Mycobacterium tuberculosis, a major threat to health in South Africa: intracellular survival after treatment with novel drugs designed against the mycothiol pathway

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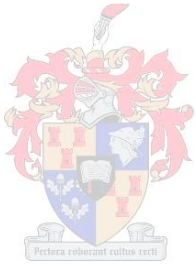

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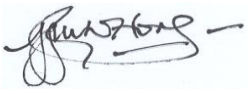
November 2010

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously, in its entirety or in part, been submitted at any university for a degree.

Signature: 

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ABSTRACT

Mycothiol (MSH) is unique to mycobacteria as the major low molecular weight cellular thiol responsible for protection of bacteria against oxidative stress. The design of drugs and inhibitors against enzymes of the mycothiol pathway was based on the premise that mycothiol is unique to mycobacteria, and is thus important for its survival. A total of 80 inhibitors designed against enzymes of the mycothiol pathway were screened for inhibition of growth on *in vitro* growing *M. tuberculosis* using the BACTEC 460^{TM} assay. The most active compounds were further tested for inhibitory potential of *M. tuberculosis* within macrophages. Initial screening in the macrophage system was done using the human-like THP1 cell line and then mouse bone marrow-derived macrophages. In this investigation we established that phenothiazine can be exploited as an inhibitor of enzymes of the mycothiol pathway. Although tunicamycin significantly inhibited the growth of *M. tuberculosis* both *in vitro* and *ex vivo;* it was found to be cytotoxic to host macrophages. To this end we provide proof-of-concept that compounds which can inhibit the expression of mycothiol enzymes have potential as anti-tubercular drugs.

The response of *M. tuberculosis* to stress conditions was assessed via LC-MS in which maximal levels of mycothiol were produced during the early time points of exposure to isoniazid. We used mycothiol-deficient (*mshA*) *M. tuberculosis* to investigate the role of mycothiol for survival as well as the resultant phenotype when such mutants are exposed to stress conditions. The *mshA* deletion mutants in *M. tuberculosis* were resistant to INH at concentrations which inhibited growth in the wild-type strains. We postulated that *katG* and *inhA*, the genes involved in INH metabolism, required mycothiol for their activation.

Morphological alterations of *M. tuberculosis* within macrophages were assessed using electron microscopy approaches. In this way we attempted to follow the fate of *M. tuberculosis* within the phagosomes, and how mycobacteria is processed in phagosomes in terms of replication, survival and degradation. The establishment of a successful infection by *M. tuberculosis* depends on the initial encounter with host

macrophages, which represent the first line of cellular defense against microbial invasion. At the interface between mycobacteria and macrophages, the complex outermost layer of the mycobacterial cell wall probably plays a role in facilitating host cell entry. Under normal conditions (*i.e.* ingestion of non pathogenic microorganisms), newly formed phagosomes intermingle contents and membrane with the successive compartments of the endocytic pathway (early endosomes, late endosomes, lysosomes) through a complex series of fusion and fission. As they are processed into phagolysosomes, they undergo gradual modifications by specific addition and removal of membrane constituents. In addition, they become acidified due to the vacuolar proton pump ATPase located in the membrane and acquire toxic constituents, including hydrolases that will ultimately destroy bacteria.

OPSOMMING

Mycothiol (MSH) is uniek aan mycobacteria as die belangrikste lae molekulêre gewig sellulêre thiol verantwoordelik vir die beskerming van bakterieë teen oksidatiewe stres. Die ontwerp van dwelms en inhibeerders teen ensieme van die mycothiol pad is gebaseer op die veronderstelling dat mycothiol uniek is aan mycobacteria, en is dus belangrik vir sy oorlewing. ‘n Totaal van 80 inhibeerders ontwerp teen ensieme van die mycothiol pad is gekeur vir die inhibisie van groei op in vitro groeiende *M. tuberculosis* met behulp van die BACTEC 460^{TM} toets. Die mees aktiewe verbindings is verder getoets vir inhiberende potensiaal van *M. tuberculosis* binne makrofage. Aanvanklike sifting in die makrofage stelsel is gedoen met behulp van die mens-soos THP1 sel lyn dan makrofage afkomstig van muis beenmurg. In hierdie ondersoek het ons vasgestel dat fenotiasien kan gebruik word as ‘n inhibitor van ensieme van die mycothiol pad. Alhoewel tunicamycin aansienlik die groei van *M. tuberculosis* beide *in vitro* en *ex vivo* inhibeer, was dit gevind word sitotoksies is vir makrofage. Om hierdie rede het ons bewys-van-konsep wat verbindings dat die uitdrukking van mycothiol ensieme inhibeer, die potensiaal het as anti-tuberkulose dwelms.

Die reaksie van *M. tuberkulose* op stress is geëvalueer deur LC-MS waarin maksimum vlakke van mycothiol gedurende die vroeë tyd punte van blootstelling aan isoniasied geproduseer is. Ons gebruik mycothiol-deficient (*mshA*) *M. tuberculosis* om die rol van mycothiol vir oorlewing sowel as die gevolglike fenotipe te ondersoek wanneer sodanige mutanten blootgestel word aan stres kondisies. Die *mshA*-weglating mutanten van *M. tuberculosis* was bestand teen INH konsentrasies wat groei geïnhibeer in die wilde-tipe-stamme. Ons veronderstel dat *katG* en *inhA*, die gene wat betrokke is in INH metabolisme, mycothiol vereis vir hulle aktivering.

Morfologiese veranderinge van *M. tuberculosis* binne makrofage is beoordeel met behulp van elektronmikroskopie. In hierdie manier waarop ons probeer om die lot van *M. tuberkulose* te volg binne die phagosomes, en hoe mycobacteria verwerk word in phagosomes in terme van replikasie, oorlewing en agteruitgang. Die vestiging van ‘n suksesvolle infeksie deur *M. tuberculosis* hang af van die aanvanklike ontmoeting met host makrofage, wat die eerste lyn ‘n sellulêre verdediging teen mikrobiese inval

verteenwoordig. Op die grens tussen mycobacteria en makrofage, speel die komplekse buitenste laag van die mikobakteriese selwand waarskynlik ‘n rol in die intog van die gasheersel.

Onder normale omstandighede (dws inname van non patogene mikroörganismes), nuutgevormde phagosomes meng inhoud en membraan met die opeenvolgende kompartemente van die endositiese roete (vroeë endosomes, laat endosomes, lisosome) deur ‘n komplekse reeks van samesmelting en fisie. Soos hulle verwerk word tot phagolysosomes, ondergaan hulle geleidelike veranderinge deur spesifieke optel en verwydering van membraan komponente. Benewens, raak hulle versuur as gevolg van die vacuolair proton pomp ATPase geleë in die membraan en verkry giftige bestanddele, insluitend hydrolase wat uiteindelik bakterieë vernietig.

TABLE OF CONTENTS PAGE

ABSTRACT i

OPSOMMING iii

LIST OF TABLES ix

LIST OF FIGURES ix

ABBREVIATIONS xi

ACKNOWLEDGEMENTS xiii

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 TUBERCULOSIS: A HISTORICAL PERSPECTIVE

1.1.1 Current trends in the global epidemiology of TB 1

1.1.2 TB control programmes in South Africa 2

1.1.3 Spread and transmission of TB 2

1.2 CURRENT ANTI-TUBERCULAR DRUGS AND EMERGENCE OF DRUG RESISTANT STRAINS

1.2.1 Anti-tubercular drug interventions: Can TB be fully controlled? 3

1.2.2 Mechanisms of action of first-line TB drugs and the evolution of antibiotic resistance 4

1.3 SURVIVAL STRATEGIES OF *M. tuberculosis* WITHIN HOST MACROPHAGES

1.3.1 Entry of *M. tuberculosis* into the host macrophage 7

1.3.2 Survival of *M. tuberculosis* occurs via arrest of phagosome maturation 7

1.3.3 Intracellular responses and rescue mechanisms from hostile internal environment 8

1.4 MYCOTHIOL AS A POTENTIAL TARGET FOR NEW ANTI-TUBERCULAR DRUGS

1.4.1 Genetic control of mycothiol biosynthesis in *M. tuberculosis* 9

1.4.2 Biochemistry of mechanisms involved in the mycothiol pathway 11

1.4.3 Hypothesis 13

CHAPTER 2: DRUG TESTING AND MYCOTHIOL QUANTITATION ON *IN VITRO* GROWING *M. tuberculosis*

2.0 INTRODUCTION 14

2.1 MATERIALS AND METHODS

2.1.1 Culturing of *M. tuberculosis* 15

2.1.2 Monitoring *M. tuberculosis* growth in the presence of inhibitors of the mycothiol pathway via BACTEC 460™ system 15

2.1.3 Stress conditions for mycothiol production in *M. tuberculosis* 15

2.1.4 Sensitivity tests of WT and Δ*mshA* deletion mutants of *M. tuberculosis* 16

2.1.5 Mycothiol extraction methods from *M. tuberculosis* 16

2.1.8 Determination of mycothiol levels by LC-MS analysis 17

2.2 RESULTS AND DISCUSSION

2.2.1 Quantitation of mycothiol via quad-pole LC-MS 18

2.2.2 Mycothiol mutants (Δ*mshA*) grow poorly/do not grow on 7H9 without OADC 23

2.2.3 Mycothiol mutants (Δ*mshA*) are resistant to INH 24

2.2.4 Mycothiol mutants (Δ*mshA*) are resistant to arsenic (V) acid 25

2.2.5 Synthesis of drugs against enzymes of the mycothiol pathway 26

2.2.6 Antimicrobial activities of phenothiazines, tunicamycin, busulfan and nikkomycin 31

2.2.7Thiozolidinone libraries (1A-12A and 1B-12B) 33

CHAPTER 3: INTRACELLULAR DRUG TESTING

3.0 INTRODUCTION 37

3.1 MATERIALS AND METHODS 37

3.1.1 Culture conditions for the THP1 cell line 37

3.1.2 Macrophage infection 38

3.1.3 Evaluating inhibitory effects of novel compounds designed towards the mycothiol pathway 38

3.2 RESULTS AND DISCUSSION FOR INTRACELLULAR DRUG TESTING

3.2.1 Drug testing in macrophage assay 39

CHAPTER 4: ASSESSMENT OF MORPHOLOGICAL ALTERATIONS OF MYCOBACTERIA IN MACROPHAGES AFTER TREATMENT WITH INHIBITORS OF ENZYMES OF THE MYCOTHIOL PATHWAY: AND THE RESULTANT CONSEQUENCES ON PHAGOSOME MATURATION

4.1 INTRODUCTION 41

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains and culture conditions 42

4.2.2 Culturing of mouse bone marrow derived macrophages 42

4.2.3 Infection of macrophages with mycobacteria and treatment with selected drugs 43

4.2.4 HRP uptake and staining for HRP 43

4.2.5 Processing of samples for transmission electron microscopy 43

4.3 RESULTS AND DISCUSSION FOR ASSESSMENT OF MORPHOLOGICAL ALTERATIONS OF MYCOBACTERIA IN MACROPHAGES

4.3.1 Morphological appearance of infected macrophages treated with selected inhibitors 44

4.3.2 Ultra-structural appearance of *M. avium* and *M. tuberculosis* infected macrophages treated with phenothiazine (AJ-4) 46

4.3.2 Quantitative analysis of phagosome processing after treatment with AJ-4 48

CHAPTER 5: GENERAL DISCUSSION AND RECOMMENDATIONS FOR FUTURE DIRECTIONS

5.1 TB Research Opportunities 51

5.2 Structural biology (e.g. elucidations of TB/host interactions) 51

5.3 Target validations/drug efficacy (e.g. validations through chemical inhibition) 52

5.4 Therapeutics 52

5.5 Diagnostics 52

5.6 REFERENCES 53

5.7 APPENDICES 60

LIST OF TABLES

Table 1.1: Anti-tuberculosis drugs and genes involved in antibiotic resistance 5

Table 2.1: Relative amounts of mycothiol for *M. tuberculosis* H37Rv and Beijing INH^R strain 19

Table 4.1: Percentages of intact intracellular bacilli aafter treatment with AJ-4 55

Table 4.2 Proportions of loner and social phagosomes that fused with lysosomes 55

Table 4.3 Proportions of loner and social phagosomes that fused with lysosomes 55

LIST OF FIGURES

Figure 1.1*:* Life cycle of *M. tuberculosis* within the human host and formation of granuloma 3

Figure 1.2*:* Pathways inhibited by first line drugs against *M. tuberculosis* 6

Figure 1.3**:** Progression from endosome to phagosome maturation 8

Figure 1.4*:* Pathway for biosynthesis, recycling of mycothiol and detoxification of xenobiotics 10

Figure 2.1*:* Structures of inhibitors of mycothiol enzymes tested (AJ-3 – AJ-8) 27

Figure 2:2: Structures of inhibitors of mycothiol enzymes tested (1A – 12A) 28

Figure 2:3: Structures of inhibitors of mycothiol enzymes tested (1B – 12B) 29

Figure 2:4: Structures of inhibitors of mycothiol enzymes tested (T1C – T12C) 30

Figure 2.5: Extracellular inhibition activities of AJ-3 – AJ-8 31

Figure 2.6**:** Extracellular inhibition activities of 1A – 12A 33

Figure 2.7**:** Extracellular inhibition activities of A, B series and AJ-8 34

Figure 2.8 Extracellular inhibition activities of T1A – T12A series 34

Figure 2.9**:** Extracellular inhibition activities of T1B – T12B series 35

Figure 2.10**:** Extracellular inhibition activities of T1C – T12Cseries 35

Figure 3.3**:** INH-sensitivity tests of wild type (WT) and *mshA* mutant 24

Figure 3.4**:** Effect of media composition on growth of wild type (WT) and the *mshA* mutant 23

