudden death is quite rare. Moreover, recent evidence has been provided that QTc prolongation takes place only in patients with a CYP2D6 genotype (LLerena *et al*., 2002). Moreover, the dosages administered to patients whose QTc is prolonged is quite high (300 mg) and not related to dosages employed for the early management of psychosis. However, QTc prolongation with a single dose of 50 mg of TZ has been reported (Thanacoody *et al*., 2007). Nevertheless, there is no data available that supports QTc prolongation with early management doses and the single dose study may not reflect patient adjustment to the drug during chronic administration. Evidence that TZ does not produce significant danger to the vast majority of patients who are free of cardiopathy has recently been put forth by Thanacoody (Thanacoody, 2007).

The essential question - to use or not to use TZ for the therapy of XDR-TB/MDR-TB infections with poor prognosis

When faced with a patient who is infected with MDR-TB or XDR-TB, and is co-infected with HIV or presents with advanced HIV or full blown AIDS, the question that the physician must answer is: What can I do for the patient that will extend his life, improve the quality of life, or perhaps even cure the patient of this deadly infection? Given the limitations of what is available to the physician, it is clear that an alternative form of therapy which is solidly based on science and which will do no harm may be preferred to the use of therapy that will yield predictable ineffectiveness. The question is a tough one? Perhaps, the question may be asked to the physician - given the same conditions, how he would treat himself. If the physician can answer this question, his selection of alternative therapy with TZ, if only for compassionate reasons, may be reached.

CHAPTER IX.

The Tuberculosis 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

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CHAPTER OUTLINE

The results from my Thesis Research up to this point have been focused on the development of new drugs that provide a potentially effective alternative for the therapy of XDR-TB/MDR-TB. As a consequence of the methods developed and applied for the study of mode of action of some of the compounds studied, the consistency of the data and its relevance to the clinical Mycobacteriology laboratory, have together provided the basis for the creation of the Tuberculosis laboratory of the future. Because of the nature of this Chapter, some of the data presented earlier will again be presented in order to facilitate the concepts that are being developed. Therefore, this chapter is focused on the **Multi- and Extensively-drug resistance** and the rapid identification of these strains**.** MDR-TB has progressed to extensively drug resistant *M. tuberculosis* (XDR-TB), defined by resistance to INH, RIF, any fluoroquinolone and at least 1 of the 3 injectable second line drugs. The TB laboratory of the future must identify rapidly antibiotic resistant TB (MDR-TB/XDR-TB). The identification of an MDR-TB infection could be accomplished within one day of receiving the clinical specimen by the application of The Faster TB Track Programme, as previously reported. However, the rationale for evaluating the intracellular killing activity of anti-tubercular agents against *M. tuberculosis* isolated from the patient's sputum and its subsequent phagocytosis by macrophages isolated from that same patient is an important subject that deserves attention. By this manner, the pulmonary TB laboratory is essential if control of TB is to be achieved. The TB laboratory of the future must assess second line of defence drugs for activity against the MDR-TB/XDR-TB strain isolate after it is phagocytosed by the patients own macrophage. The human non-killing macrophage can be easily prepared from the MDR-TB/XDR-TB infected patient's peripheral blood prior to the patient receiving any form of therapy. This is important in the evaluation of drugs for enhanced killing of intracellular *S. aureus*, which can be used as a model. Therefore, compounds that enhance the killing of intracellular *S. aureus* are usually effective against intracellular mycobacteria. The demonstration of degrees of killing activity promoted by drugs against *S. aureus* and *M. tuberculosis* involve drugs that enhance killing of intracellular *S. aureus* to a greater extent than TZ (and that can then be selected for study against intracellular *M. tuberculosis*). TZ can be used as the positive control for the demonstration of enhanced killing of intracellular bacteria. Drugs which are therefore shown to enhance killing of the phagocytosed MDR-TB/XDR-TB strain

have a high probability of achieving therapeutic success. This therapeutic strategy will be focused on the patient itself involving an individualized approach. If these approaches are to be implemented the TB laboratory of the future can be a reality today.

SUMMARY

Therapy of MDR-TB is highly problematic; that of XDR-TB even more so. Both infections result in high mortality, especially if the patient is co-infected with HIV or presents with AIDS. Selection of therapy for these infections is limited, and for most situations, it is blind. But there is a solution for the selection of effective therapy and this is presented herein. Ideal therapy of the patient infected with MDR-TB or XDR-TB can be determined *a priori* by the Mycobacteriology laboratory. This would involve the isolation of the patient's macrophages, the phagocytosis of the mycobacterial isolate and the presentation of the anti-tubercular agent to the macrophage-bacterium complex. This system is reviewed in its entirety and its potential and feasibility are supported by hard experimental demonstrations.

Multi- and Extensively-drug resistance

Multi-drug resistant *Mycobacterium tuberculosis* (MDR-TB) is resistant to the two most effective anti-tubercular drugs isoniazid (INH) and rifampin (RIF) and even under the best of circumstances, that is, under appropriate therapy, patient compliance and under direct observable therapy (DOT), it produces significant mortality (WHO, 2007a). When the infection takes place in a patient that is co-infected with HIV and has advanced to AIDS, mortality is almost always certain within a year, regardless of therapy (CDC, 2007; WHO, 2006b, 2007a). To make matters worse, MDR-TB has progressed to extensively-drug resistant *M. tuberculosis* (XDR-TB), defined by resistance to INH, RIF, any fluoroquinolone and at least 1 of the 3 injectable second line drugs (capreomycin, kanamycin and amikacin) (CDC, 2007; Shah *et al*., 2007; WHO, 2006b). Even though, individual resistance of wild type *M. tuberculosis* to these drugs exceeds the frequency of individual resistance to INH and RIF (Shah *et al*., 2007; WHO, 2006b) these drugs in combination have been recommended for the therapy of XDR-TB (Goldman *et al*., 2007).

Pulmonary tuberculosis (TB) is an intracellular infection of the alveolar macrophage. Because this cell has little killing activity of its own, the *M. tuberculosis* exists *in situ* for decades without causing any overt symptoms of infection. In fact, in the absence of immuno-incompetence, less than 10% of all infections progress to active disease (Goldman *et al*., 2007; WHO, 2007a), the phase characterized by the release of the organism from its macrophage prison which is now extracellular and eventually manifested in patient sputum. Because of the intracellular nature of the infection, in order for a given drug to be therapeutically effective it must be able to penetrate the macrophage and have activity against the *in situ* localized organism. Consequently, although literally thousands of compounds have been shown to have *in vitro* activity against *M. tuberculosis* (Spigelman, 2007) only a very few have activity against the organism at its intracellular location (Amaral *et al*., 2006, 2007c). This simple fact means that if an agent is to be therapeutically active against antibiotic susceptible *M. tuberculosis*, MDR-TB and even XDR-TB it must first be shown to be active where these strains of *M. tuberculosis* are to be found, namely, the human macrophage (Amaral *et al*., 2006, 2007b, 2007c).

It is the purpose of this chapter to present cogent support for the use of the human macrophage model in the clinical TB laboratory as the only realistic means by which effective anti-MDR-TB/XDR-TB compounds can be *a priori* selected for the therapy of the MDR-TB/XDR-TB infected patient. Moreover, because time is of essence for the identification and antibiotic susceptibility of MDR-TB/XDR-TB if this infection is to be curtailed, the system in place at our laboratory for close to five years which identifies MDR-TB within one day of receiving the specimen will also be discussed in detail.

Identification of an MDR-TB infection within one day of receiving the clinical specimen: The Faster TB Track Programme

As a consequence of the resurgence of TB and MDR-TB in the city of New York during the early 1990's, New York State Department of Health instituted the TB Fast Track Programme (Frieden *et al*., 1993; Hale *et al*., 2001; Parsons *et al*., 2004). This programme required that the clinical specimen be accompanied by a positive acid fast stained sputum after which its reception resulted in the shunting of the specimen along the most rapid manner (Fast Track) for the identification of the organism and the

determination of antibiotic susceptibilities, namely, the use of molecular probes for identification of *M. tuberculosis* complex from positive cultures and the BACTEC 460 system for determination of antibiotic susceptibility. Because the rates of new cases of pulmonary TB in Portugal in 2000 were the highest in Western Europe (Antunes *et al*., 2000; Shah *et al*., 2007) our Mycobacteriology laboratory at the Institute of Hygiene and Tropical Medicine, Universidade Nova de Lisboa, Lisbon, Portugal, adopted and modified the TB Fast Track programme to yield the required data within 12 days of receiving the specimen. Although this rapid turn-around time for complete analysis of the specimen was considered a major advancement, it was still, in our opinion, not good enough. Discussions with Max Salfinger (Hale *et al*., 2001; Parsons *et al*., 2004), then Director of Tuberculosis Laboratory of the New York Department of Health during 2003, resulted in the development and implementation of the Faster Track Programme in our Mycobacteriology laboratory. This Faster Track Programme consisted of employing the INNO-LiPA Rif.TB Line Probe Assay (Innogenetics, Zwijndrecht, Belgium) for the simultaneous identification of *M. tuberculosis* complex and detection of RIF resistance directly on the sputum specimen that was accepted into the programme due to it being accompanied by a positive acid fast stained sputum. Because resistance to RIF is almost always accompanied by resistance to INH (Hale *et al*., 2001; Viveiros *et al*., 2005b), identification of the mutated *rpoB* gene provided the identification of an MDR-TB strain. This TB Faster Track programme enabled the identification of MDR-TB strains within 8 hours of receiving the specimen, and hence, within that same day, the patient could be isolated and treated aggressively (Viveiros *et al*., 2005b). This programme, unique in Europe, has now been adopted in the USA and United Kingdom (CDC, 2007; Goldman *et al*., 2007; Sam *et al*., 2006). The employment of the TB Faster Track programme for patients presenting with clinical and radiological symptoms of pulmonary TB in Lisbon has contributed to the reduction of rates of new cases of MDR-TB from an excess of 28 per cent of all *M. tuberculosis* isolates to less than 8 per cent within a 3 year period (Antunes *et al*., 2000; DGS, 2007).

The rationale for evaluating the intracellular killing activity of anti-tubercular agents against *M. tuberculosis* isolated from the patient's sputum and its subsequent phagocytosis by macrophages isolated from that same patient

As previously stated, pulmonary TB is an intracellular infection of the pulmonary macrophage. Because this pulmonary macrophage has little killing of its own (Amaral *et al*., 2004, 2006, 2007c) *M. tuberculosis* can reside in a viable state within its phagosome prison for many decades (Saunders and Britton, 2007). If a compound is to be effective for the therapy of pulmonary TB it must be able to penetrate the macrophage and have activity against the organism at the intracellular site of the macrophage where it resides (Amaral *et al*., 2004, 2007b). If the compound is to be effective for the therapy of a specific patient that is presenting with pulmonary TB, the activity of the compound against the *M. tuberculosis* strain isolated from the patient would best shown if the macrophage that is to phagocytose the organism is obtained from that same patient. Employing this approach should result in the best predictive situation for the selection of effective anti-tubercular agents. With this consideration in mind, it is important therefore, that the assay that is to provide the means by which effective therapy can be *a priori* selected be fully mastered.

The human non-killing macrophage and its preparation from human peripheral blood

Human non-killing macrophages can be easily prepared from the MDR-TB/XDR-TB infected patient's peripheral blood prior to the patient receiving any form of therapy. The process involves obtaining peripheral blood with a syringe containing heparin, the mixing of the blood with Hank's Balanced Salt Solution (HBSS) or tissue culture medium such as RPMI 1640 (Sigma-Aldrich Química, S.A., Madrid, Spain), followed by the overlay of the diluted blood on top of Ficoll-Hypaque 1.077 (Sigma-Aldrich Química, S.A., Madrid, Spain), and subsequent centrifugation for the isolation of the layer containing the mononuclear cells. The isolated layer is washed twice with HBSS and the mononuclear cells suspended in medium and transferred to a tissue culture bottle (Leighton flask) and incubated at 37ºC for five days during which time the monocytes adhere to the bottom of the flask; the medium containing the non-adhered lymphocytes is then removed by pouring directly into a waste receptacle, and replaced

with fresh medium. The adhered monocytes are gently suspended into the medium with the aid of a sterile rubber policeman, an aliquot removed for counting the cells and aliquots of 0.8 mL containing approximately 100,000 monocytes are transferred to 1 ml wells of a 24-well microplate. The microplates are incubated at 37ºC and 5% of CO_2 until further use. During this last incubation the macrophages settle and adhere to the bottom of the wells. Details of this method have been completely described (Ordway *et al*., 2002b, 2003b) (**Chapters III and IV**).

Evaluation of drugs for enhanced killing of intracellular *Staphylococcus aureus* and *Mycobacterium tuberculosis*

We have found that compounds that enhance the killing of intracellular *Staphylococcus aureus* ATCC25923 are usually effective against intracellular mycobacteria (Amaral *et al*., 2006; Ordway *et al*., 2002a) and because the killing of intracellular *S. aureus* promoted by these drugs can be defined within two days as opposed to three or four weeks when *M. tuberculosis* is used, *S. aureus* is used for the screening of drugs that enhance intracellular killing. Drugs that enhance killing of intracellular *S. aureus* to a greater extent than our positive control (thioridazine - TZ) are then selected for study against intracellular *M. tuberculosis.* The assay for the evaluation of intracellular activity of drugs is performed as follows: To each well of the 24 wells of an duplicate microplate containing 10^5 macrophages adhered to the bottom of the well in a volume of 0.9 mL of medium, 10^6 *S. aureus*/mL in 0.1 mL of medium are added and the plates incubated at 37ºC for 30 minutes. During this time the bacteria are effectively phagocytosed. Nevertheless, to insure that no free bacteria remain in the well, each well is washed 3 times with medium to remove non-phagocytosed bacteria after the incubation period. After the replacement of the medium with 0.9 mL of fresh medium the agents to be evaluated for intracellular killing activity are to be added in a volume of 0.1 mL of medium to sets of triplicate wells. The triplicate set of absolute control wells receives an equal volume of drug free-medium (**Chapter III**). The selection of concentrations of each drug is arbitrary and may be guided as follows: Three concentrations of each compound should be empirically used: concentration #1 to be added should be equal to the *in vitro* minimum inhibitory concentration (MIC) of that drug against *S. aureus*; concentrations #2 and 3 should be 1/10 and 1/100 of the MIC, respectively. Because the macrophage has the ability to concentrate many drugs once

they penetrate into the cell (Daniel and Wojcikowski, 1999b), if the agent is effective against intracellular *S. aureus* ATCC25923, concentrations below the MIC should prove effective especially if shown to be active at 1/100 of its MIC (Ordway *et al*., 2002a, 2002b, 2003b) (**Chapter III**). The second duplicate microplate containing 10^5 adhered macrophages per well is to be used for the addition of 10^6 *M. tuberculosis*/mL obtained from the liquid BACTEC 460 cultures used for the isolation of the strain (Ordway *et al*., 2003b) (**Chapter IV**), the addition of three concentrations of the agents under investigation and processed as that containing the staphylococci as described above. The microplate containing the phagocytosed staphylococci and phagocytosed *M. tuberculosis* are to be incubated for up to 6 hours and 3 days, respectively (Ordway *et al*., 2002a, 2002b, 2003b) (**Chapter IV**). At hourly intervals of the 3 hour incubation period of the microplate containing the phagocytosed staphylococci 0.01 mL of 0.01% SDS are added to lyse the macrophage and release the intracellular bacteria and aliquots of the medium are processed for colony forming units (CFU) on plates containing a suitable solid medium. An identical approach is to be used for the microplates containing the macrophage/*M. tuberculosis* except that the intervals should be 24 hours for the duration of the incubation (Ordway *et al*., 2002a, 2002 b, 2003b). Using the two microorganisms in a combined macrophage assay allows the rapid screening of new anti-TB drugs, the evaluation of effectiveness of combined therapies and patient directed selection of effective therapies. For the latter, the macrophage-*S. aureus* assay is of lesser importance since our attention will be focused in the intelligent selection of the most effective therapy against TB and/or XDR/MDR-TB.

Demonstration of degrees of killing activity promoted by drugs against *S. aureus* and *M. tuberculosis* - Thioridazine: the positive control for the demonstration of enhanced killing of intracellular bacteria

The *in vitro* activity of TZ against *S. aureus* and *M. tuberculosis* strains has been extensively studied by us and has been shown to be consistent (Ordway *et al*., 2002a; 2003b). Briefly, the MIC of TZ against *S. aureus* and *M. tuberculosis* strains is 20-30 and 15 mg/L, respectively (Ordway *et al*., 2002a, 2003b) (**Chapter III**). At concentrations just below the MIC, TZ produces major alterations of the bacterium's ultrastructure such as: blebbing and thickening of the cell wall; bizarre cross-walls; marked vacuolization of the cytoplasm and destruction of the internal compartments of

the cell (Amaral *et al*., 2007b; Martins *et al*., 2004) (**Chapter III**). Figure 1 is an example where all of these alterations can be found in a single *S. aureus* cell that has been exposed *in vitro* to TZ for 18 hours. Although these alterations can be considered to be severe-they do not cause cell death inasmuch as the minimal bactericidal concentration of TZ exceeds 60 mg/L (Ordway *et al*., 2002a, 2002b).

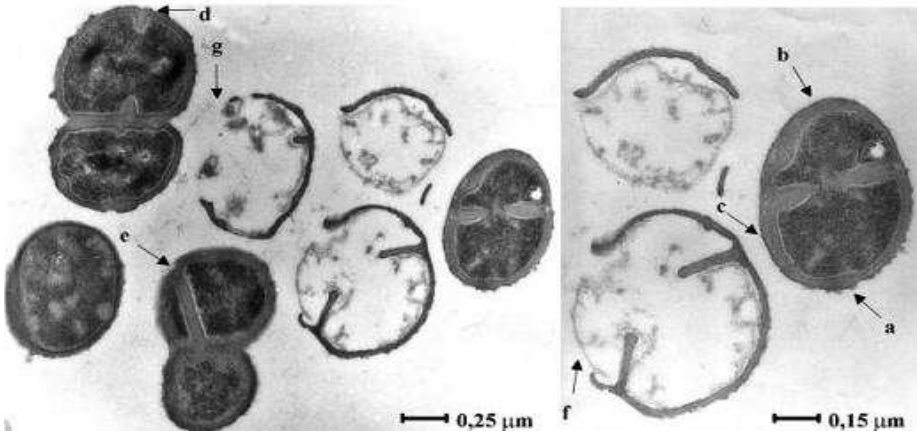


Figure 1. *In vitro* effect of TZ on the ultra-structure of MRSA after 18 hours of culture. The alterations on the ultra-structure of MRSA are: blebbing of the cell wall (a); thickening of the cell wall and cross-walls (b); partial separation of the internal portion of the cell wall (c); continued production of cross-walls without subsequent separation of the replicated cells (d); asymmetrical cross-wall formation (e); partial loss of cell wall (f) and evidence of lysis (g) (Martins *et al*., 2004).

The concentrations of TZ that inhibit the growth of *M. tuberculosis* or *S. aureus* exceed that which produces toxicity to the macrophage (Ordway *et al*., 2002a). However, because the human macrophage concentrates the phenothiazine at least 100 fold over that non-toxic concentration-*i.e.* below 0.5 mg/L, exposure of macrophages containing phagocytosed bacteria to concentrations of TZ as small as 0.1 mg/L reproduces *in situ* all of those alterations of the ultrastructure produced *in vitro* at a concentration near the MIC (Ordway *et al*., 2002a, 2002b, 2003b) (**Chapter IV**). An example of this is presented by Figure 2.

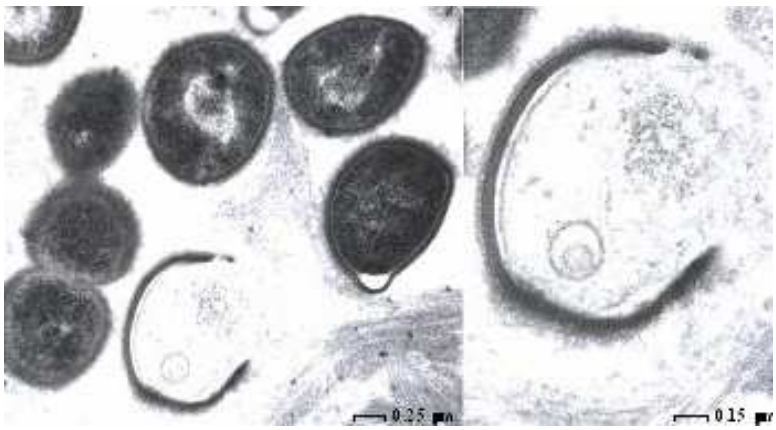
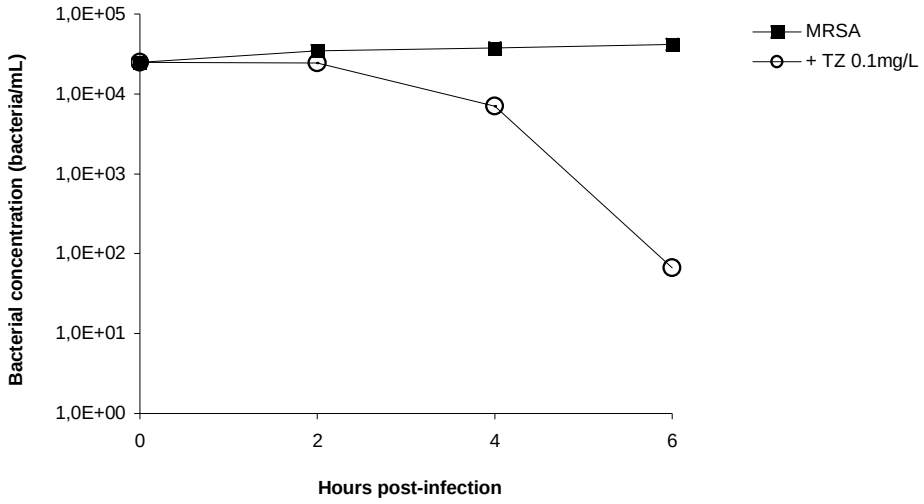


Figure 2. *Ex vivo* effect of TZ (0.1 mg/L) in phagocytosed *S. aureus* exposed for 6 hours. Control (A) and cells treated with 0.1 mg/L of TZ after 6 hours of phagocytosis (Martins *et al*., 2004).

The ultrastructural changes produced by TZ after the bacterium has been phagocytosed are accompanied by an enhanced killing activity of the non-killing human macrophage. As shown by Figure 3 exposure of the macrophage containing the phagocytosed bacterium to a concentration of TZ as low as 0.1 mg/L enhances the killing of Methicillin-Resistant *S. aureus* (Fig 3A) and Multi-Drug Resistant *M. tuberculosis* (Fig 3B).

These assays have been repeated at least 25 times and the results have been consistent when one donor serves as the source of peripheral blood employed for the derivation of human macrophages (Ordway *et al*., 2002a, 2002b, 2003b). When peripheral blood comes from a different donor source, although the results are similar, the degree of killing varies. This variability is to be expected due to age and sex of the donor as well to underlying pathology and is therefore the reason why the patient's own peripheral blood is to serve as the source for the preparation of macrophages.

A



B

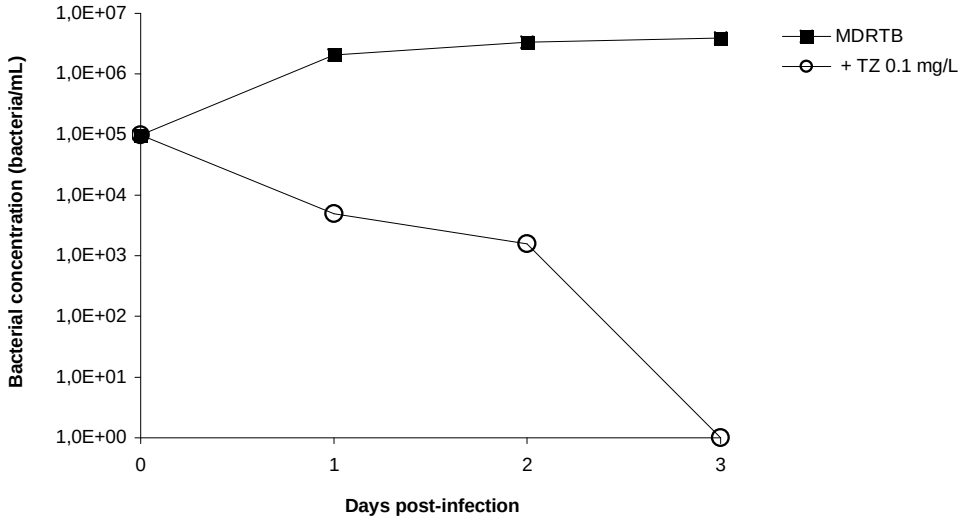


Figure 3. Effect of TZ in the killing activity of macrophages infected with MRSA (A) and MDR-TB (B). The effect of TZ on the intracellular growth of MRSA (A) and MDR-TB (B) strains was evaluated. TZ (0.1 mg/L) was separately added to human macrophages cultures. Control cultures received no drug. Control and TZ containing cultures were infected for 30 and 60 minutes with MRSA and MDR-TB strains, respectively, at concentrations of 10 CFU per macrophage (10:1). Results are expressed as the mean CFU of the strains tested in three independent experiments. MRSA – Methicillin-Resistant *S. aureus*; MDR-TB – Multi-Drug Resistant *M. tuberculosis*; TZ – Thioridazine (Martins *et al*., 2004; Ordway *et al*., 2003b).

The enhanced intracellular killing activity promoted by TZ resulted in studies that evaluated the ability of TZ to cure the mouse infected with *M. tuberculosis* (Martins *et al*., 2007a) (**Chapter V**). As shown by Figure 4 mice that have been infected with a massive dose of *M. tuberculosis* (excess of one million cells) can be cured of this infection with daily TZ injections (1200 mg/Kg mouse body weight) (**Chapter V**).

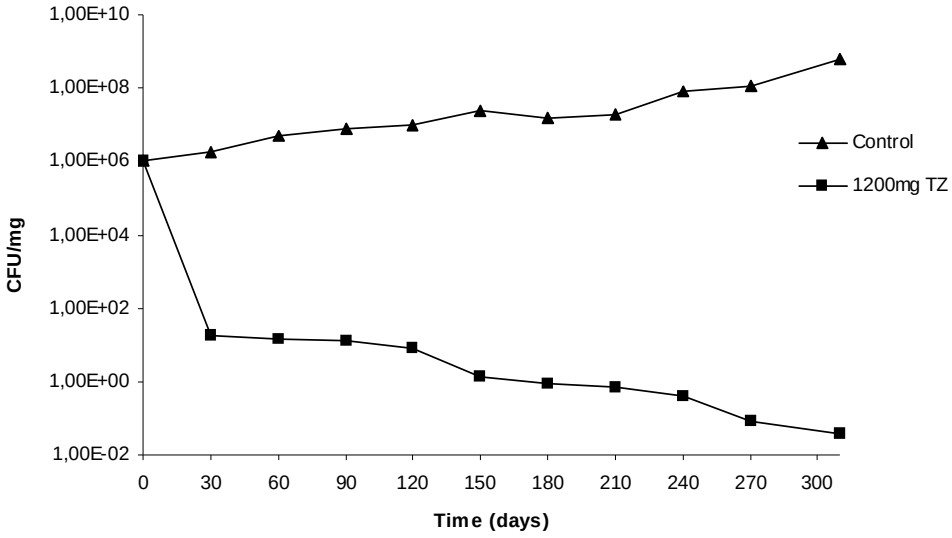


Figure 4. Effect of TZ treatment of Balb/C mice infected with *M. tuberculosis*. Four groups of eight mice were infected with approx. 10^6 CFU of *M. tuberculosis* H37Rv ATCC 27294 and treated with daily doses of TZ (1200 mg/kg). The control group received no drug. At monthly intervals animals from each group were sacrificed, their lungs removed and mycobacteria released from the cellular debris and plated; TZ – Thioridazine; CFU - Colony Forming Units (Amaral *et al*., JAC 2007b; Martins *et al*., 2007a).