Figure 3.5**:** Arsenic acid (V) sensitivity tests of wild type (WT) and the *mshA* mutant 25

Figure 3.6: Mycothiol pathway, mycoredoxin system, and resistance mechanisms to arsenate 26

Figure 2.1**:** Mycothiol levels at different time points and different INH concentrations 20-22

Figure 3.1**:** Intracellular inhibition activities of AJ-3 – AJ-8 39

***Figure 3.2*:** Intracellular inhibition activities of TA-series 40

Figure 4.1: EM of control mouse MDM + *M. avium* + tunicamycin and phenothiazine 47

Figure 4.4: EM of *M. tuberculosis*-infected macrophages + phenothiazine (AJ-4) treatment 50

***Figure 4.5*:** Fate of loner and social phagosomes on maturation 53

Figure: 4.6 Fate of loner and social phagosomes on the maturation block 54

LIST OF ABREVIATIONS

ABC	ATP-Binding Cassette
AIDS	Acquired Immunodeficiency Syndrome
DAB	3,3`-Diaminobenzidine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ETH	Ethionamide
FAD	Flavin Adenine Dinucleotide
FBS	Foetal Bovine Serum
GlcN	glucosamine
GlcN-Ins	1-D-*myo*-inosityl-2-amino-2-deoxy-α-glucopyranoside
GSH	Glutathione (reduced form)
GSSG	Glutathione (oxidised form)
HIV	Human Immunodeficiency Virus
HMDM	Human Monocyte-Derived Macrophages
HRP	Horse Radish Peroxidase
INH	Isonicotinic acid (Isoniazid)
MDR-TB	Multi-drug Resistant Tuberculosis
MDG	Millennium Development Goal
MOI	Multiplicity of Infection
mRNA	messenger ribonucleic acid
MSH	Mycothiol (reduced form)
MSSM	Mycothiol (oxidised form)
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PZA	Pyrazinamide

RNA	Ribonucleic acid
RIF	Rifampicin
STR	Streptomycin
TB	Tuberculosis Bacilli
WHO	World Health Organisation
XDR-TB	Excessively Drug Resistant Tuberculosis
µg	micro gram
µL	micro litre

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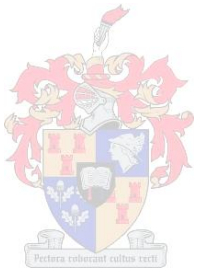
"In a global economy where the most valuable skill you can sell is your knowledge, a good education is no longer just a pathway to opportunity, it is a pre-requisite. That is why it will be the goal of this Administration to ensure that every child has access to a complete and competitive education – from the day they are born to the day they begin a career."

American President **Barack Obama** in his address to Joint Session of Congress, February 24, 2009



CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 TUBERCULOSIS: A HISTORICAL PERSPECTIVE

1.1.1 Current trends in the global epidemiology of TB

Mycobacterium tuberculosis is the etiological agent for pulmonary tuberculosis (TB) and contributes significantly to global mortality (Smith *et al*, 2004). The estimated worldwide death toll of approximately two million deaths every year is attributed to TB and is the highest mortality rate produced by a single microorganism (World Health Organisation (WHO) Report, 2010). Robert Koch discovered the *M. tuberculosis* pathogen in 1882, and by that time the disease was common in every part of the world, even in Europe (Mathema *et al*, 2006). TB has been reported to occur since time immemorial, and has been documented as one of the oldest diseases affecting man at a large scale today.

The global incidence of TB is predominantly reported in developing countries whose economies are characterized by poor incomes and low Gross Domestic Products. Currently TB is prevalent in Africa and Asia, and has been closely linked to general poverty and poor hygiene in these regions. The compounding association between TB and Human Immunodeficiency Virus (HIV) has aggravated the health situation because of disintegrated service delivery systems in the developing world (Zager and McNerney, 2008). Africa remains at the fore-front of the global TB epidemic, and the trend has been exacerbated by high prevalence of HIV-AIDS co-infection.

One of the most challenging feats that the health sector faces today is total control and eradication of the TB epidemic (WHO Report, 2010). The emergence of drug resistant strains of *M. tuberculosis* has complicated efforts directed at combating the disease (Holtz, 2007; Harris *et al*, 2001). The WHO 2010 Report recommended that surveillance efforts be extended to allow a forecast of prevalence and incidence of *M. tuberculosis*-resistant strains within a given population.

The eradication of TB requires a concerted effort from policy-makers down to the monitoring and evaluation of disease cases. Such monitoring of disease patterns requires that Governments dedicate significant amounts of their resources to surveillance programmes. One of the United Nations Millennium Development Goals (MDG number 6) is to reduce the prevalence of TB infection by 70% through early detection intervention strategies by 2015 (Dye and Floyd, 2006). This target, however, seems unrealistic, especially in view of the ever-increasing global trends of TB prevalence.

1.1.2 TB control programmes in South Africa

With the third highest incidence rate and second highest mortality rate in the world, South Africa is facing a TB problem of such magnitude that this disease has been declared a national health emergency (www.doh.gov.za). The rampant HIV co-epidemic plaguing South Africa has exacerbated the problem enormously (Harris *et al*., 2001) as well as the emergence of multi- and extremely-drug resistant strains (MDR-TB and XDR-TB strains, respectively) (Zager and McNerney, 2008).

Although some improvements have been made in controlling TB through reform of existing control programs, it is widely acknowledged that a quantum leap in the quality of tools for prevention and treatment of TB will be required (if there is to be any hope at all) to eradicate this devastating disease. Of all the new and re-emerging infections per annum in South Africa, MDR-TB and XDR-TB strains have been estimated at 4.3%, thus further complicating the existing epidemic control efforts.

1.1.3 Spread and transmission of TB

M. tuberculosis is transmitted from one person to the other when the pathogen is transferred from an infected person through air-borne inhalation, which normally occurs during sneezing and coughing (Rhode *et al*, 2007). Transmission of the TB pathogen results in it being taken up by macrophages of the alveoli, leading to either its destruction or to an active disease state. When inhaled *M. tuberculosis* reaches the lungs and is phagocytosed by alveolar macrophages to induce a local proinflamatory response which leads

to the recruitment of mononuclear cells. (*reviewed in* de Chastellier, 2009; Russell *et al*, 2010, *figure 1.1* below).

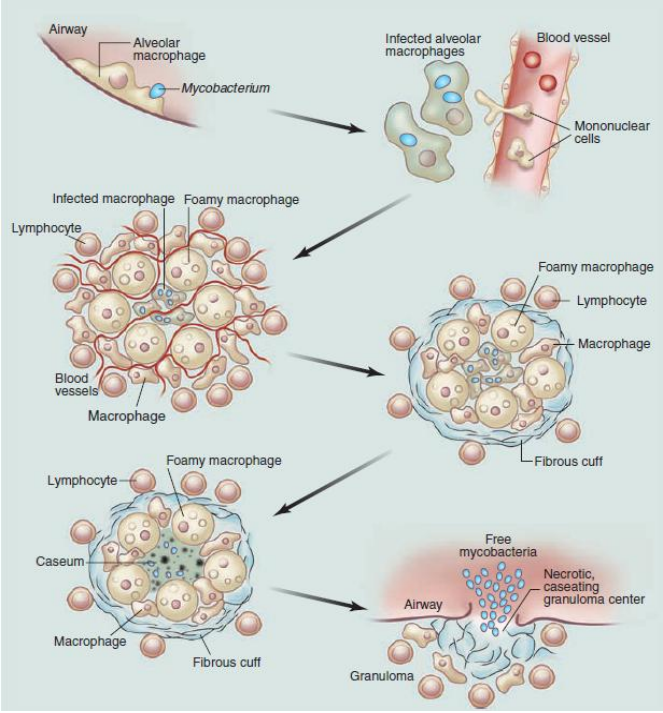


Figure 1.1: Life cycle of *M. tuberculosis* within the human host and the resultant formation of granuloma within the lung. When *M. tuberculosis* is inhaled and gains entry into the host via phagocytosis, it persists in the macrophages of the lung tissues. A sub-population of the infected macrophages undergo differentiation, and become filled with lipid droplets, referred to as foamy macrophages. *M. tuberculosis* eventually escapes when granuloma caveates, resulting in transmission to other hosts (Adapted from Russell *et al*, 2010).

1.2 CURRENT ANTI-TUBERCULAR DRUGS AND THE EMERGENCE OF DRUG RESISTANT STRAINS

1.2.1 Anti-tubercular drug interventions: *Can TB be fully controlled?*

One of the complications with TB is the latent stage in which the pathogen is either slow-growing or not growing at all; this stage is normally resistant to anti-TB drugs. Conventional TB drugs normally target biochemical pathways such as cell-wall synthesis and chromosomal replication. Total eradication of TB is complicated by those patients who are latently infected, and have the possibility of re-activation of the disease (Holtz, 2007). TB treatment drugs consisting of isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA) have been used as prescription for two months, followed by a continuation phase in which only INH and RIF are prescribed.

The emergence of MDR-TB and XDR-TB strains that are resistant to the existing drug regimen has posed serious challenges to the global control and eradication of TB (Zager and McNerney, 2008). Hoek *et al* (2008) defined MDR-TB as: "… disease caused by *M. tuberculosis* that is resistant *in vitro* to both isoniazid and rifampin with or without resistance to other anti-TB drugs." According to Holtz (2007), XDR-TB is: "…the occurrence of TB in persons whose *M. tuberculosis* isolates are resistant to isoniazid and rifampin plus any fluoroquinolone and at least one of the three injectible second-line drugs (amikacin, kanamycin, capreomycin)".

1.2.2 Mechanisms of action of first-line TB drugs and the evolution of antibiotic resistance

When administered into a patient, INH causes the infecting mycobacteria to undergo alterations in the cell wall structure such as the disruption of internal organization, development of wrinkles on the surface as well as buldging (Zager and McNerney, 2008). Such ultrastructural alterations have been confirmed by electron microscopy examination of *M. tuberculosis* upon exposure to INH (Takayama *et al*, 1973). In addition, INH effects a loss in the property of mycobacteria known as acid fastness (Takayama *et al*, 1972; Vilcheze *et al*, 2000). Precise mechanisms by which INH induces ultrastructural distortions of internal organization are still under investigation, 50 years after its discovery as an anti-mycobacterial agent.

Antibiotic resistance genes code for defense strategies through which bacteria survive in an environment where there are antibiotics that are supposed to be inhibiting bacterial growth. Several mechanisms have been suggested to play a role in conferring antibiotic resistance (Al-Haroni, 2008), and these include structural alteration of the target molecules, cellular exclusion and drug efflux. Mycobacteria have developed mechanisms through which antibiotics are inactivated through enzymatic action. A typical example is the expression of β-lactamases which degrade the β-lactam antibiotics, thereby conferring drug resistance (van Bambeke *et al*, 2000).

Drugs such as INH and RIF have been used in combination to fight TB for a long time, but the emergence of drug resistant TB strains prompted researchers on the urgent need to develop and test the efficacy of other drug formulations. INH is a pro-drug that functions through its intermediates to block pathways

involved in mycolic acid biosynthesis. Treatment of TB with INH therefore, is based on its ability to inhibit the biosynthesis of mycolic acids, long chain fatty acids which make up the bulk of mycobacterial cell wall, killing the pathogen in the process (*reviewed by* Vilcheze and Jacobs, 2007). Different genes encoding for drug resistance in *M. tuberculosis* have been characterized, and gene knockout mutants have been used to gain further knowledge on the molecular mechanisms that underlie these processes as shown in *Table 1.1* below (Sharma and Mohan, 2004, Vilcheze *et al*, 2008).

Table 1.1: Anti-tuberculosis drugs and the gene(s) involved in their susceptibility in *M. tuberculosis*. Mutations in the indicated genes confer resistance to generic antibiotics that are normally effective as first line defense drugs. In cases of MDR-TB and XDR-TB, mycobacteria become resistant to more than two antibiotics due to concurrent expression of these genes.

Drug	**Mutations in these genes result in drug resistance**
Isoniazid	Enoyl acp reductase *(inhA)*
	Catalase-peroxidase *(katG)*
Rifampicin	RNA polymerase subunit B *(rpoB)*
Pyrazinamide	Pyrazinamidase *(pncA)*
Streptomycin	Ribosomal protein subunit 12 *(rpsL)*
	16s ribosomal RNA *(rrs)*
	Aminoglycoside phosphotransferase gene (*strA)*
Ethambutol	Arabinosyl transferase (*emb A, B* and *C*)
Fluoroquinolones	DNA gyrase *(gyr A* and *B)*

Adapted from Sharma and Mohan, (2004)

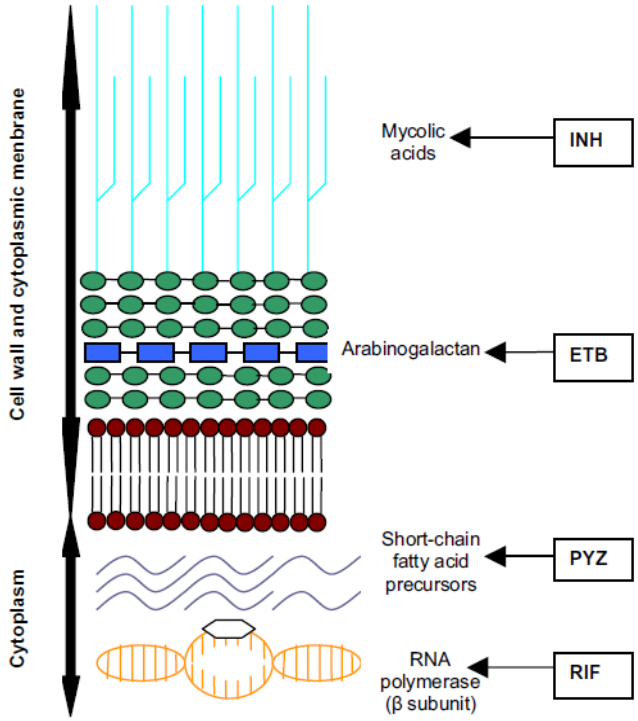


Figure 1.2: Pathways inhibited by first line drugs against *M. tuberculosis*.