The macrophage assay has been extended for the evaluation of many compounds (Martins *et al*., 2005; Ordway *et al*., 2003a), other phenothiazines (Ordway *et al*., 2002a, 2002b) and derivatives of TZ (Martins *et al*., 2007b) for activity against intracellular bacteria. The data obtained have provided direction for the selection of which compounds should be further studied for their curative activity (**Chapters IV.2, VI and VII**).

CHAPTER X.

The Macrophage Model

This chapter contains data published in:

Amaral, L., M. Martins, and M. Viveiros. 2007. Phenothiazines as Anti-Multi-Drug Resistant Tubercular Agents. Infect. Disord. Drug Targets **7**:257–265; **Martins, M., M. Viveiros, and L. Amaral.** 2008. Enhanced killing of intracellular pathogenic bacteria by phenothiazines and the role of K^+ efflux pumps of the bacterium and the killing macrophage. Anti-Infective Agents In Medicinal Chemistry **7**:63–72; **Amaral, L., M. Martins, and M. Viveiros.** 2007. Enhanced killing of intracellular multi-drug resistant *Mycobacterium tuberculosis* by compounds that affect the activity of MDR efflux pumps: a review. J. Antimicrob. Chemother. **59**:1237–1246.

CHAPTER OUTLINE

In sequence with the previous chapters, this chapter is now focused on the characteristics of MDR-TB and identifies a variety of compounds that address these characteristics and that show potential for managing these infections. The enhanced killing of intracellular multi-drug resistant *M. tuberculosis* by compounds that affect the activity of MDR efflux pumps was assayed. The mechanism by which these agents enhance the killing of intracellular bacteria is extremely important for the design of new anti-tubercular agents. From the results obtained, a hypothetical model was constructed. This model describes the mechanisms by which efflux pumps of the phagosome-lysosome complex are inhibited by agents known to inhibit the K^+ flux.

X.1 Phenothiazines as Anti-Multi-Drug Resistant Tubercular Agents

SUMMARY

Pulmonary tuberculosis (TB) has again become a global problem: it infects 2.2 billion people world-wide, caused the deaths of over 3 million last year and will produce over 8 million new cases of TB this coming year. Although effective therapy is widely available for antibiotic susceptible strains of *Mycobacterium tuberculosis*, current drugs are relatively useless against multi-drug resistant infections (MDR-TB). Mortality is almost complete within two years regardless of therapy, and in the case of co-infection with HIV/AIDS, mortality is 100% within a few months of diagnosis especially the *M. tuberculosis* strain is XDR-TB. As of the time of this writing no new effective anti-TB drugs have been made available by the pharmaceutical industry and XDR-TB. Because TB is an intracellular infection of the non-killing macrophage of the lung, any agent that is to prove effective must have activity against MDR-TB and XDR-TB strains that have been phagocytosed by the human macrophage. This chapter intents to provide cogent *in vitro*, *ex vivo* and *in vivo* evidence that supports the use of a variety of commonly available phenothiazines for the therapy of MDR-TB and XDR-TB, especially when the prognosis of the infection is poor and the use of the recommend agents can take place along lines of "compassionate therapy". In addition, it will describe the macrophage assay as indispensable if an agent is to be further studied for its effectiveness as an anti-TB drug. *In vitro* studies if not pursued by *ex vivo* studies will for the most be dead-ended since few agents that have activity *in vitro* have any activity against phagocytosed *M. tuberculosis*.

If hundreds of antibiotics are available for therapy of bacterial infections why are there none for the therapy of MDR-TB?

The last effective anti-TB drug was RIF and this drug came into use in the early 1960's. Since TB has remained a serious medical problem in Africa, South-East Asia and South America (WHO, 1997, 2000) why are there no new anti-TB drugs available? Monetary incentive is a major cause for the lack of new anti-TB drugs inasmuch as the infection is most frequent in countries that are economically disadvantaged (Amaral *et al*., 2004). However, resurgence of TB in Western urban centers, and more importantly, the

appearance and escalation of MDR-TB and XDR-TB worldwide has caused sufficient concern to the extent that a number of international programs have been created which make funds available for the development of new anti-TB drugs (WHO, 2007b). Although these programs may in time alleviate the problem, it should be understood that the creation of new and effective anti-TB drugs is far more difficult than that for the therapy of other bacterial infections, inasmuch as the drug must not only penetrate the cell housing the trapped mycobacterium but it must be active at that site when non-toxic concentrations of the drug are administered to the patient. Consequently, although literally thousands of compounds can inhibit the replication of mycobacteria *in vitro*, very few are effective at the intracellular site where the mycobacterium resides.

What is meant by an "effective" anti-TB drug? The answer is simple-it must kill the bacterium *in situ.* Remarkably, neither INH nor RIF alone can readily accomplish this task and hence the need to use these two agents in tandem. Why are they effective when administered together? Firstly, both penetrate the macrophage and retain their activity at the site where mycobacterium resides (Amaral *et al*., 2007b; Martins *et al*., 2007b). Secondly, whereas RIF inhibits the replication of the organism by binding to the RNA polymerase sub-unit β (Vattanaviboon *et al*., 1995), INH kills by interfering with the physical chemistry of the cell wall after its is converted to an active form (*i.e.* INH is a pro-drug) (Cappelletty, 2007). The separate action of each drug, inhibition of replication and killing is highly effective if, and only if, the organism is susceptible to both compounds (Chauca *et al*., 2007).

Will future therapies for MDR-TB involve the use of two or more compounds? Most would agree that the use of two or more effective drugs would be more effective than the use of only one, if only because the latter would be rendered ineffective within a short period of time due to the advent of spontaneous mutation whereas the simultaneous development of two separate mutations would be less probable. It would be ideal if only one compound were to be required that would not be subject to eventual mutation-based resistance. Is the ideal possible?

Phenothiazines as anti-MDR-TB agents

The antimicrobial activity of phenothiazines has been known since the time Paul Ehrlich studied the effects of methylene blue on the mobility of bacteria (Amaral *et al*., 2004; Kristiansen and Amaral, 1997). However, because the dye was shown to cause cats to become lethargic (McMahon and Kadowitz, 1992), the evolution of this first phenothiazine progressed towards the development of the first neuroleptic, CPZ in 1953 (Amaral and Kristiansen, 2001; Amaral *et al*., 2001b) and little attention was paid to its potential as an antimicrobial agent (Kristiansen and Amaral, 1997). Nevertheless, because of worldwide use of CPZ for therapy of psychoses and severe neuroses, clinicians noted that patients treated with this agent were cured of a variety of infections (Amaral and Kristiansen, 2000; Amaral *et al*., 2001b). Regardless, because these observations were made during the Golden Age of Antibiotics (1950s-1970s) there was no need for another antibiotic, especially for one that produced a high frequency of serious side-effects (Amaral and Kristiansen, 2000). However, as antibiotic use became more and more common, the opportunity for misuse increased, and misuse resulted in the appearance of bacterial strains that were resistant to one or more antibiotics (Amaral and Kristiansen, 2001; Amaral *et al*., 2004, 2007b; Kristiansen and Amaral, 1997). The appearance of MDR strains of bacteria provided the impetus for a search for non-antibiotic compounds, *i.e.,* drugs that were used for the therapy of non-infectious pathology, that could either be used directly (Kristiansen and Amaral, 1997), or as possible adjuncts with conventional antibiotics, or, serve as lead compounds for the syntheses of new agents (Martins *et al*., 2007b) (**Chapter IV**). Consequently, phenothiazines were shown to inhibit the replication of Gram-negative (Amaral and Lorian, 1991) and Gram-positive bacteria (Kristiansen *et al*., 2003, 2006; Martins *et al*., 2004; Ordway *et al*., 2002a, 2002b), mycobacteria (Amaral and Kristiansen, 2000; Amaral *et al*., 2001b, 2007b; Martins *et al*., 2007b; Viveiros *et al*., 2005c) (**Chapters III and IV**), a large variety of microorganisms (Amaral *et al*., 2004, 2006, 2007a; Grácio *et al*., 2003; Viveiros *et al*., 2003; Wainwright and Amaral, 2005), parasites that cause malaria (Amaral *et al*., 2001b; Wainwright and Amaral, 2005), sleeping sickness (Boda *et al*., 2006; Khan *et al*., 2000; Page and Lagnado, 1995; Wainwright and Amaral, 2005), and leishmania (Amaral *et al*., 2004; Kristiansen and Amaral, 1997; Leandro and Campino, 2003; Wainwright and Amaral, 2005) and even parasitic worms (Grácio *et al*., 2003). However, because all of these activities were shown to take place

in vitro at concentrations that were well beyond any that could be achieved in the patient, their use as direct antimicrobial agents was out of the question (Amaral and Kristiansen, 2000; Amaral and Lorian, 1991; Amaral *et al*., 2001a, 2001b, Kristiansen and Amaral, 1997; Kristiansen *et al*., 2003, 2006; Martins *et al*., 2004, 2007b; Ordway *et al*., 2002a, 2002b, 2003b) even though some reports showed their *in vivo* effectiveness (Amaral *et al*., 2001a, 2001b). Furthermore, because some phenothiazines were shown to enhance the activity of antibiotics to which the bacterium was susceptible and could also render antibiotic resistant bacteria susceptible to the antibiotic to which they were initially resistant (Amaral *et al*., 1992; Bettencourt *et al*., 2000), interest in phenothiazines continued (Amaral *et al*., 2006, 2007b). This interest would rise dramatically when Crowle and his group demonstrated that a concentration of CPZ in the medium that corresponded to one that would be expected in the plasma of a patient chronically managed with this phenothiazine would enhance the killing of intracellular *M. tuberculosis* (Crowle *et al*., 1992). However, because CPZ produces so many serious side-effects this demonstration remained limited to academic interest until a similar demonstration of enhanced killing of intracellular *M. tuberculosis* by the far less noxious phenothiazine, TZ, was reported (Amaral *et al*., 2001a). Killing of phagocytosed *M. tuberculosis* is enhanced by concentrations of TZ that are below those found in the plasma of patients chronically treated with this neuroleptic. Because TZ may on rare occasions can even cause sudden death (1 sudden death per ten thousand man years of use (Crowle *et al*., 1992), its use for the therapy of MDR-TB has not been seriously considered, despite its potential for the therapy of terminal MDR-TB, if only for compassionate reasons (Amaral *et al*., 2006). Regardless, because TZ is very effective in enhancing the killing of intracellular MDR-TB (Amaral *et al*., 2001a; Martins *et al*., 2005, 2007b; Ordway *et al*., 2003b; Viveiros *et al*., 2005c) (**Chapter IV, Section IV.1**) the opportunity for the syntheses of TZ derivatives was pursued and the derivatives studied for their intracellular effectiveness (Martins *et al*., 2007b; Yano *et al*., 2006) (**Chapter IV, Section IV.2**). The derivatives that were proven to have significant *in vitro* activity against *M. tuberculosis* were selected for potential enhancement of killing of phagocytosed *M. tuberculosis.* It is important to note that these derivatives produced no cytotoxicity as per trypan blue exclusion criteria (Ordway *et al*., 2002a, 2003b) (**Chapter IV, Section IV.2**). During the time that the derivatives were being made, TZ was studied for its ability to cure the mouse of a severe pulmonary TB infection (Amaral *et al*., 2007a, 2007b). Although normally the study of an agent

with the use of a mouse model requires doses of the agent to be at least 10 fold higher than those used in the human (Amaral *et al*., 2007b; Jia *et al*., 2005; Wieland *et al*., 2006), the highest dose of TZ used in the mouse study was 0.5 mg/day and is equivalent to about twice that given to a human who is chronically treated with this drug. A dose of 0.5 mg/day practically eliminated within 30 days all traces of *M. tuberculosis* that could be retrieved from the lungs of the infected mouse (**Chapter V**). During the time of this study, others have reported that derivatives of CPZ can also cure the mouse of pulmonary TB (Weinstein *et al*., 2005).

How do phenothiazines inhibit the *in vitro* growth of bacteria?

All phenothiazines studied to date have the ability to inhibit the binding of Ca^{2+} to specific proteins (Amaral *et al*., 2007a), such as calmodulin (Mayur *et al*., 2006), calmodulin-like proteins of bacteria (Fry *et al*., 1986) and Ca^{2+}-dependent ATPases (Bhatnagar and Singh, 2004). Because of these effects they can also inhibit a variety of processes which are dependent upon Ca^{2+}-dependent ATPase derived energy (Wieczorek *et al*., 1999; Zakharov *et al*., 1996). Therefore, the motility of flagellate-bacteria (Molnar *et al*., 1992a), parasites (Boda *et al*., 2006; Jori, 2006) and cells in tissue cultures (Gil-Ad *et al*., 2004), processes of endocytosis (phagocytosis and pinocytosis), processes that activate the entrance of viruses (Hewlett *et al*., 1997) and bacteria (Molnar *et al*., 1982) into a eukaryotic cell are all inhibited by phenothiazines. However, these processes are not significantly affected at concentrations of the phenothiazine which correspond to those that are clinically achievable.