In this illustration, PYZ = pyrazinamide, INH = isoniazid, and RIF = rifampicin, ETB = ethambutol are drugs that target different pathways that are essential for the survival of *M. tuberculosis* (adapted from du Toit *et al*, 2006).

INH is the most important antimicrobial agent that has been used in the treatment of *M. tuberculosis*. When *M. tuberculosis* is exposed to INH, the mycolic acid pathway is inhibited, leading to the accumulation of fatty acids and eventually cell death (Vilcheze and Jacobs, 2007). The activation of INH is accomplished through the activity of catalase-peroxidase *katG* which results in the formation of an isonicotinoyl radical. The activated INH reacts with NAD, forming an INH-NAD adduct which inhibits the FASII enoyl-ACP reductase *inhA* (Vilcheze *et al*, 2000). The inhibition of FASII *inhA* leads to cell death of the *M. tuberculosis*.

Mutations in catalase-peroxidase *katG* have been reported to be critical in conferring drug resistance in *M. tuberculosis* (Johnson, 2007). These mutations in *katG* have been observed to prevent the formation of INH-NAD complex. The sub-cellular location of the INH-NAD adduct also needs to be established in an effort to develop effective drugs against *M. tuberculosis*. Several gene mutations have been implicated in the INH resistance mechanisms, but the dominant phenotype mutations are due to *inhA* while other mechanisms of resistance such as *katG, msh, nat* and *ndh* have been found to be recessive (*reviewed in*

Vilcheze and Jacobs, 2007). As more studies on *M. tuberculosis* drug resistance continue, attempts have been made to identify alleles for resistance and their possible mechanisms of action.

1.3 SURVIVAL STRATEGIES OF M. tuberculosis WITHIN HOST MACROPHAGES

1.3.1 Entry of M. tuberculosis into the host macrophage

The establishment of a successful infection by *M. tuberculosis* depends on the initial encounter with host macrophages, which represent the first line of cellular defense against microbial invasion. Ingestion of mycobacteria occurs through a receptor-mediated, actin-dependent process called phagocytosis. At the interface between mycobacteria and macrophages, the complex outermost layer of the mycobacterial cell wall probably plays a role in facilitating host cell entry. Several envelope molecules have been shown to interact with macrophage phagocytic receptors (Astarie-Dequeker *et al*, 2009). For instance lipoarabinomannan binds to the mannose receptor which is one of the major receptors for mycobacterial entry (Schlesinger *et al*, 1994). Another important phagocytic receptor of macrophages, the complement receptor 3, recognizes outer mycobacterial capsular polysaccharides through its lectin site.

1.3.2 Survival of M. tuberculosis occurs via arrest of phagosome maturation

Under normal conditions (*i.e.* ingestion of non pathogenic microorganisms), newly formed phagosomes intermingle contents and membrane with the successive compartments of the endocytic pathway (early endosomes, late endosomes, lysosomes) through a complex series of fusion and fission events (shown in *figure 1.3*, Vieira *et al*, 2002). As they are processed into phagolysosomes, they undergo gradual modifications by specific addition and removal of membrane constituents. In addition, they become acidified due to the vacuolar proton pump ATPase located in the membrane and acquire toxic constituents, including hydrolases that will ultimately destroy bacteria. One of the major strategies used by endoparasites such as pathogenic mycobacteria, but by no means the only one, is to modulate these interactions.

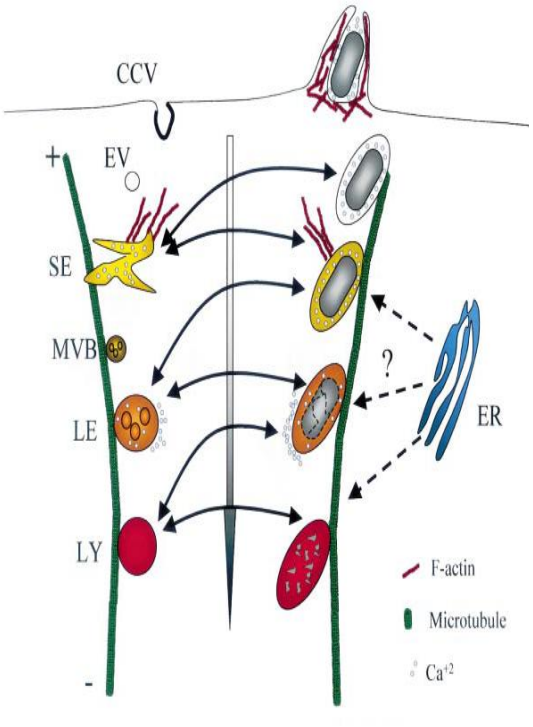


Figure 1.3: Phagosome maturation (adapted from Vieira *et al*, 2002).

In this illustration, pathogenic bacteria are engulfed from the cell surface and channelled through a series of maturation stages until the final fusion with lysosomes. Within the endocytic pathway, there is continuous exchange of membranes and membrane contents through fusion and fission events. Actin is important in the endocytic pathway, and is found in abundance at the phagocytic cup. Abbreviations used in this figure: CCV = clathrin-coated vesicle; EV = endocytic vesicle; SE = sorting endosome; LE = late endosome; LY= lysosome; ER = endoplasmic reticulum.

A striking feature of *M. tuberculosis* is its ability to prevent maturation of the phagosome in which it resides. In this manner, it avoids the acidic and hydrolytically active phagolysosome compartment (Armstrong and Hart, 1971; Clemens and Horwitz, 1995; de Chastellier *et al*., 1995; Via *et al*., 1997). Recently, de Chastellier and colleagues have shown that a necessary requirement for the block of phagosome maturation is the establishment of a close apposition of the phagosome membrane with the entire mycobacterial surface all around (de Chastellier and Thilo, 1997; 2006; de Chastellier *et al*, 2009).

1.3.3 Intracellular responses and rescue mechanisms from hostile internal environment

Work done by de Chastellier (2009) suggests that, whatever the molecular mechanisms involved in the block of maturation of the mycobacterium-containing phagosome, a necessary requirement is the establishment and maintenance of a close apposition of the phagosome membrane with the entire bacterial surface all around (de Chastellier and Thilo, 1997; Pietersen *et a*l, 2004; de Chastellier and Thilo, 2006). Whenever several mycobacteria are enclosed in the same phagosome, close apposition is not maintained in

the regions where the phagosome membrane spans adjacent bacteria. Such phagosomes systematically mature and fuse with lysosomes (de Chastellier *et al*., 1995; Clemens and Horwitz, 1995; de Chastellier and Thilo, 1997; Pietersen *et al*., 2004). Interestingly, mycobacteria do not die in this hostile environment but are instead rescued by a process that remains to be determined (de Chastellier, 2009).

1.4 MYCOTHIOL, A POTENTIAL TARGET FOR NEW ANTI-TUBERCULAR DRUGS

1.4.1 Genetic control of mycothiol biosynthesis in M. tuberculosis

A wide variety of bacterial constituents that play an important role in the pathogenicity of *M. tuberculosis* could be potential targets for novel therapeutic strategies. Mycothiol has recently emerged as a potential candidate because it performs critical functions within mycobacteria. Mycothiol is a major low molecular weight thiol present in a wide range of actinomycetes, and protects mycobacteria against oxidative stress (Newton *et al*, 1996). It acts as an antioxidant by reacting with exogenous or endogenous reactive oxygen intermediates, thereby maintaining an intracellular redox homeostasis (Dosanjh-Nirpjit *et al*, 2005). During this reaction, two equivalent mycothiols are oxidized to one molecule of mycothiol disulfide (MSSM).

Mycobacteria produce mycothiol at very low levels of up to milli-molar per mL quantities, and the biosynthesis of mycothiol occurs via five gene-controlled stages (Rawat *et al*, 2003). The genes that code for the proteins involved in the mycothiol pathway are designated *mshA, mshA1, mshB, mshC* and *mshD*, coding for glycotransferase, deacetylase, ligase, and acetyltransferase, respectively (Newton and Fahey, 2002; *figure 1.4*). The formation of GlcNAc-Ins is the first key step, regulated through the expression of *mshA* (Rv0486), which codes for the enzyme N-acetylglucosamine transferase (Anderberg *et al*, 1998).

The second step in mycothiol biosynthesis is regulated by the *mshB* gene (Rv1170) which codes for a de-acetylase which results in the removal of the acetyl group from N-acetylgulcosamine, resulting in GlcN-Ins (Fan *et al*, 2009). The third step is the ligation of cysteine with GlcN-Ins though the action of a ligase coded for by *mshC* (Rv2130c). Mycothiol is finally formed through the acetylation of Cys-Glc-Ins, a reaction controlled by *MshD* acetyltransferase (Rv0819) (Newton *et al*, 2008). *M. tuberculosis* gene

knockout mutants and recombinants of mycothiol biosynthesis genes and related proteins have been studied (Park *et al* 2006), and have helped understand mycothiol biosynthesis and regulation.

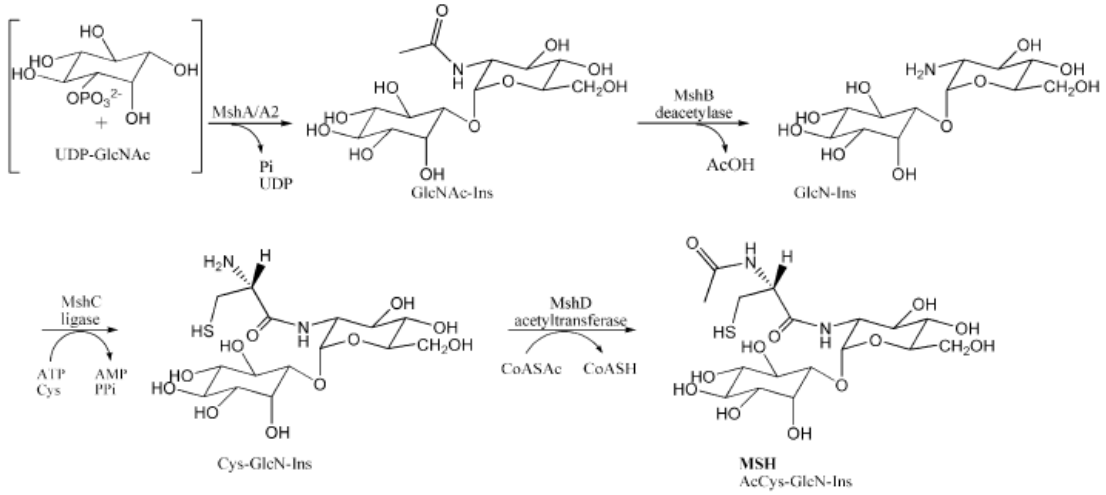


Figure 1.4: The pathway for biosynthesis, recycling of mycothiol and detoxification of xenobiotics (adapted with permission from Vilcheze *et al*, 2008).

Enzymes involved in mycothiol metabolism were chosen as attractive targets for designing new antituberculous drugs because most of these enzymes and in particular, MshC is essential for *M. tuberculosis* survival (Fan *et al*, 2009). In contrast however, inactivation of MshB is non-lethal since the Mycothiol S-conjugate amidase (Mca) production of GlcN-Ins (1-D-myo-inosityl-2-amino-2-deoxy-alpha-D-glucopyranoside) results in the mycobacteria still being able to synthesize MSH and survive (Rawat *et al*, 2003). Inactivation of mycothiol reductase inhibits the growth of *M. tuberculosis* (Sassetti *et al*, 2003; Hayward *et al*, 2004).

In order to develop new drugs through rational drug design, we analysed the expression of all important enzymes of the mycothiol pathway. We postulated that mycothiol biosynthesis drug targets may be important for the treatment of tuberculosis since mycothiol and MSSM establish a thiol buffer system in mycobacteria. Gene knockout studies done on *M. tuberculosis* Erdman mycothiol genes (Sareen *et al*, 2003) have suggested that *mshC* is a potentially good candidate for drug targeting.

1.4.2 Biochemistry of mechanisms involved in the mycothiol pathway

Within the intracellular environment, mycothiol protects the cell against antibiotics, electrophiles and other reactive substances which react with the thiol groups (Fan and Blanchard, 2009). In the presence of antibiotics or under electrophilic attack, the mycothiol-S-conjugate is degraded to produce GlcN-Ins which will then help to recycle mycothiol for cellular defense (Jothivasan and Hamilton, 2008). The mycothiol pathway is closely regulated through the action of the mycothiol reductase enzyme, Mtr that helps to maintain the reduced form, MSH (Patel and Blanchard, 1999).

The gene that codes for the mycothiol reductase enzyme has been identified (Newton and Fahey, 2002) and cloned, and has been found to be very similar to glutathione oxidoreductase. Mycothiol is involved in the replenishment of cysteine, another anti-oxidant molecule found in mycobacteria. A pathway in which cysteine is regenerated from mycothiol has been proposed by Anderberg *et al* (1998), and has been shown to suggest that acetyl-cysteine is converted to cysteine. Mycothiol functions as a protective molecule against reactive oxygen species within actinomycetes, and the conjugation of two MSH molecules results in MSSM. Mtr activity helps maintain a high MSH:MSSM ratio through the reduction of MSSM to MSH. The activity of Mtr depends on NADH and NADPH as reducing cofactors, but NADPH is more effective as a substrate, with >20 fold greater k_{cat}/k_m value than NADH (Jothivasan and Hamilton, 2008).