The *in vitro* inhibitory activity of phenothiazines against bacteria and mycobacteria (Kristiansen *et al*., 2003, 2006; Martins *et al*., 2007b; Viveiros *et al*., 2005c) at the lowest concentrations, *i.e.*, at their MIC is always bacteriostatic (Amaral *et al*., 2004; Kristiansen *et al*., 2006). At substantially higher concentrations, phenothiazines are bactericidal (Amaral and Lorian, 1991; Amaral *et al*., 1992; Kristiansen *et al*., 2003). The means by which the inhibitory effects are produced are the result of the effects of the agent on Ca^{2+}-dependent ATPase processes which when affected reduce the amount of cellular energy needed to maintain active transport processes over an extended period of time (Amaral *et al*., 2007a). Most of these processes are of an ABC transporter type and hence, the transport of vital substances from the medium into the bacterium is

reduced or inhibited (Marquez, 2005). In addition, specialized transport processes that extrude metabolic-toxic compounds to the environment are also affected (Langton *et al*., 2005). These latter export processes are efflux pumps, named Multidrug And Toxic Extrusion (MATE) and which recognize a wide number of unrelated metabolic products and extrude them to the environment (Cattoir, 2004). In a manner of speaking, MATE efflux pumps serve as the "renal system" of the bacterium. The third grouping of active transport processes that may be affected by phenothiazines at concentrations equivalent to those that inhibit the replication of bacteria *in vitro* are clinically relevant, inasmuch as these are energy dependent processes (efflux pumps) and are responsible for MDR phenotypes of Gram-negative (Viveiros *et al*., 2005a, 2007) and Gram-positive pathogenic bacteria (Kristiansen *et al*., 2003, 2006; Martins A *et al*., 2007; Martins *et al*., 2006a). Recently, Rubin and his team have proposed another mechanism by which phenothiazines exert their anti-tubercular activity (Yano *et al*., 2006). These studies demonstrate that the activity of Type-II NADH-menaquinone oxidoreductase which is an essential respiratory enzyme of *M. tuberculosis* is significantly inhibited by analogues of phenothiazines. The ability of the phenothiazine to reduce or reverse the resistance of the bacterium to a given antibiotic means that the phenothiazine could be used as an adjuvant with the conventional antibiotic to which the bacterium was initially resistant. This remarkable ability suggests that if these phenothiazine agents reach clinical use, many antibiotics that have fallen by the wayside due to MDR phenotypes of clinically relevant bacteria may again be used. The need for new antibiotics, which will in time, encounter efflux-mediated resistance may be highly reduced or even obviated. The mechanism by which phenothiazines exert bactericidal activity is not entirely understood. However, at concentrations greater than the MIC, the agent reaches the DNA and via intercalation (Cremieux *et al*., 1995) binds to sites of the DNA that are rich in guanosine and cytosine bases (Rohs *et al*., 2001). When intercalation takes place, it is irreversible and therefore inhibits all DNA based processes (Amaral *et al*., 2007a). This alone may be the cause for the bactericidal effects of the phenothiazine.

How do phenothiazines enhance the killing of intracellular *Mycobacterium avium* and *Mycobacterium tuberculosis?*

Macrophages are cells rich in lysosomes that concentrate phenothiazines as much as one-hundred fold over the concentration in the medium (Amaral *et al*., 2007a; Wojcikowski and Daniel, 2002). It was at first believed that the concentration of the phenothiazine by the lysosome would be at least equal to that required to kill the bacterium *in vitro* (Ordway *et al*., 2002a, 2002b, 2003b) (**Chapter IV**). However, recent studies have shown that the killing of intracellular bacteria by neutrophils is dependent upon ATP (Matsumoto *et al*., 1991), K^+ (Reeves *et al*., 2002) and Ca^{2+} (Ahluwalia *et al*., 2004) and that these substances are required for the acidification of the lysosome which in turn activates lysosomal hydrolases (Pillay *et al*., 2002). Because phenothiazines inhibit directly or indirectly the transport of K^+ (Mazumder *et al*., 1990) and Ca^{2+} (Williams *et al*., 1997), the possibility that killing of intracellular bacteria was not due to the phenothiazine being concentrated by the lysosome but due to the inhibition of the transport of K^+ and Ca^{2+} was considered (**Chapter VI**).

Agents which inhibit the transport of Ca^{2+} and K^+ directly or indirectly, and which have no activity against bacteria at the concentrations used in the study (Amaral *et al*., 2007b; Martins *et al*., 2007b) cause non-killing macrophages to kill *S. aureus* (Ordway *et al*., 2002a) (**Chapter III**) and *M. tuberculosis* (Martins *et al*., 2007b) (**Chapter IV**). The results obtained in these studies suggest the following: Bacteria bind to receptors present at the surface of the plasma membrane of the macrophage (Sharon *et al*., 1981). Binding activates invagination of the plasma membrane thereby forming the phagosome (Amaral *et al*., 2007b). The Ca^{2+} and K^+ transporting pumps, which are present in the plasma membrane and which prior to the formation of the phagosome pumped Ca^{2+} and K^+ into the cell against a concentration gradient, will now pump these cations from the phagosome to the cytoplasm of the cell (Amaral *et al*., 2007b). The penetration of the phenothiazine, ouabain, verapamil and reserpine, all inhibitors of Ca^{2+} and K^+ transport, takes place via pinocytosis. The pinocytic vesicles fuse with the lysosome that had fused with the phagosome creating the phagolysosome. The cation pumps at its surface are inhibited by phenothiazines, ouabain, reserpine or verapamil, and Ca^{2+} and K^+ are retained inside the phagolysosome (**Chapter VI**). This Ca^{2+} and K^+ retention will induce a raise in the intracellular concentrations of these cations that in turn will activate

other ATP-driven membrane pumps, namely, vacuolar-type proton ATPases (V-ATPases). The V-ATPases are enzymes responsible for the import of H^+ to the phagolysosome. This import is accomplished by the exchange of cations from the inside of the phagolysossome for H^+ from the cytoplasm. Therefore, the acidification of the phagolysosome will activate the hydrolases to destroy the bacterium (Amaral *et al*., 2007b). The enhanced killing of *M. avium* by phenothiazines (Viveiros *et al*., 2005c) as well as by substances extracted from plants (Martins *et al*., 2005; Ordway *et al*., 2003a) is most probably due to the same mechanism (**Chapter VI**).

What are the clinical implications for enhanced killing of intracellular bacteria?

It is generally accepted that with time resistance to any given antibiotic will take place (Poole, 2007). Agents that enhance the killing of intracellular bacteria by non-killing macrophages afford the advantage over conventional antibiotics inasmuch as the problem of bacterial resistance is by-passed. Therefore, perhaps it may be prudent to design agents which have the ability to inhibit K^+ transport and evaluate their ability to kill intracellular bacteria. If this is to be taken, there would be the need to establish a routine working macrophage-bacterium model that would yield the type of data needed for establishing the potential of a given compound for the therapy of MDR-TB.

The monocyte-derived macrophage/*Staphylococcus aureus* model for the evaluation of enhanced killing by agents that inhibit Ca^{2+} and K^+ transport

The enhancement of killing of intracellular *S. aureus* by agents which we have studied provided a reliable prediction of whether that same agent would also enhance the killing of intracellular *M. tuberculosis* (Ordway *et al*., 2003b). Because the results can be obtained within a few days when *S. aureus* is used, as opposed to 3 or more weeks when *M. tuberculosis* is used, we recommend that the former bacterium be employed as a "screening" tool for the selection of agents that are to be evaluated for similar activity against intracellular *M. tuberculosis.* The assay is identical whether staphylococci or mycobacteria are employed-and differs with respect to media, to the period of incubation post-phagocytosis and of course, the time needed for CFU to form and be counted is about 1 day with *S. aureus* (Ordway *et al*., 2002a) and at least 3 weeks for mycobacteria (Ordway *et al*., 2003b) (**Chapters III and IX**).

X.2 Enhanced killing of intracellular pathogenic bacteria by phenothiazines and the role of K^+ efflux pumps of the bacterium and the killing macrophage

SUMMARY

Pulmonary tuberculosis is an intracellular infection caused by *Mycobacterium tuberculosis*. Because its intracellular site is commonly the macrophage of the pulmonary system, and that cell has little killing action of its own, an antibiotic that is to be effective against this organism must be able to penetrate the macrophage and exert its action at the intracellular site where the organism resides. The anti-tubercular drugs which are most effective against this intracellular infection and which constitute the "first line of defence" are isoniazid and rifampin, both of which have activity against phagocytosed *M. tuberculosis*. Unfortunately, resistance to both of these agents (MDR-TB) continues to increase in frequency, and regardless of therapy, mortality is very high, nearing 100% within one year if the patient is co-infected with HIV or presents with AIDS. Since no new effective drugs have been made available for almost 40 years, compounds that enhance the killing activity of monocytes against MDR-TB are obviously needed. As previously shown, monocytes can be transformed into effective killers of intracellular bacteria when exposed to clinical concentrations of a phenothiazine or to inhibitors of efflux pumps, such as reserpine and verapamil, or to the inhibitor of K^+ transport, ouabain. This section discusses the *in vitro* and *ex vivo* activity of phenothiazines, their derivatives and the mechanism by which these agents manifest their antibacterial activity. It also identifies a variety of other efflux pump inhibitors that address these characteristics and therefore have potential for managing MDR-TB. Lastly, a model describing the mechanisms by which distinct efflux pumps of the phagosome-lysosome complex are inhibited by agents that are known to inhibit K^+ flux is presented. The model also predicts the existence of a K^+ activated exchange (pump) that is probably located in the membrane that delineates the lysosome. It is this putative pump, immune to the inhibitors of K^+ flux which is identified as being the cause for the acidification of the lysosome thereby activating its hydrolytic enzymes. Because the non-killer macrophage can be transformed into an effective killer by a variety of compounds that inhibit K^+ transport, perhaps it would be wiser to develop drugs that enhance the killing activity of these cells inasmuch as this approach would not be subject to any resistance as is the eventual case for conventional antibiotics.

The macrophage and why it does not kill intracellular *M. tuberculosis*

The killing activity of the alveolar macrophage, as compared to that of the neutrophils is quite nominal (Amaral and Lorian V, 1988; Appelberg, 2007). Nevertheless, this nominal killing activity involves the initiation of a cascade of biochemical and cellular events all of which lead to the recruitment of other cells involved in cellular immunity (Akagawa *et al*., 2006; Appelberg, R. 2007; Kwiatkowska and Sobota, 1999; Riethmüller *et al*., 2006). The initial event subsequent to the phagocytosis of a bacterium by the macrophage is the release of cytokines IL-6 and IL-12 that attract T helper lymphocytes to the site of infection. At the site of infection T helper cells release IFN-gamma, a very potent macrophage-activating cytokine, that eventually results in the acidification of the phagolysosome and the subsequent activation of hydrolases which afford the complete destruction of the bacterium (Ordway *et al*., 2004; Russell, 2007). However, mycobacteria have evolved strategies that control the ability of the macrophage to kill the bacterium (Nguyen and Pieters, 2005). Firstly, unlike the events that follow the phagocytosis of a bacterium, phagocytosis of *M. tuberculosis* does not result in the fusion of the phagosome with the lysosome (Vergne *et al*., 2003). Although the mechanism by which this inhibition takes place is not fully understood, because fusion of the phagosome with the lysosome requires Ca^{2+} (Vergne *et al*., 2003) and ATP (Wittekindt *et al*., 2006), presumably for the energy provided from the activity of a Ca^{2+}-dependent ATPase (Martins *et al*., 2006a), it is most probable that the entrapped mycobacterium secretes a substance that inhibits access to Ca^{2+} by binding all of the available Ca^{2+} within its phagosome trap. This is supported by studies that demonstrate that *M. tuberculosis* contains a Ca^{2+}-binding type protein with similar properties of the Ca^{2+} binding protein calmodulin (Méndez-Samperio *et al*., 2006; Ratnakar and Murthy, 1993; Salih *et al*., 1991). The inhibition of phagolysosome-lysosome fusion obviates the destruction of the mycobacterium allowing the existence of the bacterium within its phagosomal trap for decades. Although this period has been termed to be one of latency, the reality is that the bacterium is actually undergoing replication, albeit slowly, and after an unknown number of such replications, the bacterium breaks out of its phagosomal trap and is subsequently phagocytosed by neighbouring macrophages of the alveolar structure. The repeat of the above processes results in the development of a granuloma that is large enough to be seen with the naked eye (in histological sections of affected lung and or radiographs) and may be considered the earliest evidence of TB

(Russell, 2007). Although this treatise may suggest that the infection will proceed until death, only 5 to 10% of all such infections actually result in active disease (Kamholz, 2002; Russell, 2007). The activity of the immune system has long been suspected to in some way restrict or limit the progression of infection to active disease status (Ordway *et al*., 2004, 2005a). This restriction apparently involves the creation of a "cellular block" that is formed by T lymphocytes that have responded to the secretions of IL-6 and IL-12 from the infected macrophages (Ordway *et al*., 2005a; Russell, 2007). This "cellular wall" inhibits dissemination of the mycobacteria to further sites of the lung via the capillaries or lymphatics (Ordway *et al*., 2005b; Russell, 2007). In addition, the "cellular wall" also restricts access of the bacterium to sputum thereby reducing or completely inhibiting transmission of the bacterium to others (Flynn and Chan, 2005; Russell, 2007). However, as a consequence of external factors such advanced age, malnutrition, stress, co-infections (ex: HIV), the loss of the "cellular wall" may result and the granuloma structure could caseate, *i.e.*, disrupt, therefore releasing large numbers of mycobacteria that can infect more of the lung (Nguyen and Pieters, 2005; Ordway *et al*., 2005b; Russell, 2007). The release of mycobacteria due to caseation also results in discharge of large numbers of the organism to the environment via sputum, and now renders the subject as a serious source of infection to others (Kamholz, 2002; Russell, 2007). The need to develop agents that are effective against *M. tuberculosis*, especially MDR-TB strains, is obvious and significant to the reduction of new infections.