Mycothiol-S-conjugate amidase is utilized by actinomycetes to detoxify the reactive oxygen species and other toxins through hydrolysis of glucosaminyl-amide bond of MS-congugates to release the mercapturic acid-labeled toxin and GlcN-Ins (Jothivasan and Hamilton, 2008). Mycothiol-S-conjugate amidase (Mca) is a zinc-dependent N-acyl hydrolase that is closely related to MshB. Studies have been done with monobromobimane (mBBr), a fluorescent alkylating agent that forms an adjunct that is degraded by Mca, producing mercapturic acid (AcCyS-mB) and GlcN-Ins (Rawat *et al*, 2004). The mercapturic acid derivatives are excreted from the cell while GlcN-Ins is recycled back to the MSH biosynthetic pathway. Although the mycothiol-Mca relationship has been proposed as important in the detoxification and eventual efflux of mBBr, it is the mycothiol rather than Mca that has been found to be critical for the detoxification process.

Mca is the most important enzyme in the bio-inactivation of electrophilic molecules as well as the reactive oxygen species within the mycothiol pathway. Mca is important in the cleavage and subsequent dissociation of mercapturic acid from GlcN-Ins. The role of amidase was confirmed by experimental evidence (Rawat *et al*, 2004) that *mca* gene knockout mutants become more prone to antibiotics when compared to the wild-type. However, not all antibiotics are dependent on amidase activity, for example lincomycin has a gene homologous to the *mca* in its antibiotic biosynthesis cluster.

Vilcheze *et al* (2008, and unpublished data) have been able to knockout all the genes involved in the mycothiol pathway in *M. tuberculosis*, except for the *mshC* gene. The inability to successfully generate a deletion gene knock-out studies suggests that *mshC* is important for *M. tuberculosis* growth. In fact, based on these results, one would hypothesize two alternative explanations for the inability to delete *mshC* gene in *M. tuberculosis*: (1) the *mshC* gene is part of some other essential pathway or (2) *mshC* inactivation leads to the accumulation of some toxic intermediate (Jacobs, personal communication). The *mshB* and *mshD* genes are not essential for *M. tuberculosis* as mutants in these genes still produce significant amounts of MSH or related thiols. Mycothiol-deficient bacteria show increased resistance to INH, and this resistance has been used as a selection marker when screening for mutants in mycothiol biosynthesis (Rawat *et al*, 2007). Resistance to ETH is also enhanced in MSH-deficient mutants. INH and ETH are both pro-drugs requiring catalase peroxidase (*katG*) and flavoprotein mono-oxygenase activity respectively.

Jothivasan and Hamilton (2008) acknowledge findings by Vilcheze *et al* (2008) that demonstrate the possibility of generating MSH-deficient strains of *M. tuberculosis*, which grow normally in immuno-deficient mice and only show slight defective growth in immuno-competent mice. However Vilche`ze *et al* (2008) suggest that MSH is not essential for *in vivo* growth of *M. tuberculosis* in mice. The work reported herein argues that on the basis of the findings by Vilche`ze *et al* (2008) it would be worthwhile to investigate how MSH-deficient *M. tuberculosis* survives *in vivo* when exposed to anti-TB drugs.

1.5 HYPOTHESIS

New anti-tubercular drugs have been designed towards inhibition of specific steps of the mycothiol pathway and its intermediates. The ability of mycobacteria to survive within the host is may depend on mycothiols which confer protection against reactive oxygen species that would otherwise disrupt cellular functions (Raman *et al*, 2005). In this regard, the design of drugs against TB has focused on the mycothiol biosynthetic pathway (*figure 1.4*) with a view to understand the genetic and molecular mechanisms underlying the process of mycobacterial survival within the macrophages.

Our hypothesis was that a drug inhibiting the expression of *mshA* affects synthesis of mycothiol. In the absence of mycothiol, the bacilli are killed as they cannot resist reactive oxygen species and reactive nitrogen intermediates generated during oxidative stress. Ultimately, this would affect the ability of *M. tuberculosis* treated with drugs against mycothiol to block phagosome maturation. *M. tuberculosis*-containing phagosomes would then be processed into phago-lysosomes in which bacilli would be degraded. We targeted mycothiol enzymes as the production of mycothiol has been implicated in reducing multidrug resistance (Jardine, personal communication). The development of inhibitors that target mycothiol biosynthetic or processing enzymes has been postulated to constitute important approaches towards improving tuberculosis treatments.

CHAPTER 2: DRUG TESTING AND MYCOTHIOL QUANTITATION ON *IN VITRO* GROWING *M. tuberculosis*

2.0 INTRODUCTION

Compounds that inhibit the biosynthesis of mycothiol were developed in the context of this study, and would potentially serve as useful agents for TB treatment. The fact that genes involved in mycothiol pathway have been characterized (Hayward *et al*, 2004; Park *et al*, 2006; Sassetti *et al*, 2003), and several studies on their expression and function have been reported (Patel and Blanchard, 1998; Takayama *et al*, 2005) means that enzyme-specific inhibitors of this pathway could be developed.

Drug screening experiments were initiated by obtaining the purified mshA enzyme to assay for compound binding activities (Jardine Lab, University of Cape Town, South Africa). Since all enzymes within the mycothiol pathway use substrates with the same basic structure, compounds that showed adequate binding and inhibitory activity in the enzyme assay were further derivatised and screened for mycobacterial killing effects. The role of mycothiol for the survival of *M. tuberculosis* was also investigated by using the *mshA* deletion mutant. The phenotype of the *mshA* deletion mutant of *M. tuberculosis* was investigated by exposing the mycobacteria to INH, arsenic (V) acid and different culture media compositions.

The objective of this work was to test inhibitors of enzymes of the mycothiol pathway and derivatives thereof in the BACTEC 460^{TM} system. Furthermore, assays were validated by using substrate mimetics as inhibitors. These substrate mimetics were synthesised in a linear fashion from available starting materials. Some commercially available natural product substrate analogs (such as busulfan) were also tested for inhibitory activity. A library of drug-like compounds was synthesised and tested (*figure 2.6*).

2.1 MATERIALS AND METHODS

2.1.1 Culturing of M. tuberculosis

M. tuberculosis H37Rv strain were cultured in 7H9 broth supplemented with 10% oleic acid-albumin-dextrose catalase (OADC, Difco, BD Biosciences, Mountain View, CA, USA) and 2% glycerol and 0.05%

Tween 80. Liquid cultures were grown for up to 3 weeks and stored at -80°C in 1ml aliquots with 15% glycerol. Clumps were eliminated after thawing by 25 to 30 passages through a needle (26-gauge 3/8; 0.45 x 10 for intradermal injection; BD Biosciences, USA). Before inoculation, the viability of mycobacteria, was evaluated by the propidium iodide exclusion method to ensure >90% viability (Pietersen *et al*, 2004).

2.1.2 Stress conditions for mycothiol production in M. tuberculosis

A pre-culture of *M. tuberculosis* (5 mL) was incubated at 37^{o}C for 3 weeks. Experimental cultures (25 mL) were inoculated with the pre-culture of *M. tuberculosis* and incubated for 7 days at 37ºC after which stress/drug interventions were done. INH was added to induce stress to *M. tuberculosis* (at 0.2 and 2 µg/mL for H37Rv and INH^{R}, respectively), and samples were processed at 0 hr, 4 hr, 24 and 48 hr post-drug exposure. Mycothiol produced after exposure of *M. tuberculosis* to stress conditions of INH was quantified via LC-MS triple quadpole analysis.

2.1.3 Mycothiol extraction methods from M. tuberculosis

Two different extraction methods were used to obtain mycothiol from *in vitro* growing *M. tuberculosis* under stress conditions as follows:

(i) Sonication with perchloric acid/acetonitrile/water

Steenkamp and Vogt (2004) used this method to purify mycothiol from *M. smegmatis*. In brief, cells were disrupted through sonication, and mycothiol was extracted using a buffer system of 0.25M perchloric acid and 2mM EDTA in a 40% acetonitrile/H_2O. After removal of the cell debris, the supernatant was directly lyophilized overnight. The resulting residue was re-suspended in 50% acetontrile/H_2O before LC-MS analysis.

(ii) BugBuster® extraction method

BugBuster® protein extraction reagent (Novagen, Germany) was also used to extract the analytes. Cell pellets were re-suspended in five times less extraction buffer than the original culture sample. The suspensions were incubated for 2 hours at room temperature on a shaking platform. The cell debris was

removed through centrifugation and the supernatant lyophilized overnight. The remaining residue was re-dissolved in 50% acetonitrile/H_2O and processed for LC-MS analysis.

2.1.4 Determination of mycothiol levels by LC-MS analysis

Both mycothiol (MSH, reduced form) and mycothione (MSSM, oxidised form) that were used as standards for mycothiol quantitation via LC-MS were kindly donated by Professor Spencer Knapp from the Department of Biology and Chemistry at Rutgers The State of New Jersey University in the United States of America. LC-MS analysis was performed at the Mass Spectrometry Unit of the Central Analytical Facility (www.sun.ac.za/caf), University of Stellenbosch. Samples were analyzed on an ACQUITY UPLC® system (Waters Corp., USA) with an autosampler and a Synergi 4 µM Fusion RP, 250x2mm column (Phenomenex). The sample injection volume was 20 µl and the auto sampler syringe was washed with solvent A (0.1% formic acid in water) before each injection. A gradient elution program with a flow-rate of 0.20 ml/min was used for the analysis. The gradient was as follows: 100% solvent A for 8 min; 7 min linear increase up to 95% solvent B (0.1% formic acid in acetonitrile); 95% B step for 3 min; 95% solvent A from 18 to 20 min.

Detection of mycothiol (MSH and MSSM) was performed on a Q-TOF Ultima API quadrupole mass spectrometer (Micromass, Manchester, UK). Analytes were detected in the positive ion mode. The capillary voltage was set at 3500 V. The source temperature and the nebulization gas temperature were set at 80 ºC and 250 ºC, respectively. The cone voltages were set at 35V. Data was processed using MassLynx™ software (Micromass, Manchester, UK). All statistical data analysis and graphical presentation of the data was performed with Microsoft® Office Excel 2007.

2.1.5 Sensitivity tests of WT and ΔmshA deletion mutants of M. tuberculosis

M. tuberculosis gene knockout mutants deficient in *mshA* gene were kindly provided by Dr Catherine Vilcheze from Albert Einstein College of Medicine in the USA. In this study, the role of growth conditions and media composition on the survival of wild-type *vs* the Δ*mshA* strain was investigated. Furthermore the

phenotypic responses of the WT *vs* the Δ*mshA* strain were investigated by exposure to INH (0.01, 0.05 and 0.1 µg/mL) and arsenic (V) acid (1mM, 50mM and 100mM). The plates were incubated for periods up to 4 weeks at 37^{o}C until colonies appeared.

2.1.6 Monitoring M. tuberculosis growth in the presence of inhibitors of the mycothiol pathway via BACTEC 460™ system

M. tuberculosis H37Rv (1 mL aliquots) were thawed and grown directly in BACTEC 460$^{™}$ vials with various concentrations of inhibitors (between 0 and 10 µg/mL). The inhibitors tested in this study were mainly directed towards the mshA enzyme of the mycothiol pathway. *M. tuberculosis* was cultured for 7 days before addition of inhibitors of mycothiol enzymes and BACTEC 460^{TM} measurements. Untreated *M. tuberculosis* was grown under the same conditions to serve as a control. Mycobacterial growth was monitored by the BACTEC 460$^{™}$ instrument which makes use of radioactively labelled ^{14}C triacylglycerides (Roberts *et al*, 1983). The minimal inhibitory concentrations for the drugs were calculated based on BACTEC 460^{TM} growth index readings.

2.2 RESULTS AND DISCUSSION

2.2.1 Quantitation of mycothiol via quad-pole LC-MS

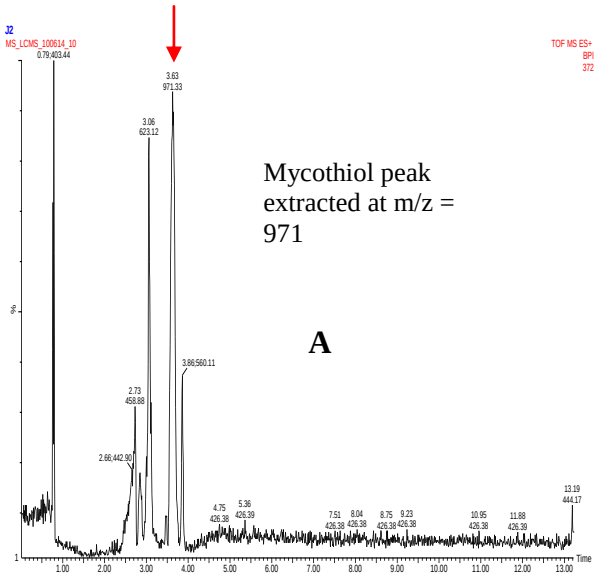
In this work, mycothiol expression was quantified via LC-MS after *M. tuberculosis* was exposed to oxidative stress induced by INH. The mycothiol peaks were integrated to provide an indication of the amounts in each sample relative to one another (refer to *Table 2.1* below). Mycothiol was maximally produced during the early time points (4 hours after drug exposure in this case) as this would allow the pathogen to survive and adapt to the conditions of stress. The levels of mycothiol (and its intermediates) have previously been determined for *M. tuberculosis* and *M. smegmatis* as a function of the growth phase (Anderberg *et al*, 1998). The levels of MSH have been found to be constant through the exponential growth and stationary phase. During the stationary phase, there appeared to be a significant drop in the intermediates *i.e.* GlcN and GlcN-Ins, and an increase in H_2S levels (data not shown). In this study mycothiol levels were found to be around 80% in the *M. tuberculosis* H37Rv strain during the early

exponential phase, and dropped slightly to around 70% in the stationary phase upon exposure to INH (at 0.2 µg/mL).

Table 2.1: Changes in mycothiol levels over the growth phase. Table summarises mycothiol levels after different treatments of *M. tuberculosis* upon exposure to INH (at 0.2µg/mL) for different time points (4 hrs, 24 hours and 48 hrs). Mycothiol was extracted as described in the *Materials and Methods* section (*sections 2.1.3*), and quantified via LC-MS using the triple quad-pole method (described in *section 2.1.4).*

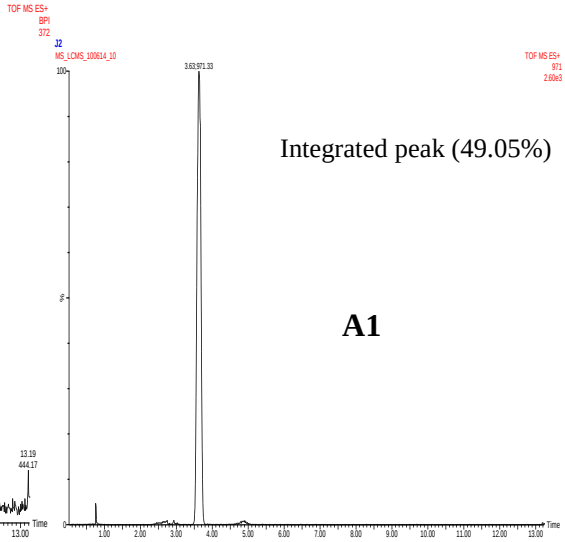
Sample ID	***M. tuberculosis* strain**	**INH concentration (µg/mL)**	**Time of drug exposure/hrs**	**% mycothiol**
A	*M. tuberculosis* Beijing INH^R	- control	- control	49.1
B	*M. tuberculosis* Beijing INH^R	0.2 µg/mL	4 hrs	46.9
C	*M. tuberculosis* Beijing INH^R	0.2 µg/mL	24 hrs	47.4
D	*M. tuberculosis* Beijing INH^R	0.2 µg/mL	48 hrs	52.7
E	*M. tuberculosis* H37Rv	0.2 µg/mL	4 hrs	84.1
F	*M. tuberculosis* H37Rv	0.2 µg/mL	24 hrs	70.5
G	*M. tuberculosis* H37Rv	0.2 µg/mL	48 hrs	72.7

Mycothiol levels were followed over the growth phase in the Beijing INH^R strain and was found to be half the levels in the *M. tuberculosis* H37Rv strain (between 40 – 50%) at INH 0.2 µg/mL. If this data is corroborated with INH resistance phenotype of the *mshA* of *M. tuberculosis*, this might suggest that the Beijing INH^R strain had a defect in mycothiol expression. Mycothiol levels were found to be in the same range for *M. tuberculosis* Beijing INH^R strain at INH concentration of 2µg/mL (data not shown). Mycothiol quantitation was done by following the levels of the oxidised form (since this is the predominant form in which mycothiol exists in the cell). The equilibrium between reduced and oxidised forms of mycothiol is maintained by mycothionine reductase system; during conditions of oxidative stress MSSM is actively reduced to MSH which scavenges for free radical generated from antibiotics, ROI or RNI within the environment.



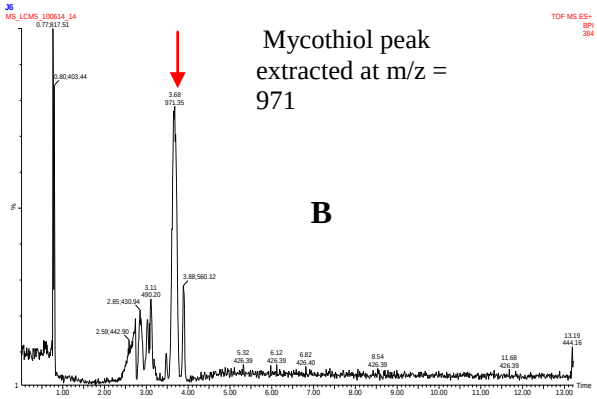
Mycothiol peak extracted at m/z = 971

A



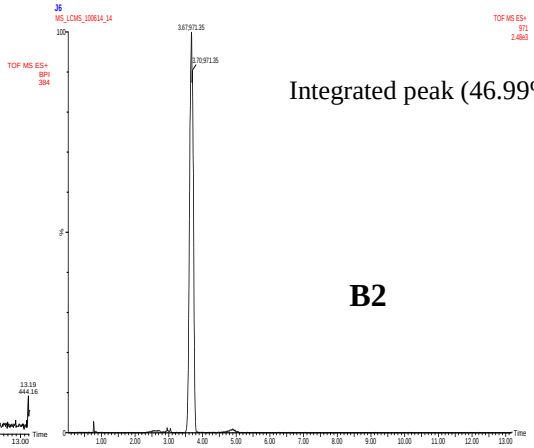
Integrated peak (49.05%)

A1



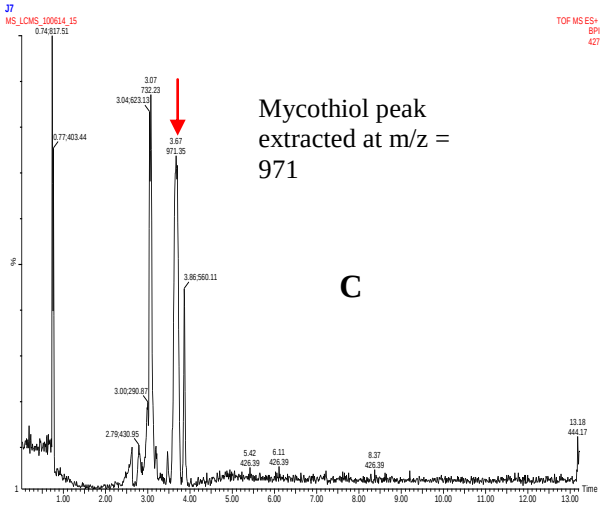
Mycothiol peak extracted at m/z = 971

B



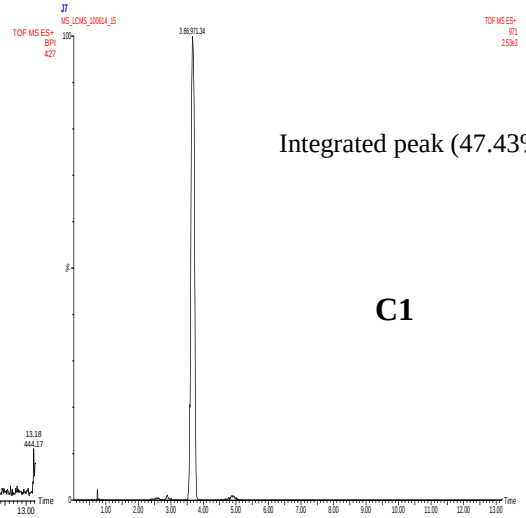
Integrated peak (46.99%)

B2



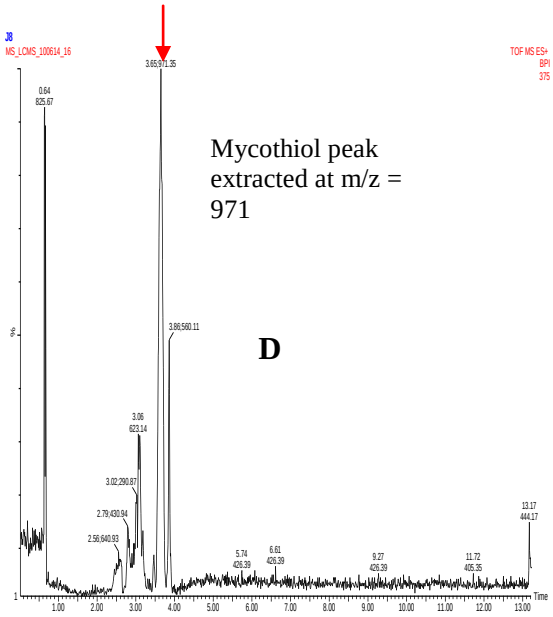
Mycothiol peak extracted at m/z = 971

C



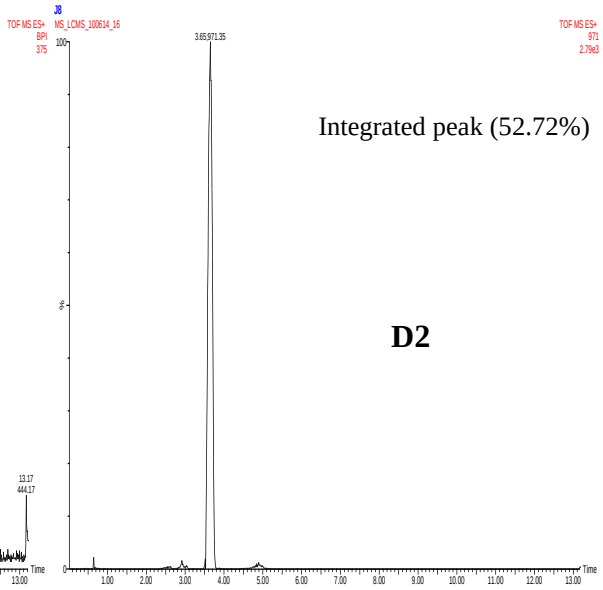
Integrated peak (47.43%)

C1



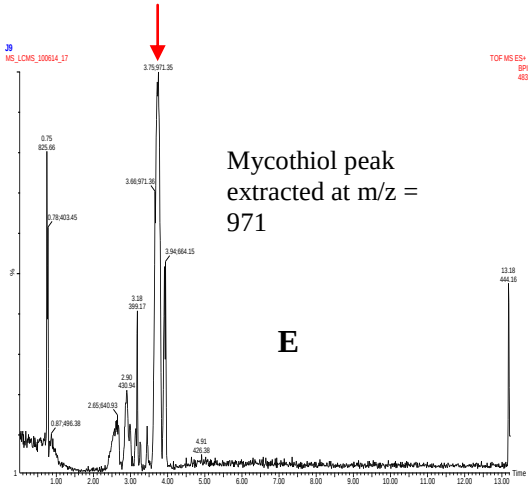
Mycothiol peak extracted at m/z = 971

D



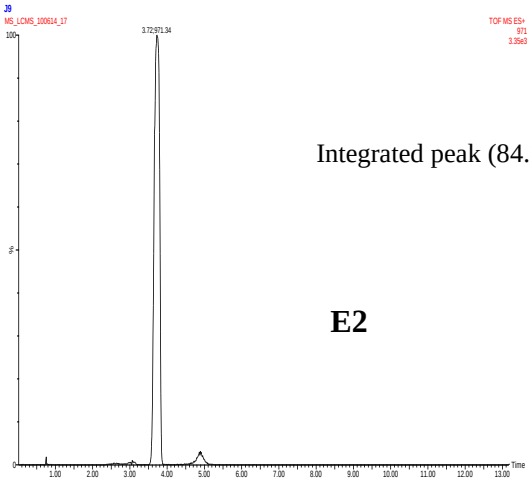
Integrated peak (52.72%)

D2



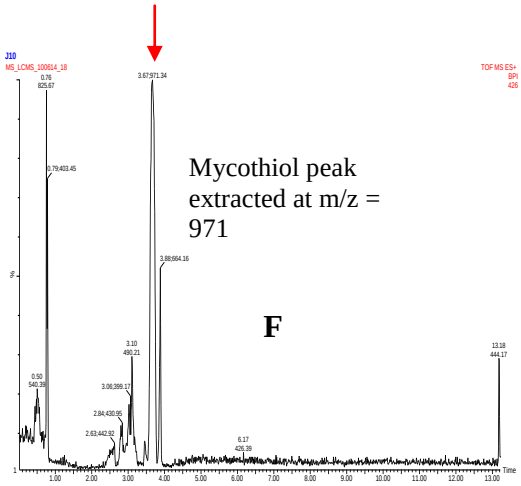
Mycothiol peak extracted at m/z = 971

E



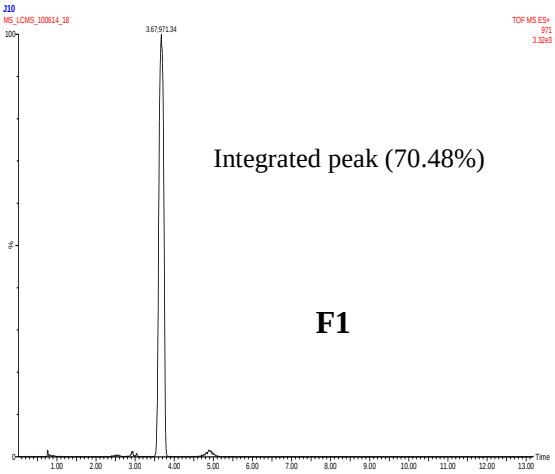
Integrated peak (84.14%)

E2



Mycothiol peak extracted at m/z = 971

F



Integrated peak (70.48%)