Limitations of drugs

Although hundreds of compounds are reported each year that have activity against TB, with few exceptions, activities are restricted to *in vitro* only-and the need to evaluate these compounds for *ex vivo* activity is essential if a compound is to be used for therapy. We have been studying phenothiazines and their derivatives for *in vitro* activity first (Amaral and Kristiansen, 2000, 2001; Amaral and Lorian, 1991; Amaral *et al*., 1992, 2000, 2001a, 2001b, 2004, 2006; Bettencourt *et al*., 2000), and when amply present, evaluated their activity against bacteria that were phagocytosed by monocyte-derived macrophages (MDM) (Amaral *et al*., 2001a, 2006, 2007b; Amaral and Kristiansen, 2000; Martins *et al*., 2006b, 2007b; Ordway *et al*., 2002a, 2002b, 2003b) which have little killing activity of their own (Ordway *et al*., 2002b, 2003b; Smith *et al*., 2000;

Theus *et al*., 2004). These studies, carried over a period of 15 years were based on the observations of Crowle *et al* who demonstrated that the *in vitro* activity of CPZ against *M. tuberculosis* could be reproduced against the phagocytosed organism at concentrations of the agent that were far below those needed for *in vitro* activity (Crowle *et al*., 1992). Although the *in vitro* activity of CPZ against mycobacteria had been known for many years (Amaral and Kristiansen, 2000, 2001; Amaral *et al*., 2001a, 2001b, 2004, 2006), its use as an anti-TB agent was never seriously considered due to the wide availability of effective anti-TB compounds (Amaral and Kristiansen, 2000, 2001; Amaral *et al*., 2001a, 2001b, 2004, 2006; Radhakrishnan *et al*., 1999) and, perhaps of greater significance, three decades of global experience with the use of CPZ for the therapy of psychosis indicated a large number of serious side effects (Amaral and Kristiansen, 2000, 2001; Amaral *et al*., 1996, 2001a; 2006). However, TZ, a specific derivative of CPZ and the equal of CPZ for the effective control of psychoses, is a much milder drug, producing as its major side effect somnia although it is now known to produce Torsades de Pointes (Amaral and Kristiansen, 2000, 2001), albeit, at a very low frequency (1 sudden death over 10,000) man years of use (Amaral and Kristiansen, 2000; Amaral *et al*., 2001b). Because of these facts, we have studied the *in vitro* activity of TZ against antibiotic susceptible strains as well as against strains that were mono, poly-drug and MDR, including strains that were resistant to all five of the drugs used as the "first line of defence" (Amaral and Kristiansen, 2000; Amaral *et al*., 1996, 2001a, 2004; Bettencourt *et al*., 2000; Martins *et al*., 2007b; Ordway *et al*., 2003b; Viveiros and Amaral, 2001) (**Chapter IV**). In addition, the results of other investigators are similarly presented in order to demonstrate that phenothiazines, in general, have *in vitro* activity against antibiotic susceptible and, more importantly, antibiotic resistant strains. Nevertheless, the activity of TZ takes place at concentrations which are clinically irrelevant, inasmuch as the highest plasma concentration that can be safely achieved in a patient that is chronically treated with the agent is about 0.4 mg/L and the lowest *in vitro* active concentration is of the order of 15 mg/L.

Ex vivo activity of phenothiazines

Agents which have *in vitro* activity against MDR-TB, if they are to be effective anti-TB compounds, must also have activity against these bacteria at their intracellular sites within the non-killing macrophage (Amaral and Kristiansen, 2000; Amaral *et al*., 1996,

2001a, 2004, 2006, 2007b; Martins *et al*., 2005, 2006b, 2007b; Ordway *et al*., 2002a, 2002b, 2003b; van den Broek *et al*., 1999; Viveiros and Amaral, 2001). In addition, they must be essentially non-toxic (Kristiansen and Amaral, 1997; Wainwright and Amaral, 2005). Our studies and those conducted by Crowle *et al* demonstrate that phenothiazines enhance the killing of *M. tuberculosis* that has been phagocytosed by macrophages at concentrations well below those that have activity *in vitro* (Bettencourt *et al*., 2000; Crowle *et al*., 1992; Martins *et al*., 2004; Ordway *et al*., 2002a, 2002b, 2003b) (**Chapter V**). These concentrations are clinically acceptable in as much as they are within the ranges anticipated in the plasma of patients treated with these agents (Ordway *et al*., 2002a, 2002b, 2003b). Because CPZ and to a far lesser extent, TZ, produce undesirable side effects (express levels of toxicity), we have studied newly synthesised derivatives of TZ for *in vitro* activity, and when present, studied those that were significantly active relative to TZ for activity against antibiotic susceptible and antibiotic resistant strains of *M. tuberculosis.* Whereas *in vitro* active derivatives of TZ are also active against phagocytosed mycobacteria, derivatives #1867, #1870 and #1875 present higher activity than the parent compound (TZ) (**Chapter IV**). Evaluation of these compounds for cellular toxicity indicate that substantially higher concentrations than those which have intracellular activity are devoid of any cellular toxicity. The agents are good candidates for the therapy of MDR infections of TB. However, animal studies are still needed prior to clinical trials.

How does a Phenothiazine enhance the killing of intracellular bacteria including mycobacteria?

Phenothiazines have been shown to inhibit the transport of K^+ from external to internal cellular compartments such as transport channels of cardiac Kir2.1 cells and red blood cells (Kim and Kim, 2005; Plishker, 1984; Sun *et al*., 2006;) and between intracellular compartments (*ex.* diencephalic neurons; rat liver mitochondria) (Chukhlova *et al*., 1984; Tolón *et al*., 2000;). They also inhibit the binding of calcium to calmodulin, the calcium binding protein of mammalian cells (Weiss *et al*., 1980). The binding of calcium to calmodulin-like proteins of bacteria has been amply demonstrated (Dhople, 1999; Fry *et al*., 1986; Nagai *et al*., 1994, 1997; Reddy *et al*., 2003; Rigden *et al*., 2003; Salih *et al*., 1991; Sarma *et al*., 1998; Sarkisova *et al*., 2005; Yonekawa *et al*., 2005). The inhibition of calcium access to Ca^{2+} dependent-ATPase inhibits transport processes

such as those performed by influx and efflux pumps (Kaatz *et al*., 2003; Molnar *et al*., 1997; Nacsa *et al*., 1998). Because phenothiazines inhibit access to calcium, they inhibit the activity of calcium-dependent ATPase and hence, the transport processes (Kaatz *et al*., 2003; Kristiansen *et al*., 2003, 2006; Molnar *et al*., 1997; Nacsa *et al*., 1998; Viveiros *et al*., 2002, 2003, 2005a).

Bacteria as well as mammalian cells contain efflux pumps that extrude noxious agents from the periplasm and cytoplasm of the former (Piddock, 2006a) and from the cytoplasm of the latter (Hoffmann and Kroemer, 2004). Understanding the effects of a phenothiazine on calcium dependent transport processes of the bacterium or the mammalian cell predict that efflux pumps of the bacterial or mammalian cell will be affected by a phenothiazine. This prediction has been shown to be correct whenever studied (Kaatz *et al*., 2003; Kristiansen *et al*., 2003, 2006; Molnar *et al*., 1997; Nacsa *et al*., 1998; Viveiros *et al*., 2002, 2003, 2005a). Since the first contact of the phenothiazine with the bacterium takes place at the surface of the cell envelope, it would be expected that although the phenothiazine could penetrate this structure as a consequence of a concentration gradient and its amphipathic structure, one of the many efflux pumps of the bacterium would recognize this molecule and extrude it once it reached the periplasm of the bacterium. Although this expectation has yet to be fully studied, the effect of the phenothiazine on the efflux system of the bacterial cell has been shown to one of inhibition (Kristiansen *et al*., 2003, 2006; Molnar *et al*., 1997; Viveiros *et al*., 2002, 2003, 2005a). Phenothiazines readily intercalate between nucleic bases of the DNA helix (de Mol *et al*., 1983; Rohs and Sklenar, 2004; Sinha *et al*., 2006; Webb *et al*., 1979, 1984). The degree of intercalation is dependent upon the number of guanosine-cytosine residues (Rohs and Sklenar, 2004). When phenothiazines intercalate into DNA they inhibit all DNA based processes as well as the degree of coiling and uncoiling of DNA promoted by gyrases (Boshoff *et al*., 2004). The inhibition of the efflux pump by a phenothiazine would result in large numbers of phenothiazine molecules entering the cell, reaching their intercalative sites of the DNA and thereby inhibiting the replication of the bacterium. The concentrations of the phenothiazine in the medium required for the inhibition of bacterial replication vary greatly with respect to the bacterium: the MIC for Gram-negative bacteria ranges from 100 to 200 mg/L whereas for Gram-positive bacilli or cocci the MIC ranges from 10 to 40 mg/L (Kristiansen and Amaral, 1997). Although not proven, there seems to be a

strong correlation between bacteria that contain a highly effective system of efflux pumps and the concentration of the phenothiazine required for the *in vitro* inhibition of replication. As an example, as much as 35% of the genome of Gram-negative bacteria codes for influx/efflux pumps (transporters) and with respect to *E. coli*, as many as twenty mdr efflux pumps have been genetically characterized (Piddock, 2006a). Although this organism has an obvious redundancy of efflux pumps-the major efflux pump that accounts for multi-drug resistance in this organism is coded by the *acrAB-TolC* operon (Viveiros *et al*., 2005a; Yu *et al*., 2003). When this operon is deleted, the organism becomes extremely sensitive to antibiotics and the phenothiazines CPZ and TZ (Viveiros *et al*., 2005a). When both the *acrAB* intact and *acrAB* deleted strains are induced to high level of resistance to tetracycline, the genetic expression of the acrAB efflux pump of the former has been increased six-fold whereas the acrEF efflux pump of the latter strain has been increased eighty-fold (Viveiros *et al*., 2005a). The increased genetic expression of these pumps renders both strains significantly more resistant to chlorpromazine and thioridazine as opposed to their parents. These results support the contention that the phenothiazines chlorpromazine and thioridazine are substrates of the AcrAB and AcrEF efflux pump systems and that when the ability of the pumps to extrude the agents is exceeded, the agents reach their intercalative targets and bacterial replication is inhibited. Because the concentration of either phenothiazine in the medium that enhances the killing of intracellular bacterium is about one hundredth of that needed to kill the bacterium *in vitro* (Ordway *et al*., 2003b, 2002a, 2002b; Weinstein *et al*., 2005; Yano *et al*., 2006), the killing activity appears to be related to the phenothiazine being concentrated by the non-killing macrophage to levels comparable to those required for killing *in vitro* (Crowle *et al*., 1992). Electron microscopy studies of the effects of phenothazines on phagocytosed *Staphylococcus aureus* demonstrate that the *in vitro* effect of these agents on the bacterium's morphology is reproduced when the bacterium is phagocytosed by macrophages that are subsequently exposed to concentrations of the agent (see Figure 1) that produce neither an inhibition of replication nor a change in the morphology of the organism (Martins *et al*., 2004) (**Chapter III**).

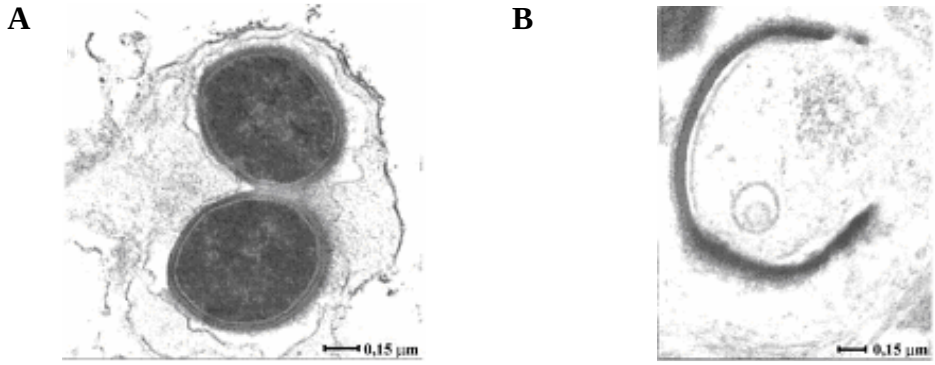


Figure 1. The *ex vivo* effects of Thioridazine on the ultrastructure of *Staphylococcus aureus* phagocytosed by monocyte derived macrophages. Control (A) and Thioridazine treated (B) 6 hours post phagocytosis. Thioridazine (0.1 mg/L) added to the medium 30 minutes after phagocytosis (Martins *et al*., 2004).

The requirement of K^+ for intracellular killing

The killing activity of neutrophils, although highly complex, has been shown by a series of elegant studies conducted by Segal's group (Reeves *et al*., 2002) to depend upon the availability of K^+ (Ahluwalia *et al*., 2004) to the phago-lysosome and the dependence of this process on active Ca^{2+} channels of the phago-lysosome unit. The essential need for Ca^{2+} required for the availability of K^+ involves a Ca^{2+} dependent ATPase that is employed for the generation of energy required in K^+ transport. Because the K^+ concentration needed to trigger the acidification process required for the activation of hydrolases (Pillay *et al*., 2002) is higher than that present in the cytoplasm (Ahluwalia *et al*., 2004; Munsiff *et al*., 2003) one would have to assume that the membrane of the phago-lysosome complex would contain the required energy driven efflux pumps. Because phenothiazines do transform non-killing macrophages into effective killers (Crowle *et al*., 1992; Ordway *et al*., 2002a, 2002b, 2003b) and because phenothiazines are potent inhibitors of K^+ transport processes that are dependent upon Ca^{2+} dependent ATPase, it seems probable that killing is enhanced due to the inhibition of efflux of K^+ from the phago-lysosome by the phenothiazine. If this hypothesis is correct, then one would predict that inhibitors of K^+ transport would also enhance the killing activity of non-killing macrophages. As is evident from Figure 2 ouabain, verapamil and reserpine,

inhibitors of K^+ transport processes, also enhance the killing activity of non-killer macrophages (Martins *et al*., 2006b) (**Chapter VI**).