F1

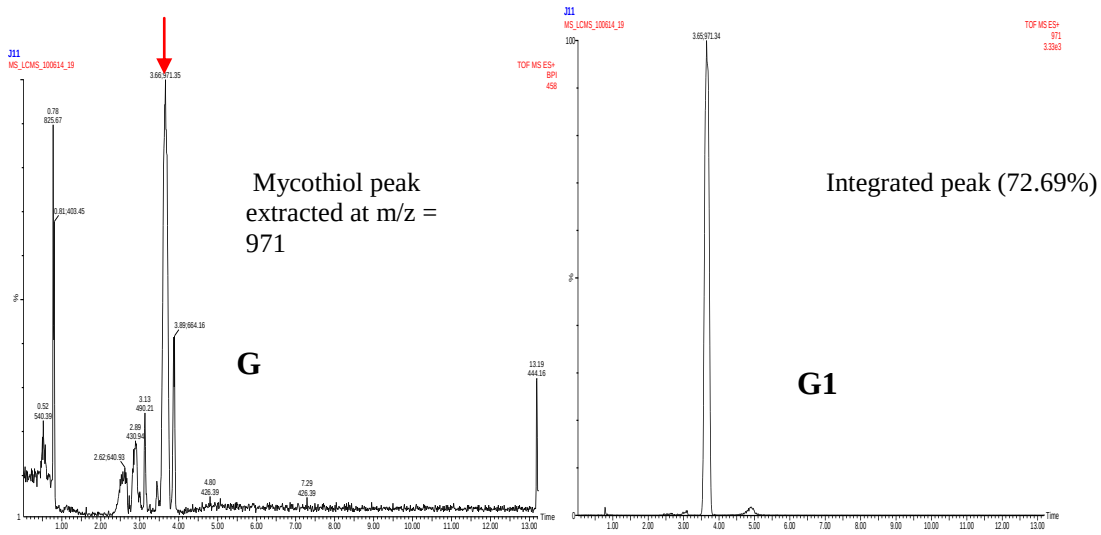


Figure 2.1: LC-MS quantitation of mycothiol levels in *M. tuberculosis* H37Rv and Beijing INH^R at different time points after treatment with INH using different concentrations. Figures on the left (**A - G**) are chromatograms showing extraction of mycothiol in the H^+ ion mode at *m/z* = 971 (shown by red arrow on the chromatograms), and figures on the right (**A1 - G1**) are integrated chromatograms showing relative amounts of mycothiol as percentage points.

In this study mycothiol levels were quantified via LC-MS and shown to increase under antibiotic stress conditions. Mycothiol confers protection against reactive oxygen species and electrophiles to mycobacteria (Anderberg *et al*, 1998). The effect of electrophilic moieties and reactive oxygen species on cellular antioxidant status is that they destroy the biological integrity of the molecules through disruption of S-groups in proteins and DNA base-pairing. In the mycothiol pathway, the reverse reaction which maintains a constant MSH:MSSM ratio is catalyzed by mycothiol disulfide reductase which is encoded by the *Rv2855* (*mtr*) gene (Patel and Blanchard, 1998). As for other functionally homologous enzymes such as glutathione and trypanothione reductase, it depends on bound FAD and NADPH for the reduction of the disulfide bond of MSSM to produce two equivalents MSH (Patel and Blanchard, 1999). Interestingly, mycothiol and the enzymes involved in its metabolism are sensitive to free radical generating antituberculosis drugs (This study; Hayward *et al*, 2004).

2.2.2 Mycothiol mutants (ΔmshA) grow poorly/do not grow on 7H9 without OADC

Growth on solid plates indicated that the Δ*mshA* mutant required OADC supplement to grow (*figure 2.2*). Vilcheze *et al* (2008), investigated the same phenomenon, and implicated catalase as a prerequisite for the survival of the Δ*mshA* mutant. It would be interesting, however, to investigate how the Δ*mshA* strain would survive when first grown in the presence of catalase (up the late log-phase, for example), then remove the catalase from the growth media. In this way, the requirement for catalase for the survival of the Δ*mshA* strain would be ascertained.

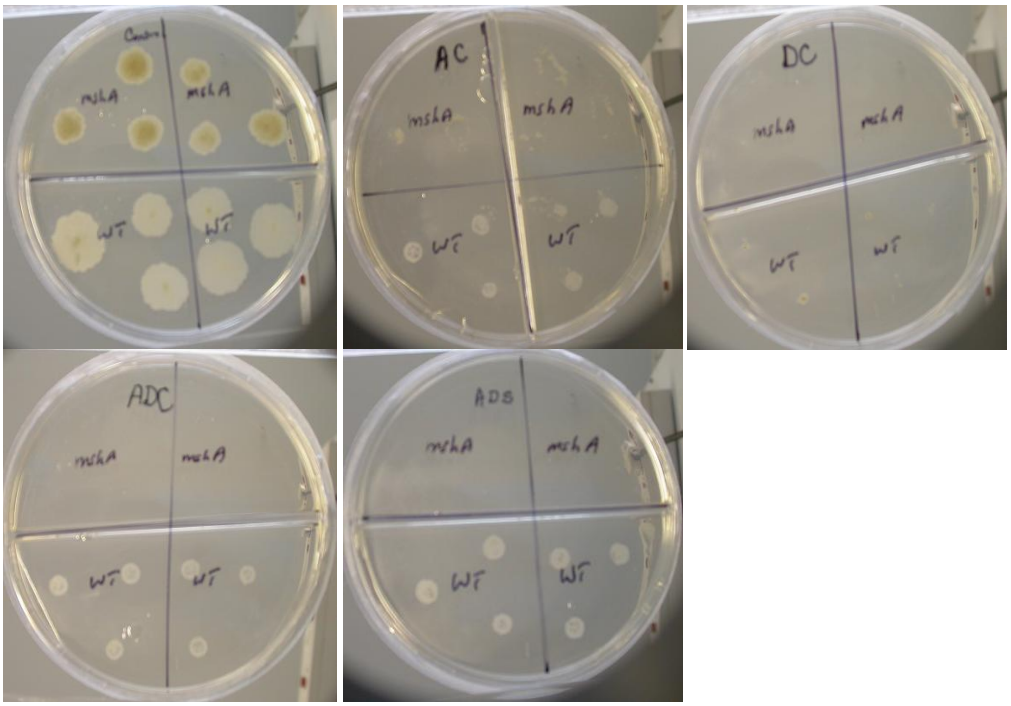


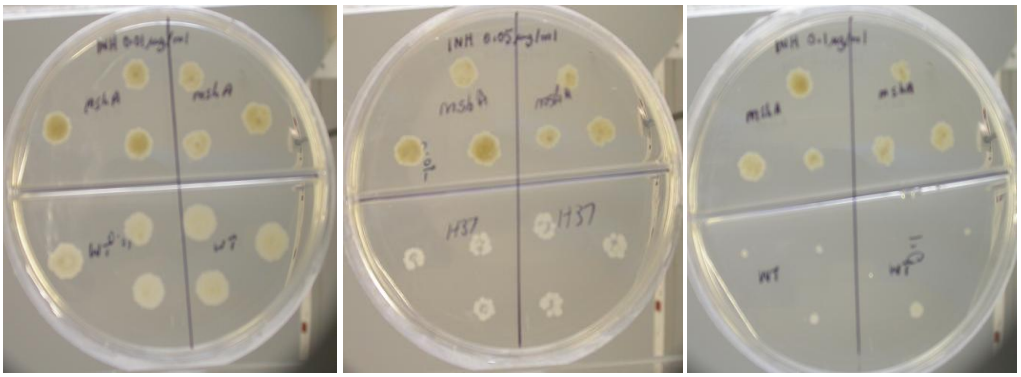
Figure 2.2: Growth of WT *M. tuberculosis* H37Rv and the Δ*msh*A gene knock-out mutant of *M. tuberculosis* H37Rv on solid media of Middlebrook 7H10 agar supplemented with 0.2% glycerol and OADC, AC, DC, ADC and ADS. [A = albumin, O = oleic acid, D = dextrose S = sodium chloride and C = catalase].

The *mshA* strain did not grow in the presence of ADS (bovine albumindextrose-sodium chloride), but grew normally under OADC supplementation. Catalase has been implicated in the detoxification of any reactive oxygen species or peroxides in the growth media. Mycothiol is important in detoxification, hence its absence in the *mshA* mutant explains the poor/no growth in the absence of catalase within the growth

media. Vilcheze *et al* (2008) hypothesised that (i) the mouse *in vivo* environment was not conferring significant oxidative stress or (ii) that the *in vivo* environment resulted in the production of other thiols that compensated the absence of mycothiol. Since Vilcheze *et al* (2008) had reported that *mshA* was not essential *in vivo*, we sought to generate the *mshC* conditional deletion mutant in *M. tuberculosis* (on-going project, data not reported herein).

2.2.3 Mycothiol mutants (ΔmshA) are resistant to INH

The *mshA* gene knockout mutant was used to investigate the phenotype in the presence and absence of conditions of stress, such as INH (see *figure 2.3*).



(A) (B) (C)

Figure 2.3: Sensitivity tests of *M. tuberculosis* H37Rv and Δ*mshA* to INH. In this figure, wild type (WT) *M. tuberculosis* H37Rv and the Δ*msh*A gene knock-out mutant of *M. tuberculosis* H37Rv were cultured on solid media of Middlebrook 7H10 agar supplemented with 0.2% glycerol and OADC. INH was added at different concentrations: (A) 0.01 µg/mL, (B) 0.05 µg/mL and (C) 0.1 µg/mL.

As shown in *figure 2.3* above the *mshA* deletion mutant of *M. tuberculosis* was resistant to INH at concentrations which inhibited growth of the WT. At 0.1 µg/mL INH Δ*mshA* still survived, but the growth of the WT was significantly reduced. Vicheze *et al* (2008), investigated this resistance mechanism, and proposed that mycothiol was required for the activation of the *katG* and *inhA* in *M. tuberculosis*. The *mshA* deletion mutant of *M. tuberculosis* is defective in mycothiol production, and in the absence of mycothiol *M. tuberculosis* became resistant to antibiotics due to reduced activities of both *katG* and *inhA*.

2.2.4 Mycothiol mutants (ΔmshA) are resistant to arsenic (V) acid

The importance of arsenate resistance in this investigation dates back to one of the oldest known antibiotic, *Salvarsan 606* which was based on the toxicity of arsenic acid. In *M. tuberculosis* arsenate reductases (important for protection against arsenate) use mycothiol and mycoredoxin in a thiol/disulfide redox cascade (Ordo´nez *et al,* 2009). Enzymes involved in mycothiol biosynthesis as well as these arsenate reductases have been suggested to be involved in arsenate resistance.

In this work, we used the Δ*mshA* mutant to investigate resistance patterns to arsenic acid (V). Indeed, the *mshA* strain showed increased resistance to arsenic acid (V) *vs* the WT strain (*figure 2.4*). Reduction of arsenate (V) to arsenite (III) is catalysed by arsenate reductase. The role of mycoredoxin is to reduce the thiol-arseno bond and form arsenite and mycothiol-mycoredoxin mixed disulphide. Mycoredoxin is then recycled by a second molecule of mycothiol, resulting in the formation of reduced mycothiol (MSSM) that, in turn is reduced by mycothionine reductase (model for this system is shown in *figure 2.5*).

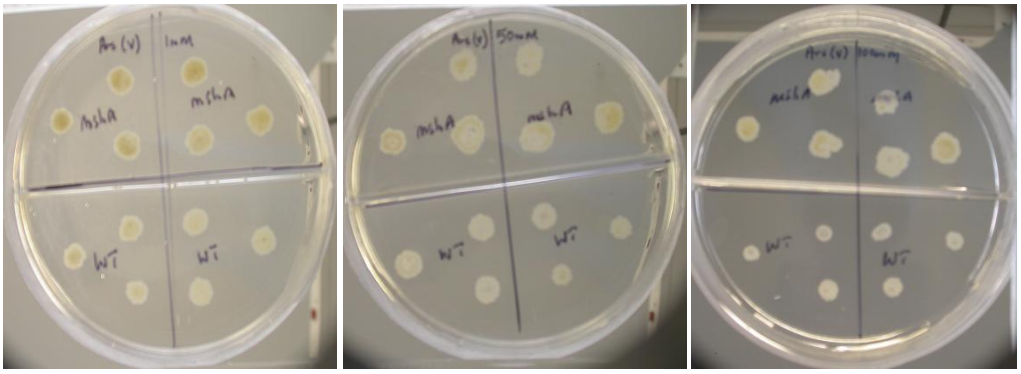


Figure 2.4: Growth of wild type (WT) *M. tuberculosis* H37Rv and the Δ*mshA* gene knock-out mutant of *M. tuberculosis* H37Rv on solid media of Middlebrook 7H10 agar supplemented with 0.2% glycerol and OADC. Arsenic acid (V) was added different concentrations of 1 mM, 50 mM and 100 mM.

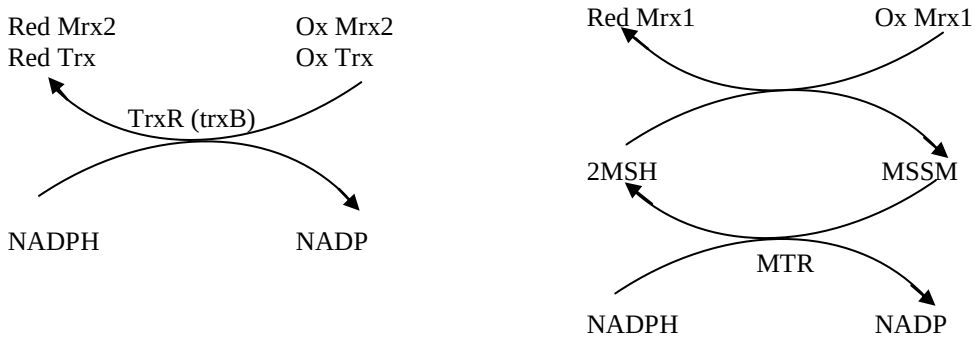
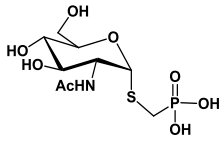


Figure 2.5: The proposed link between mycothiol pathway and the mycoredoxin system, and resistance mechanisms to arsenate. In this illustration, Mrx = mycoredoxin system, MSH = reduced mycothiol, MSSM = oxidised mycothiol, mtr = mycothionine reductase, Trx = thioredoxin system, Red = reduced form, Ox = oxidised form.

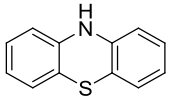
In further work it would be desirable to assess how the *mshA* gene-knockout would behave within infected macrophages, and then analyse replication/survival/death of this strain via electron microscopy and colony forming unit counts. Although the BACTEC 460^{TM} assay was used in this study, colony forming units would be useful to investigate *M. tuberculosis* survival after treatment with different inhibitors over time

2.2.5 Synthesis of drugs against enzymes of the mycothiol pathway

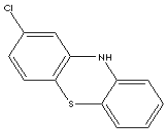
All the inhibitors and drug-like compounds directed against enzymes of the mycothiol pathway (specifically mshA) were synthesised by Dr Anwar Jardine at the University of Cape Town in South Africa. Expression and purification of enzymes crucial to the mycothiol pathway as well as subsequent enzyme inhibition tests were done at the University of Cape Town. In order to inhibit mshA (a *glycosyltransferase* involved in the first step of mycothiol biosynthesis), the substrate analogs of uridine diphosphate-N-acetyl glucosamine, UDP-GlcNAc (URID) were synthesised in a total of 10 steps from glucosamine. Likewise, the dipolar addition product of a thiopropargyl group and azide, gave the "CLICK" substrate analog in a total of 12 steps from glucosamine and uridine respectively. Commercially available substrate analogs were also required for testing (structures shown in *figure 2.6*). The most active compounds that were selected include the known glycosyl transferase inhibitors, nikkomycin (AJ7) and tunicamycin (AJ-8).



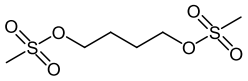
AJ-3



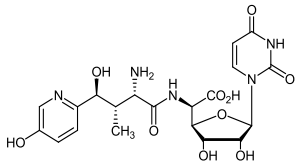
AJ-4, phenothiazine



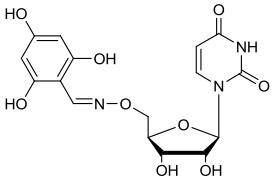
AJ-5, 2-chlorophenathiazine



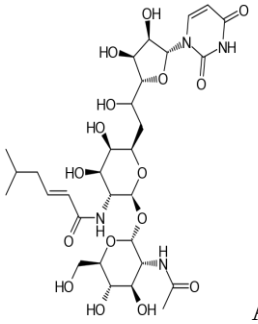
AJ-6, Busulfan



AJ-7, Nikkomycin



YY1



AJ-8, Tunicamycin

Figure 2.6: Structures of compounds that were tested in this study for anti-tubercular activity with specific reference to mycothiol biosynthesis**.**

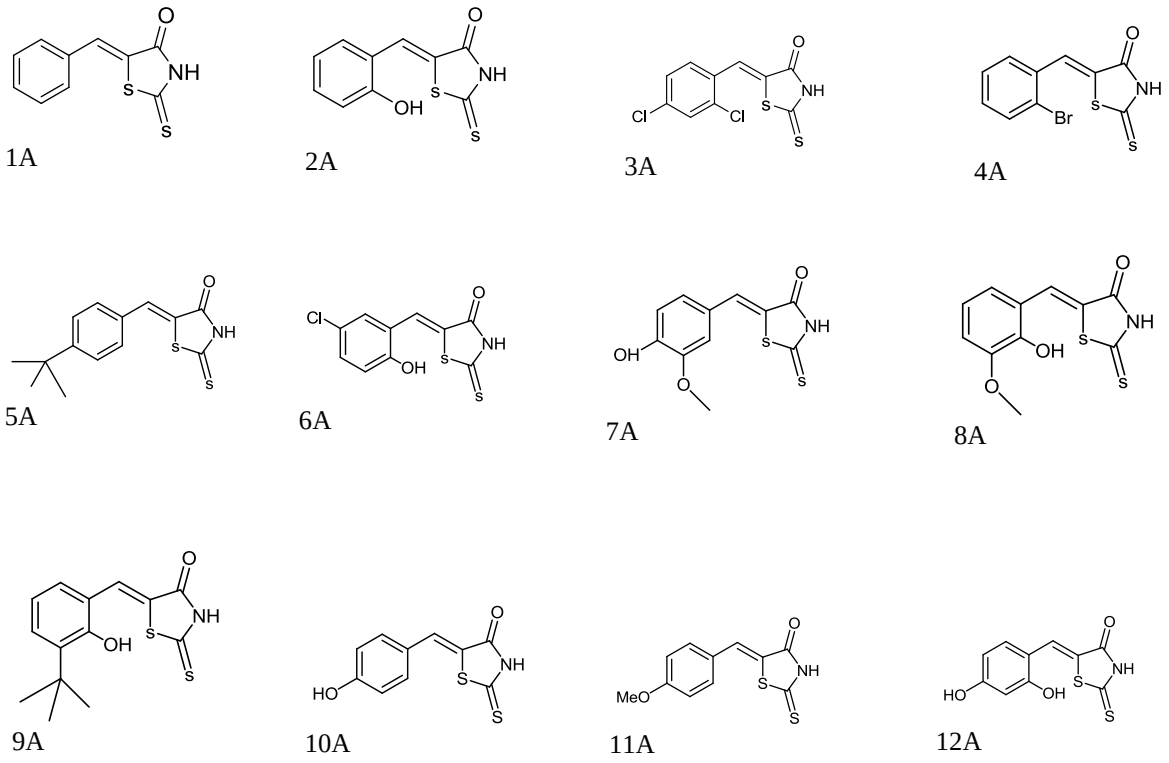


Figure 2.7: Structure of inhibitors of mycothiol enzymes that were tested on *in vitro* growing cultures of *M. tuberculosis*.

The treatment of aldehydes with rhodamine and rhodamine-3-acetic acid gave a library of compounds. Thiazolidinone scaffold is known to demonstrate widespread biological activity. The thiazolidine 3-acetic acid library was further coupled to URID to give a series of relatively stable, active ester intermediates, T1A-T12A. Longer reaction times of the T1A – T12A series of inhibitors gave less active compounds T1C-T12C as shown by extracellular activities in *figure 2.9*.

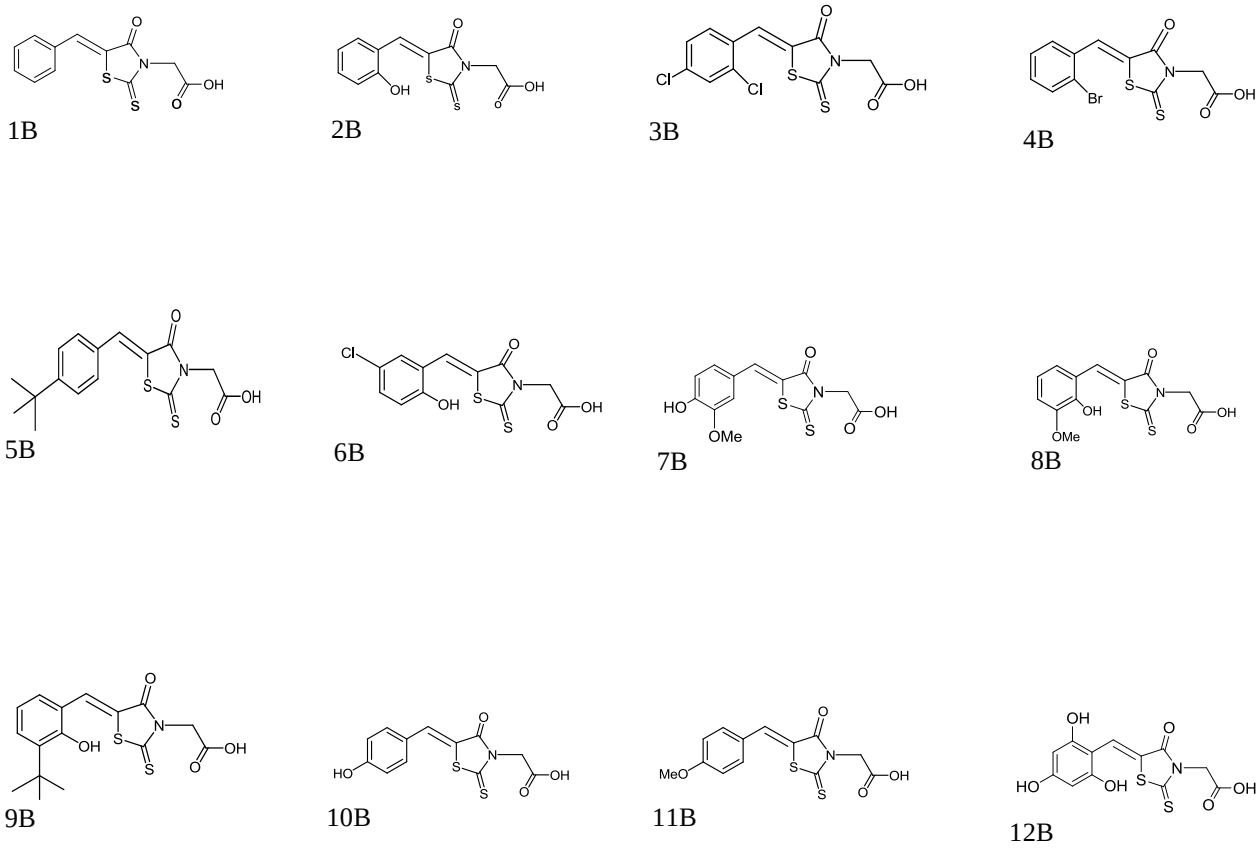
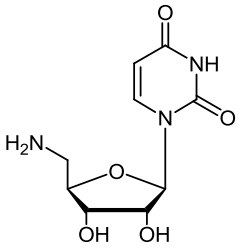
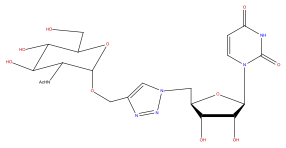


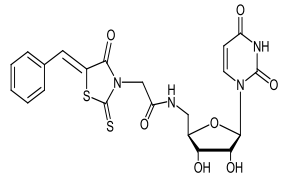
Figure 2.8: Structures of inhibitors of mycothiol enzymes that were tested on *in vitro* growing cultures of *M. tuberculosis*.



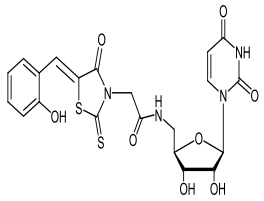
URID



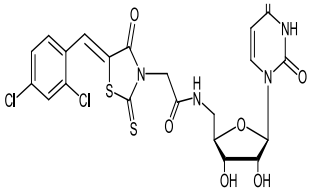
CLICK



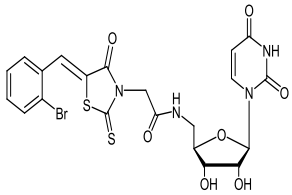
T1C



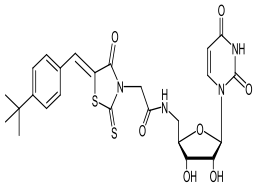
T2C



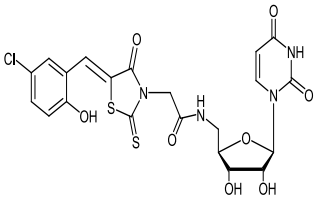
T3C



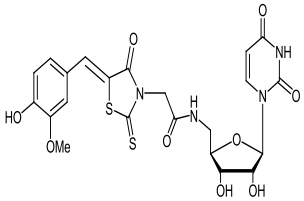
T4C



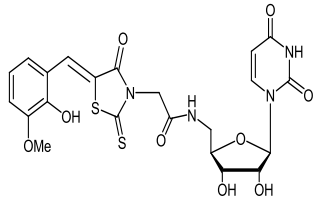
T5C



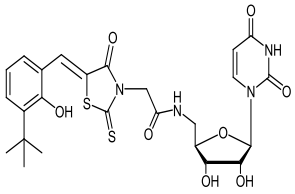
T6C



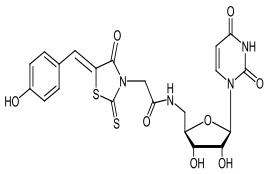
T7C



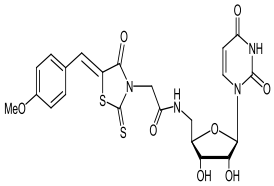
T8C



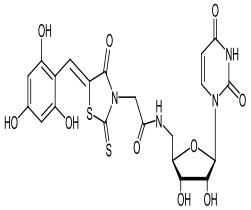
T9C



T10C



T11C



T12C

Figure 2.9: Structures of inhibitors derived from longer reaction times of the thiazolidine 3-acetic acid library (T1A - T12A series of inhibitors) after coupling to URID giving active ester intermediates.