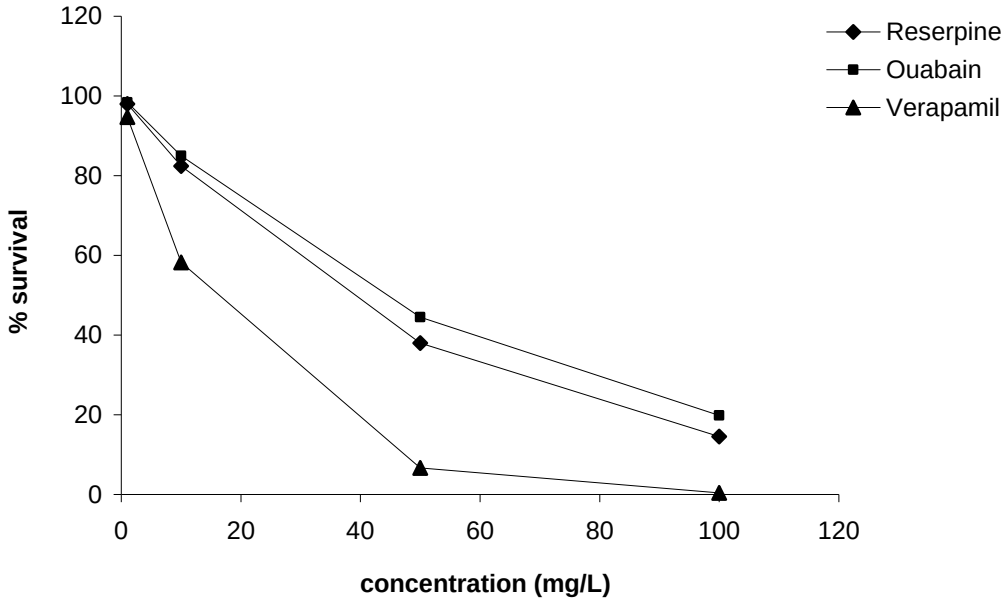


Figure 2. Effects of ouabain, verapamil and reserpine in the killing activity of human monocyte-derived macrophages (Martins *et al*., 2006b).

The question of whether the K^+ efflux pump units pre-exist in the lysosome or are part of the phagosome unit and hence have their origins in the plasma membrane of the macrophage has not yet been addressed. Nevertheless, with the exception of smooth muscle (Aydemir-Koksoy and Allen, 2001) the plasma membrane of most mammalian cells has a plethora of K^+ transport units (Terreros and Tiedemann, 1991), in all likelihood the plasma membrane that delineates the phagosome probably contains many K^+ transport units whose origins are from the plasma membrane of the macrophage and which due to the invagination process that results in the phagosome, would pump K^+ from the lumen of the phagosome to the cytoplasm, a region of the cell that is known to have a high concentration of the ion (Gribble *et al*., 2000) (Figure 3A – I to III). It is at these K^+ transport sites of the phagosome where ouabain, verapamil, reserpine and phenothiazines inhibit K^+ efflux and thereby the concentration of K^+ within the phagosome would be maintained to levels needed for the acidification of the phago-lysosome, and ensuing activation of the hydrolases (Ahluwalia *et al*., 2004; Reeves *et al*., 2002) (Figure 3A – IV). However, recent studies have shown that the killing activity of monocyte-derived macrophages which are identical to those used in the experiments

described by Figure 2, is dependent upon extracellular Ca^{2+} and extracellular ATP (Fairbairn *et al*., 2001; Kusner and Barton, 2001) both of which are under transport control mediated by Ca^{2+} dependent ATPase. As is the case for the K^+ transport systems, the transport process for Ca^{2+} would be reversed in the phagosome if this system were to be retained in the plasma membrane that delineates the phagosome unit. Phagocytosis of the bacterium by macrophages should result in a phagosome that contains K^+ and Ca^{2+} transport systems that pump out these ions to the cytoplasm of the cell. It is hypothesized that failure to kill bacteria present in the phagosome-lysosome is due to the low concentration of these ions in the complex that results from their extrusion to the cytoplasm. The inhibition of either one or both of these processes by a phenothiazine may establish conditions by which the concentration of these ions can be increased to a point where other ATP driven membrane exchange systems involved in the cytosolic homeostasis are activated leading to an intense hydrolytic activity of the phagosome-lysosome ATPases, in particular the V-ATPases (Fairbairn *et al*., 2001; Kusner and Barton, 2001; Pillay *et al*., 2002) (Figure 3B - I to VII). The import of H^+ thereby creating the acidification of the lysosome component that had fused with the phagosome and the activation of the hydrolases needed for the killing of the bacterium. These ATP driven membrane exchange systems must also be immune to ouabain, phenothiazines, *etc*. Figures 3A and B summarizes the hypothetical model which describes the aforementioned events that ultimately transform non-killer macrophages into effective killers. The phagocytosis of the bacterium (Figure 3A) results in a phagolysosome whose pumps extrude K^+ and Ca^{2+} from the vacuole to the cytoplasm (IV). When a phenothiazine is presented to the macrophage that has phagocytosed the mycobacterium (Figure 3B), the efflux of K^+ and Ca^{2+} from the phagolysosome is prevented, the build up of H^+ protons takes place, the hydrolases are now activated and the bacterium is digested (Figure 3B-VII).

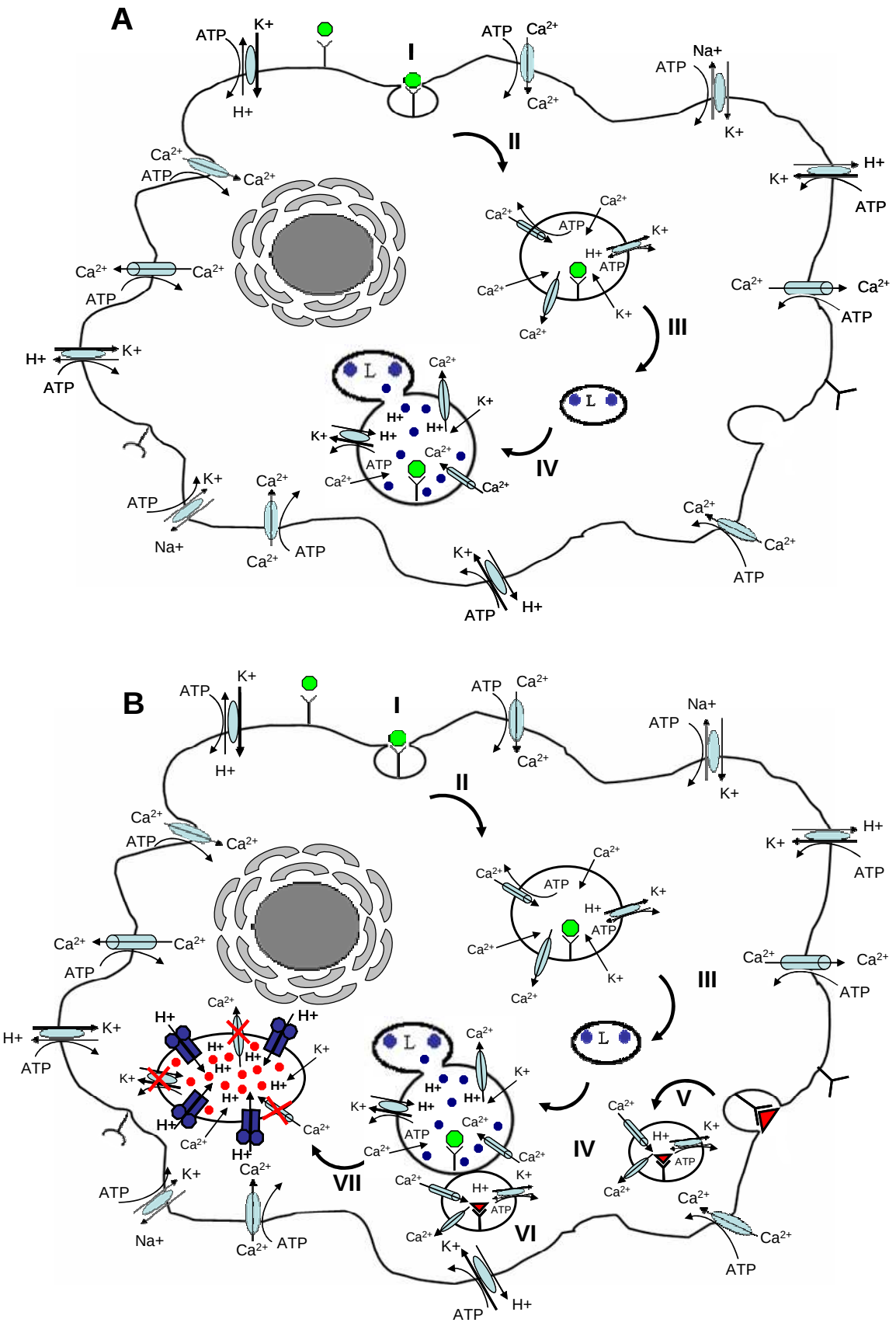
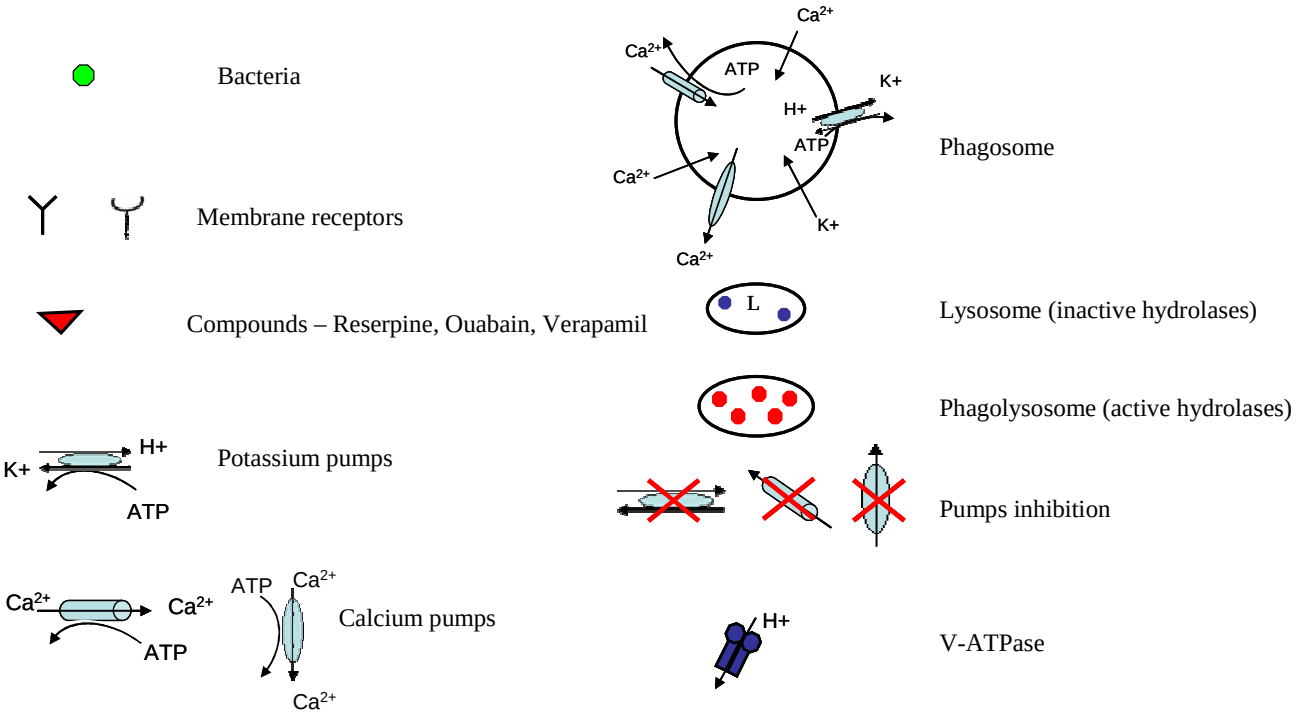


Figure 3. Hypothetical model suggested for the enhancement of killing of human monocyte-derived macrophages by phenothiazines and other efflux pumps. (A) Infected and untreated macrophage and (B) Infected and treated macrophage.

Legend: I: Binding of bacteria (●) to plasma membrane. **II:** Invagination and formation of phagosome. **III and IV:** Maturation of phagosome and fusion of the lysosome with the phagosome. **V:** Binding of inhibitor of efflux pump (▼) and formation of vesicle. **VI:** Fusion of vesicle containing the inhibitor of efflux pumps with matured phago-lysosome. **VII:** Inhibition of Ca^{2+} and K^{+} efflux by inhibitor of efflux pump; K^{+} leaks into phago-lysosome with cytosolic homeostasis mechanisms activated leading to an increased activity of the ATPases; acidification; activation of hydrolases; killing of bacteria.

Key:



The search for inhibitors of efflux pumps which may also enhance the killing activity of non-killing macrophages

Our previous studies demonstrated unusual properties of an extract made from the nuisance plant *Carpobrotus edulis* (Martins *et al*., 2005; Ordway *et al*., 2003a). Among these were: 1) the ability to invoke Th1 responses of a variety of T cell subsets as well as to stimulate the production of cytokines that are involved in the immune response to an infectious agent; 2) the ability to render highly resistant mouse lymphoma cells that contain the mdr efflux pump transport pg1 completely susceptible to cytotoxic agents; 3) the ability to inhibit the mdr efflux pump of these mouse lymphoma cells as evident from the increased retention of the efflux pump substrate rhodamine 1,2,3; and 4) the ability to enhance the killing of intracellular (phagocytosed) *M. tuberculosis* as well as

Mycobacterium avium (Viveiros *et al*., 2005c) whereas the highest concentration of the extract was devoid of any *in vitro* activity against these mycobacteria. Although it is not yet known whether the activities noted for the *C. edulis* extract are due to one or more substances, because the extract and the inhibitors of K^+ transport (ouabain, reserpine and verapamil) enhance the killing of intracellular bacteria by non-killing macrophages whereas they do not have any *in vitro* activity against these bacteria, it seems possible that the activity noted for the extract is due to one agent, and that this agent manifests its effects via the inhibition of K^+ transport (**Chapter VII**). Other plant extracts as well as plant derived compounds that have been shown to inhibit efflux pumps of cancer cells also have activity against phagocytosed bacteria. The suggestion that inhibitors of the paraglycoprotein of mammalian cells may also inhibit the efflux pump of bacteria receives support from the studies that used piperidine, an alkaloid isolated from the fruits of *Piper longum* (Lee *et al*., 2005) and its derivative piperine, to inhibit the paraglycoprotein of Caco-2 cells (Bhardwaj *et al*., 2002) as well to increase the retention of ethidium bromide by *S. aureus* by the inhibition of efflux activity (Khan *et al*., 2006). Polyphenols obtained from plant sources have been shown to inhibit efflux pumps of Caco-2 cells (Sergent *et al*., 2005) and have been shown to enhance the killing of intracellular *M. tuberculosis* (Anand *et al*., 2006).

Other plant derived agents that have activity against efflux pumps of cancer cells may also enhance the killing activity of macrophages against bacteria, perhaps even mycobacteria. Voacamine, a bisindolic alkaloid from *Peschiera fuchsiaefolia,* induces a significant increase of drug retention of cancer cells by its ability to inhibit the mdr transporter protein P-glycoprotein (Meschini *et al*., 2005). Irofulven, a novel anticancer agent derived from the mushroom reverses the resistance of cancer cells to cytotoxic agents by inhibiting the mdr efflux pump responsible for this resistance (Poindessous *et al*., 2003). Curcumin mixture and three major curcuminoids purified from turmeric (curcumin I, II, and III) have been shown to modulate the function of MRP1, the mdr protein 1 (MRP1) of HEK293 cells stably transfected with MRP1-pcDNA3.1 (Chearwae *et al*., 2006). These and many other plant derived compounds that are active against efflux pumps of cancer cells may serve as lead compounds for the synthesis of new agents that have activity against intracellular bacteria, and will be explored in the continuation of this work as important sources of new antimicrobial agents.

CHAPTER XI.

Concluding Remarks and Future Perspectives

Tuberculosis continues to be considered the major cause for worldwide morbidity and mortality caused by an infective organism. Since TB remains a serious medical problem around the world, the need for new anti-TB drugs is more and more prominent. The resurgence of TB in some urban centers, allied to the appearance and escalation of MDR-TB and XDR-TB worldwide has become the cause of increased medical and political concern. Since these intracellular infections target the alveolar macrophage, compounds presenting activity against these bacteria have to reach its intracellular site. However, most of the compounds reported to have activity against these infections, are most of the time restricted to *in vitro* activity and by this manner, ineffective. In order to obtain new compounds for the treatment of these infections, a novel approach in the drug design should be considered. It would be wiser to develop compounds that enhance the killing activity of the infected macrophages instead of the traditional approach implemented until the present time. If we design drugs that target and activate the phagocytic cells instead of acting on the bacteria itself, then the problem of resistance could also be avoided.

Phenothiazines are a group of tricyclic compounds whose antimicrobial activity has been known for over a century. The resistance associated to its clinical use is mainly due to the side-effects associated with its long-term use. However, toxic side-effects occur infrequently with new phenothiazine neuroleptics, such as TZ, that is less toxic than CPZ and that is also used for the treatment of psychosis. In fact, the major side effect is somnia although it is now known to produce torsades de pointes (Vieweg and Wood, 2004), but at a very low frequency (1 sudden death over 10,000) man years of use (Glassman and Bigger, 2001). TZ has *in vitro* antimycobacterial properties, being effective against clinical strains of *M. tuberculosis* regardless of their antibiotic susceptibility status (Amaral *et al*., 1996). Based on this evidence, TZ might be useful as an adjuvant to current regimens used for the management of freshly diagnosed TB (Viveiros and Amaral, 2001). The chemical manipulation of the parent phenothiazine sometimes yields a molecule that is more active than the parent compound (Csiszar and Molnar 1992; Molnar *et al*., 1991). Based on this, alternative strategies such as TZ derivatives should be considered. This could constitute a good approach to obtain more efficient compounds that lack the problems previously stated for the parental molecule.

Clinical Concentrations of Thioridazine Enhance the Killing of Intracellular Methicillin-resistant S*taphylococcus aureus*: an *In Vivo, Ex Vivo* and Electron Microscopy Study (detailed in Chapter III)

The results presented in this study showed that TZ kills intracellular MRSA at concentrations that are well within those clinically achieved for the management of psychosis. MRSA exposed to *in vitro* concentrations of TZ just below its MIC showed ultrastructural changes of the bacterial cells. TZ inhibited the replication of phagocytosed MRSA, affecting the structure of the cell envelope that resulted in the lysis of the bacterium 6 hours post-phagocytosis. The effects of this agent on the ultrastructure of phagocytosed MRSA parallels that observed *in vitro*.

Macrophages are cells rich in lysosomes and these organelles are known to concentrate compounds. When the bacteria are phagocytosed by the macrophage it resides within phagosomes and when this organelle fuses with the lysosome the bacteria is killed. Furthermore, because lysosomes are known to concentrate the phenothiazine and the phagocytosed organisms are clearly within cell organelles that have the characteristics of lysosome-phagosomes, it was postulated that it is in the lysosomal-phagosome structure that the microbe is killed. Support was provided for the contention that TZ has potential for the management of MRSA intracellular infections that are difficult to treat due to the privileged site offered by the macrophage, which can serve as sources for subsequent recurrent infections. By this manner, TZ, as well as other phenothiazines, has potential for the management of MRSA intracellular infections that are difficult to treat.

In vitro* and *ex vivo* activity of TZ and its derivatives against *Mycobacterium tuberculosis* and *M. avium (detailed in Chapter IV)

Clinical concentrations of thioridazine kill intracellular multidrug-resistant *Mycobacterium tuberculosis*

TZ has equal *in vitro* activity against antibiotic-sensitive and -resistant *M. tuberculosis*. However, it has not been used as anti-*M. tuberculosis* agent because its *in vitro* activity takes place at concentrations that are beyond those clinically achievable. We have

performed studies to evaluate if TZ kills *M. tuberculosis* that has been phagocytosed by human macrophages. The results obtained demonstrated that when TZ was used at concentration in the medium well below that present in the plasma of patients treated with this agent, it killed intracellular antibiotic-sensitive and -resistant *M. tuberculosis*. This *in vitro* concentration was not toxic to the macrophage and complete killing was obtained after 3 days post-infection at a concentration of 0.1 mg/L of TZ. When *M. tuberculosis* strains were exposed to *in vitro* concentrations of TZ, the minimum concentration required to completely inhibiting growth ranged from 8 to over 30 mg/L and the MBC ranged from 20 to 50 mg/L. The concentration of these compounds required to kill *M. tuberculosis in vitro* can be achieved inside the macrophage since these cells have the ability to concentrate compounds inside their lysosomes and phagosomes (structures were the entrapped phagocytosed bacterium is located). It was also demonstrated that clinically relevant concentrations of TZ kill phagocytosed mycobacteria. These results support the use of TZ in the management of intracellular *M. tuberculosis* infections. However, its use has some risks associated such as the case of cardiac failure or episodes of torsade de pointes that might result in sudden deaths (10 to 15 such events in 10,000 person-years of observation (Glassman and Bigger, 2001). However, these episodes are rare and usually associated with prolonged therapy. In this sense, the use of TZ for the management of an active pulmonary TB infection caused by antibiotic-susceptible *M. tuberculosis* strains provides no advantage over the use of present therapies. However, due to the severity of MDR *M. tuberculosis* in some parts of the world where effective therapy is not possible, the use of TZ deserves consideration. Therefore, TZ can be used as an adjunct to conventional therapy during initial management of the patient, until the susceptibility data for the causative organism are available.

In vitro and *ex vivo* activity of thioridazine derivatives against *Mycobacterium tuberculosis*

TZ has been shown by us to have *in vitro* and *ex vivo* activity against antibiotic-susceptible and MDR-TB. The current therapy of MDR-TB is highly problematic even when all the ‘first line of defence’ drugs are employed. Therefore, the need for effective antituberculosis drugs is obvious. Twenty-two new derivatives of TZ were synthesised and their *in vitro* activity against a reference strain of *M. tuberculosis* evaluated. The

results obtained showed that all of the TZ derivatives tested had activity against the *M. tuberculosis* reference strain H37Rv and derivatives #1867, #1870 and #1875 had similar activity to that of TZ. Toxicity of the derivatives, assayed by the Trypan Blue exclusion method, showed that at concentrations equivalent to that associated with TZ, no significant toxicity was detected. Derivatives that showed significant *in vitro* activity were then evaluated by *ex vivo* studies and were shown to enhance the killing of intracellular *M. tuberculosis* at a concentration of 0.1 mg/L. Some of the derivatives tested even demonstrated higher *ex vivo* activity than TZ, since after one day no phagocytosed *M. tuberculosis* could be retrieve from the cultures. These results demonstrate the *ex vivo* potential of TZ derivatives against *M. tuberculosis* strains.

The *in vitro* activity of phenothiazines against *Mycobacterium avium*: potential of thioridazine for therapy of the co-infected AIDS patient

Patients presenting with AIDS are predisposed to co-infection with *M. avium*. In order to manage these infections there is the need for effective and non-toxic compounds. Since *M. avium* presents a very high resistance to most of the available compounds, we evaluated the effect that phenothiazines have on these strains. Therefore, we assessed the *in vitro* activity of CPZ, TZ, promazine, promethazine and desipramine against *M. avium* strains. The results obtained showed that CPZ, TZ, PMZ, PMTZ and DSP have *in vitro* activity against *M. avium*, showing MICs that ranged from *ca*. 10 to >50 mg/L. These results had previously been obtained with *M. tuberculosis*. TZ was the most active against *M. avium*, having an MIC of 10 mg/L. These results highlight the importance of TZ to treat these infections. However, at the present time it is not recommended for the management of AIDS patients co-infected with *M. avium* since more studies are needed, namely with infected macrophages.

The Curative Activity of Thioridazine on Mice Infected With *Mycobacterium tuberculosis* (detailed in Chapter V)

Animal studies were initiated to establish the effectiveness of TZ. BALB/C mice were infected with *M. tuberculosis* ATCC H37Rv strain. After thirty days, treatment with TZ was initiated, except for the control group. Animals were then treated with different concentrations of TZ ranging from 0.05 to 0.5 mg/day (at equivalent concentrations to

that used in the humans). The results showed that a daily dose of 0.5 mg/day of TZ reduced the number of CFU retrieved from the lungs of the infected mice within one month in comparison to the control group. These results reflect the effectiveness of TZ. However, by the end of the 300 days of therapy, mycobacteria could still be recovered from the organs of the infected animals. Hence, the infection persisted, albeit at a very low level. This could be explained by the increase in the number of CFU obtained from the liver and spleen of the infected animals that could be a continuous source of mycobacteria. However, considering the massive dose of infection, it is important to stress the effectiveness of TZ therapy. Nevertheless, it is important to note that although TZ is effective for the therapy of pulmonary TB of the mouse, other than anecdotal evidence and laboratory studies that support the use of TZ, its effectiveness for therapy of human pulmonary TB remains to be performed. More studies are needed not only using TZ but also the TZ derivatives that demonstrated a higher activity than TZ on the infected macrophages.

Inhibition of the *Carpobrotus edulis* methanol extract on the growth of phagocytosed multidrug-resistant *Mycobacterium tuberculosis* and methicillin-resistant *Staphylococcus aureus* (detailed in Chapter VI)

We tested a methanolic extract of *C. edulis* against MRSA and MDR-TB strains. Because fivefold concentrations of the plant extract which produce toxicity against monocyte-derived macrophages does not inhibit the growth of methicillin-resistant *S. aureus* or multidrug-resistant *M. tuberculosis*, we conclude that the effectiveness of the highly diluted extract (tenfold) on the growth of phagocytosed methicillin-resistant *S. aureus* and multidrug-resistant *M. tuberculosis* may be due to the ability of the monocyte derived macrophages to concentrate the extract beyond the highest concentration employed *in vitro*. Our results correlate with the antimicrobial effects observed with the traditional uses of *C. edulis* described (Van Wyk *et al*., 1997) and suggest that this plant may serve as a source of new antimicrobial agents that are effective against problematic drug-resistant intracellular infections.

Reserpine, ouabain and the calcium channel blocker verapamil, cause intracellular killing of *Staphylococcus aureus* (detailed in Chapters VI and X)

Monocyte-derived macrophages obtained from the peripheral blood have little killing activity of their own. However, these cells can be transformed into effective killers of intracellular bacteria by *in vitro* exposure of the cells to clinically relevant concentrations of TZ and other phenothiazines. Some studies had also reported that the neutrophils killing activity is highly dependent upon the availability of K^+. Because mechanisms dependent upon the availability of Ca^{2+} are inhibited by phenothiazines, we investigated the possibility if that these and other compounds presenting similar activity also have the ability to enhance the killing of bacteria by the macrophage derived monocytes. The compounds selected were inhibitors of Ca^{2+} and K^+ flux, namely, ouabain, reserpine and verapamil. In this study we showed that the presence of increasing concentrations of ouabain, reserpine or verapamil in the medium have the capacity to enhance the killing of *S. aureus* that have been phagocytosed by monocyte derived macrophages. From our previous studies (Ordway *et al*., 2002a, 2003b) the enhancement of the macrophage killing activity in the presence of phenothiazines was suggested to be due to the concentration of the compounds inside the macrophage. If this takes place, the compounds could reach concentrations comparable to those achieved *in vitro* and presenting bactericidal activity. However, these concentrations of ouabain, verapamil and reserpine had no activity on the replication or killing of the *S. aureus*, and by this manner, the killing was deemed to be due to the macrophage itself. The results obtained suggest that these agents have similar activity to phenothiazines and we attribute their intracellular activity with respect to their effects on the inhibition of the transport of K^+ and Ca^{2+}. A model was developed describing the mechanism by which these agents and phenothiazines indirectly activate lysosomal enzymes as a result of the inhibition of K^+ efflux pumps. However, more studies were also conducted on macrophages infected with *S. aureus* and *M. tuberculosis* strains in order to clarify the enhancement of the macrophage killing activity by these K^+ and Ca^{2+} inhibitors.

Inhibitors of Ca^{2+} and K^+ Transport Enhance Intracellular Killing of *M. tuberculosis* by Non-Killing Macrophages (detailed in Chapters VI and X)

Our previous studies demonstrated that monocyte derived macrophages kill intracellular *S. aureus* when cultured in the presence of inhibitors of Ca^{2+} and K^+ efflux pumps. The aim of this study was to evaluate the effect of ouabain, reserpine and verapamil in the killing activity of macrophages infected with *M. tuberculosis*. After three days post-infection, macrophages treated with the inhibitors demonstrated an enhancement of the killing activity destroying the phagocytosed mycobacteria.

These results suggest a different approach for the therapy of TB, and especially, MDR-TB and XDR-TB. This approach involves the design of compounds that target the macrophage and transform it into an effective bacterial killer. Compounds which target the bacterium are predicted to become ineffective due to mutations of the bacterial target, however, agents that enhance the killing activity of the infected macrophage will not present this disadvantage. By this manner, these compounds that enhance killing by macrophages but that normally do not kill mycobacteria may yield a more effective form of therapy of infections caused by MDR and XDR-TB. This is a viable and innovative approach that could be applied for the therapy of other intracellular infections such as malaria, leishmaniasis, among others. These results highlight the effectiveness of inhibitors of Ca^{2+} and K^+ transport processes that could be applied for the therapy of intracellular infections.

SILA compound 421, an inhibitor of efflux pumps of cancer cells, enhances the killing of intracellular MDR-TB (detailed in Chapter VII)

As already mentioned Multi-Drug Resistant (MDR) and Extensively-Drug Resistant (XDR) Tuberculosis (TB) infections are problematic to manage. Therefore, there is a dire need for effective anti-TB agents. From the studies conducted by our team, we have shown that agents that inhibit efflux pumps of MDR bacteria and cancer cells also enhance the killing of intracellular mycobacteria. This killing is possibly associated with the increasing of K^+ and Ca^{2+} needed for the activation of lysosomal enzymes. We have evaluated a newly synthesized and recently patented SILA compound 421 for its *in vitro* and *ex vivo* activity against an XDR-TB strain. The minimum *in vitro*

concentration that inhibits the growth of the strain was determined, as well as the effect of this compound on the killing activity of human monocyte-derived macrophages infected with the XDR-TB strain. SILA compound 421 was shown to have *in vitro* activity against XDR-TB, with an MIC below 3.5 mg/L, and also to transform non-killing macrophages into effective killers of phagocytosed XDR-TB. From the toxicity studies performed no toxicity was demonstrated at the concentrations used on the studies. The fact that this compound is devoid of any cytotoxic activity makes it a very good candidate for the treatment of XDR-TB and MDR-TB infections.

An instrument-free method for the demonstration of efflux pump activity of bacteria (detailed in Chapter VII)

The search for new compounds that present activity against MDR infections is one main goal of all the work developed during this Thesis. However, most of the times, this search is associated with methods that are expensive and non-reproducible. With this in mind, we have developed a simple, inexpensive and reproducible method – the ethidium bromide (EB)-agar based method. This method requires no specialized instrumentation and allows the demonstration of efflux pump activity, responsible for antibiotic resistance of bacteria. Since EB is a well known substrate for efflux pumps, the method is based on a series of agar plates containing varying concentrations of EB. The plates are then swabbed with the bacterial strains that are to be studied and the plates incubated at different temperatures and time-points. The fluorescence obtained from the strains is then used to evaluate the efflux activity of each culture. From the results obtained we where able to identify the efflux of EB in different bacteria, namely *E. coli* and *S. aureus* strains. The minimal concentration of EB that produced fluorescence was significantly greater at 37ºC than at 4ºC, suggesting the presence of an energy-dependent pump. It was also possible to identify strains of a mixed culture that differed from each other with respect to the activity of their efflux pumps. This is a simple, inexpensive and reproducible method that may be employed for inter-laboratory standardization of efflux pump activity of MDR clinical isolates, allowing the fast screening of a large collection of bacteria. A new improved version of this method (Carthweel-agar EB method) is being currently developed and tested at the Unit of Mycobacteriology and the results are currently the subject of a manuscript to be submitted to an International Peer-reviewed journal.

The future of chemotherapy (detailed in Chapter VIII)

The discovery of new anti-microbial agents that are known inhibitors of efflux pumps is determinant in the therapy of MDR bacteria, such as, MDR-TB. To treat these infections, new approaches are urgently needed. The use of these agents in conjunction with the classical antibiotics could provide the basis for a novel strategy implemented in the management and control of MDR infections. The group of compounds termed 'non-antibiotics' exhibit properties that render them important for the therapy of MDR infections. However, with the exception of infections caused by MDR-TB and XDR-TB where some of these have promise, non-antibiotics are at this time best considered as 'helper compounds' to be co-administered with conventional antibiotics to which the MDR organism was initially resistant. Nevertheless, regardless of how certain we may feel regarding their use, it still remains for clinical trials to demonstrate their importance in the therapy of some of the most serious bacterial infections. Because TZ has *in vitro* and *ex vivo* activity against XDR-TB and MDR-TB and cures the mouse of a pulmonary infection produced by *M. tuberculosis,* it is recommended that this phenothiazine be considered for the management of XDR-TB and MDR-TB infections with poor prognosis if only for compassionate reasons. Taking into account the certainty of mortality produced by XDR-TB infections that are immune to available antibiotics, the risks associated with TZ are minimal.

The Tuberculosis 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 (detailed in Chapter IX)

The global resurgence of pulmonary TB, the resulting development of MDR-TB, and now, the progression of antibiotic resistance to practically any available antibiotic as is the case for XDR-TB strains, is due in large part to the failure to recognize laboratories as a corner-stone of TB control policy (Goldman *et al*., 2007; WHO, 2006b). Newly developed molecular methods for the direct screening of TB from smear-positive samples, which also detect the mutations most frequently associated with RIF and INH resistance, are now referred by the WHO, in order to early detect MDR-TB cases. The guidelines recently proposed at the WHO Laboratory Strengthening Task Force recommend rapid molecular methods for detection as an essential tool for TB and

MDR/XDR-TB control (WHO, 2006a). Rapid identification of pulmonary TB and XDR/MDR-TB makes it possible to provide effective therapy and hence interfere with the cycle of transmission from patient to patient that is currently experienced. Hence, there is a need for new and effective biotechnological strategies to detect TB and XDR/MDR-TB as well as develop alternative therapies (Pai *et al*., 2006; Palomino, 2006). The biotechnological approach presented in this Thesis aimed to identify XDR/MDR-TB rapidly and predict the most effective therapeutic combination to maximize a favorable outcome in the patient that is being treated. However, given the fact that resistance of antibiotic sensitive *M. tuberculosis* to each of the compounds of the "second line of defence" exceeds resistance to INH and RIF; and, given the fact that XDR-TB strains are far more resistant to these compounds (Hamilton *et al*., 2007; Migliori *et al*., 2007), it is obvious that there is a dire need for new and effective anti-tubercular compounds. It is therefore strongly suggested that the macrophage model be used during drug development phase. Moreover, and of more immediate importance, we recommend that the model as described be used for the evaluation of "second-line of defence" drugs for activity against intracellular mycobacteria (Martins *et al*., 2008). This latter recommendation will, we believe, provide the eventual basis for the intelligent selection of drugs to be used for the therapy of MDR-TB/XDR-TB infections.

Enhanced killing of intracellular pathogenic bacteria by phenothiazines and the role of K^+ efflux pumps of the bacterium and the killing macrophage (detailed in Chapter X)

The determination of anti-TB properties of any given agent must be proven by the use of the macrophage model presented regardless of its *in vitro* activity. Although few published studies have made the leap from *in vitro* activity to a successful demonstration of the effectiveness of an agent in an *M. tuberculosis* infected mouse, most studies that have gone directly from an *in vitro* to an *in vivo* model fail, and are therefore not published. These failed studies are expensive and time consuming. Hence, we recommend the use of the staphylococcus-macrophage model, and if successful, proceed with mycobacteria, and only if the agent is significantly effective and produces no cytotoxicity, proceed to the *M. tuberculosis* infected mouse model.

The demonstration that inhibitors of Ca^{2+} and K^{+} transport cause the killing of phagocytosed mycobacteria significantly alters the approach employed for the design of anti-TB agents. Because phenothiazines enhance the killing of intracellular mycobacteria, they should be seriously considered as sources for new anti-TB agents. Because these agents by-pass the possibility of mutation of a mycobacterial target, they have the potential to be used for considerable periods of time. Nevertheless, it remains for future studies to determine whether these considerations are to be realised.

Phenothiazines as Anti-Multi-Drug Resistant Tubercular Agents (detailed in Chapter X)

The *in vitro* activity of phenothiazines against *M. tuberculosis* is now well known. Nevertheless, these activities take place at concentrations which are beyond clinical relevance. The effectiveness of any compound against pulmonary TB, regardless of its *in vitro* activity, is dependent upon the ability of the agent to kill the bacterium where it normally resides post-infection. That phenothiazines have activity *in situ* against intracellular mycobacterium at concentrations that are comparable to those expected in the plasma of subjects treated with these agents provide a degree of prediction of its effectiveness when the agent is to be used for the therapy of active disease. That this prediction is probable is supported by recent studies that employed TZ successfully for the therapy of mice presenting with active pulmonary TB (Weinstein *et al*., 2005; Yano *et al*., 2006). Although we do not recommend that TZ of any other phenothiazine be used for therapy of antibiotic susceptible active TB, inasmuch as existing antibiotics are effective, we do recommend that this agent be considered for the therapy of MDR-TB if and when all other agents have failed and mortality is certain. In other words, to be used as "compassionate therapy" (Amaral and Kristiansen, 2000; Amaral *et al*., 2001a; 2001b; 2006). When employed as compassionate therapy, we recommend that the dose and schedule employed for the therapy of psychoses be used. Because therapy would be limited to months as opposed to years, rare but serious side effects associated with chronic use are not anticipated (Amaral and Kristiansen, 2000; Amaral *et al*., 2006). Lastly, we cannot stress enough the need for studies that evaluate the effectiveness of an active *in vitro* agent via *ex vivo* methods. The use of the macrophage-*Mycobacterium* assay provides distinctive advantages that are not evident with *in vitro* assays alone. Because the *ex vivo* assay is very inexpensive and yields results quickly as compared to

the use of *in vivo* (mice studies), we urge that they be employed prior to the use of *in vivo* assays.

Enhanced killing of intracellular multi-drug resistant *Mycobacterium tuberculosis* by compounds that affect the activity of MDR efflux pumps: a review.

The global rise of MDR-TB continues and the need for new effective agents is obvious. Because MDR-TB is an intracellular infection of the non-killing macrophage of the lung, any drug that is to be effective must have activity at this site. Conventionally, anti-tubercular drugs are designed to have direct activity against intracellular MDR-TB. And as has been the case for all other antibiotics, resistance would ensue with usage of the compound. Because the non-killer macrophage can be transformed into an effective killer by a variety of compounds that inhibit K^+ transport, perhaps it would be wiser to develop drugs that enhance the killing activity of these cells inasmuch as this approach would not be subject to any resistance as is the eventual case for conventional antibiotics. A model describing the mechanisms, by which distinct efflux pumps of the phagosome-lysosome complex are inhibited by agents that are known to inhibit K^+ flux, was developed and presented. This macrophage model predicts the existence of a K^+ activated exchange putative pump, probably located in the membrane that delineates the lysosome and that is immune to the inhibitors of K^+ flux which is identified as being the cause for the acidification of the lysosome thereby activating its hydrolytic enzymes. Since those non-killer macrophages could be transformed into effective killers by a variety of compounds that inhibit K^+ transport, it would be wiser to develop drugs that enhance the killing activity of these cells inasmuch as this approach would not be subject to any resistance as is the eventual case for conventional antibiotics.

The data obtained in this Thesis, have provided direction for the selection of which compounds should be further studied for the curative activity of MDR infections. It is therefore strongly suggested that the macrophage model be used during drug development phase. Moreover, and of more immediate importance, we strongly recommend that the model described be used for the evaluation of “second-line of defence” drugs for activity against intracellular mycobacteria. We believe that this latter recommendation will provide the eventual basis for the intelligent selection of drugs to be used for the therapy of MDR-TB and XDR-TB infections.

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