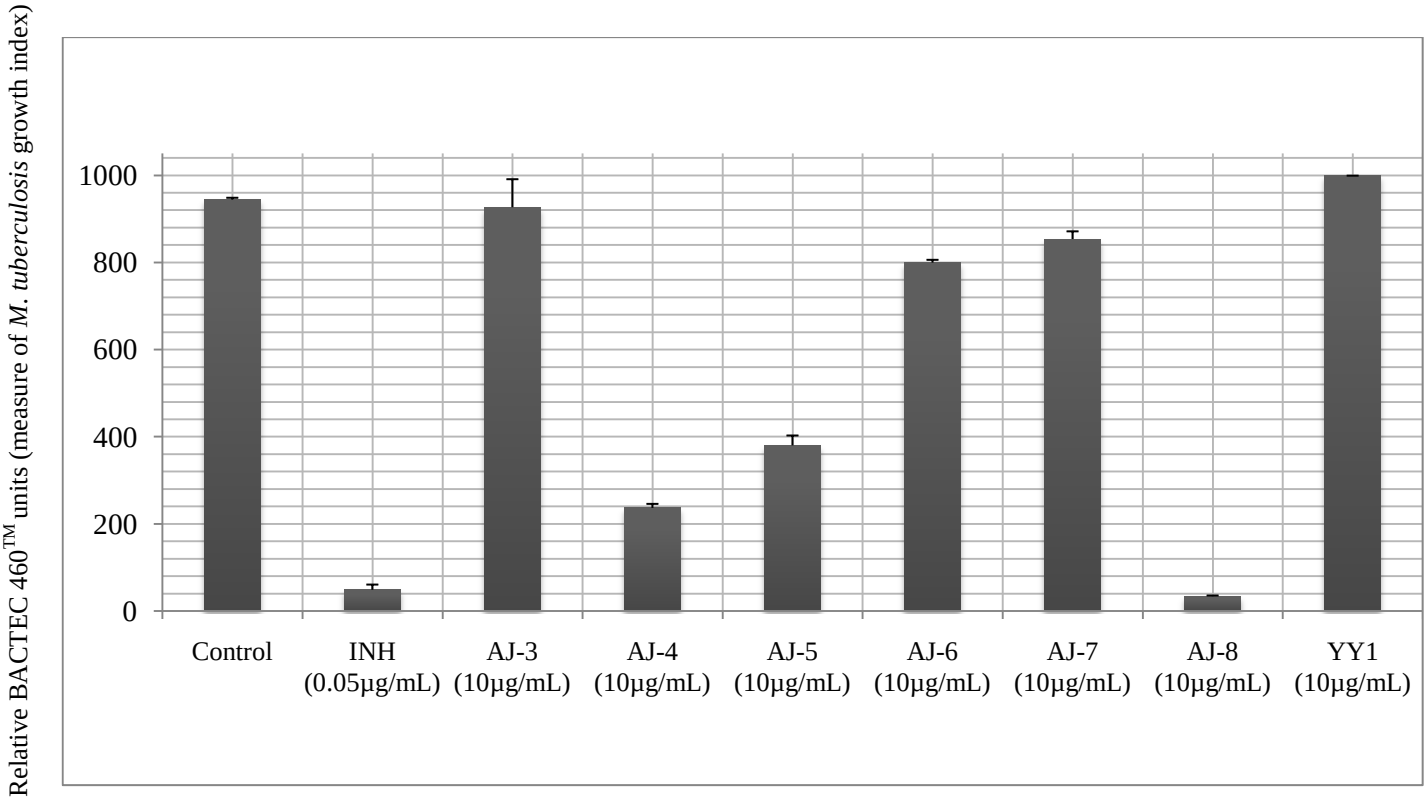


Figure 2.10: Extracellular drug testing at 1 x 10^7 *M. tuberculosis* per BACTEC 460™ vial containing 4 mL Middlebrook 7H12 medium. For control, *M. tuberculosis* was inoculated into BACTEC 460™ vials with no inhibitor or drug added.

2.2.6 Antimicrobial activities of phenothiazines, tunicamycin, busulfan and nikkomycin

Phenothiazines have been reported as inhibitors of type II NADH:menaquinone oxidoreductases (Martins *et al*, 2008). It is known that N-substituted phenothiazine possess unacceptable side effects on the central nervous system at high dose. Phenothiazines are known to exert significant mycobactericidal activity against *M. tuberculosis* strains resistant to first and second line antitubercular drugs (Weinstein *et al*, 2005). Derivatives of phenothiazines were tested for binding and inhibitory activity towards mshA. From the extracellular screening shown in *figure 2.10*, it was found that phenothiazines (AJ-4 and AJ-5) and tunicamycin (AJ-8) were effective for *in vitro* inhibition of *M. tuberculosis.* These findings were in agreement with the observations by Weinstein *et al*, 2005. Phenothiazines have been shown to exhibit high activity as anti-tubercular compounds, especially against *M. tuberculosis* strains that have resistance towards INH, RIF, streptomycin, PZA, and EMB combined.

The limitation of phenothiazines was that the concentration required *in vitro* for bacteriocidal activity was much greater than the clinical concentration of drug *in vivo*. It was against this background that substrate analogues of phenothiazines with greater activity were sought through derivatisation of parent compounds. Although phenothiazines are attractive in the treatment of *M. tuberculosis,* the fact that they are concentrated within the macrophages means that the dose required for treatment would be greatly reduced (Martins *et al*, 2008).

It is reasonable to speculate that the modulation of electron transport in *M. tuberculosis* by phenothiazines may have a profound effect on the entrance and maintenance of dormancy. These compounds interfere with drug efflux pumps and mechanisms that inhibit the activation of Ca^{2+}-dependent ATPase which generates H^+ ions required for providing energy for activity of the pumps via the H^+-ion gradient (Weinstein *et al*, 2005). Phenothiazines may accelerate the course of TB treatment by interfering with bacterial persistence, thus making other anti-TB agents more effective. Although antimicrobial activities of phenothiazines have been well documented, their use against psychosis has led to complications in patients (Martins *et al*, 2008). Among the compounds tested was busulfan (AJ-6), an anti-cancer drug given *in vivo* or *in vitro* to decrease hepatocyte glutathione by 60 and 50%, respectively (Martins *et al*, 2008). It was hypothesised that busulfan becomes conjugated to glutathione with and without the assistance of glutathione–S-transferase (Jardine, personal communication). The rationale for testing busulfan was therefore to understand how it interacts with the *M. tuberculosis* homolog, mycothiol.

Nikkomycins (AJ-7) are peptidenucleoside antibiotics which competitively inhibit synthatase enzymes due to their close similarity to UDP-N-acetylglucosamine. These compounds have been reported to be non-toxic and degrade easily, and could therefore be exploited as anti-mycobacterial agents in combination therapy (Decker *et al*, 1991). Despite these attractive features of nikkomycins, they have been found to be required in high concentrations for whole cell assay inhibition.

2.2.7 Thiozolidinone libraries (1A-12A and 1B-12B)

Thiozolidinone libraries (1A-12A and 1B-12B) had moderate activity and were tested in enzyme assay, and were tested on *in vitro* growing *M. tuberculosis* (results shown in *figure 2.11*). Molecular dynamic docking experiments were performed in the mshA enzyme active site to aid further compound design. The 5'-deoxy 5-amino uridine and thiazolidinone 3-acetic acid amide coupling reaction provided a relatively unstable set of active ester intermediates (TA1-TA12) that displayed the highest potency (results shown in *figure 2.13*).

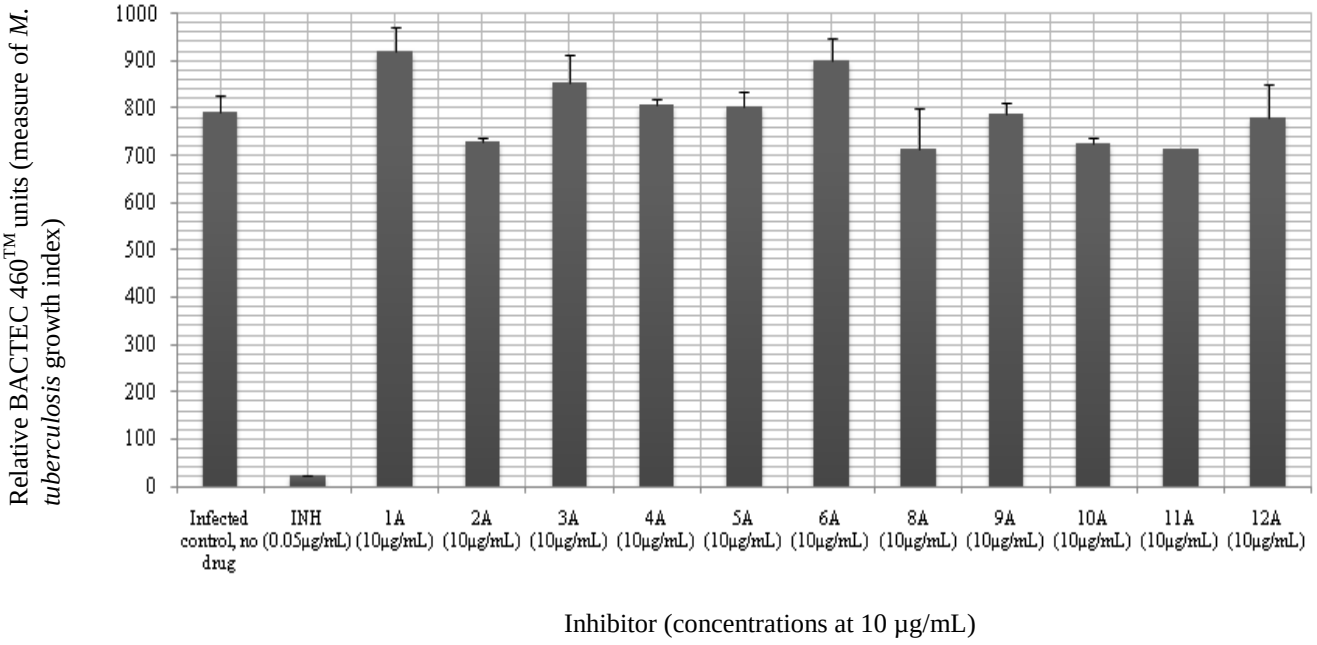


Figure 2.11: Extracellular drug testing at 1 x 10^7 *M. tuberculosis* per BACTEC 460™ vial containing 4 mL Middlebrook 7H12 medium. The relative BACTEC 460^{TM} values were used as a measure of *M. tuberculosis* growth index in Middlebrook medium.

The promising compounds (1B, 2B, 7B, 12B and AJ-8) with highest inhibitory activity were used at three different concentrations (10, 1 and 0.1 µg/mL) and inhibition was monitored over time. The MICs of these compounds were established to be between 1 an 10 µg/mL (*figure 2.7*). However these compounds lost biological activity at concetrations below 1 µg/mL.

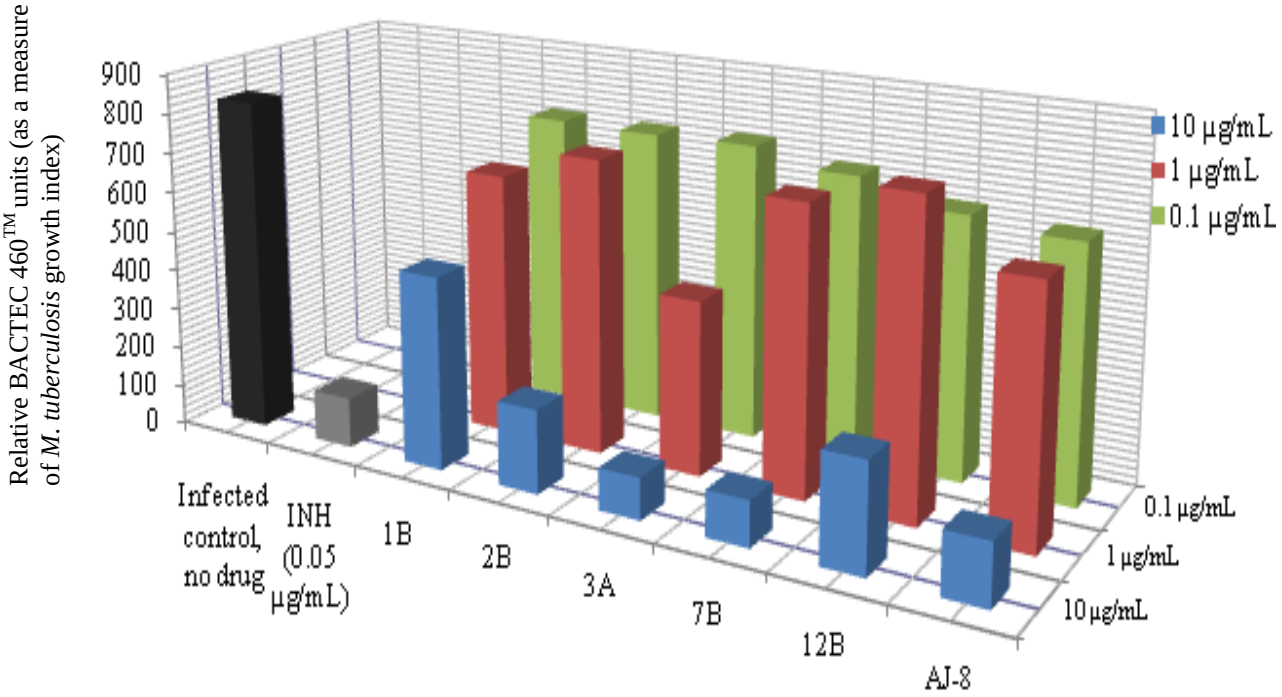


Figure 2.12: Extracellular drug testing at 1 x 10^7 CFUs *M. tuberculosis* per BACTEC 460™ vial containing 4 mL 7H12 Middlebrook medium.

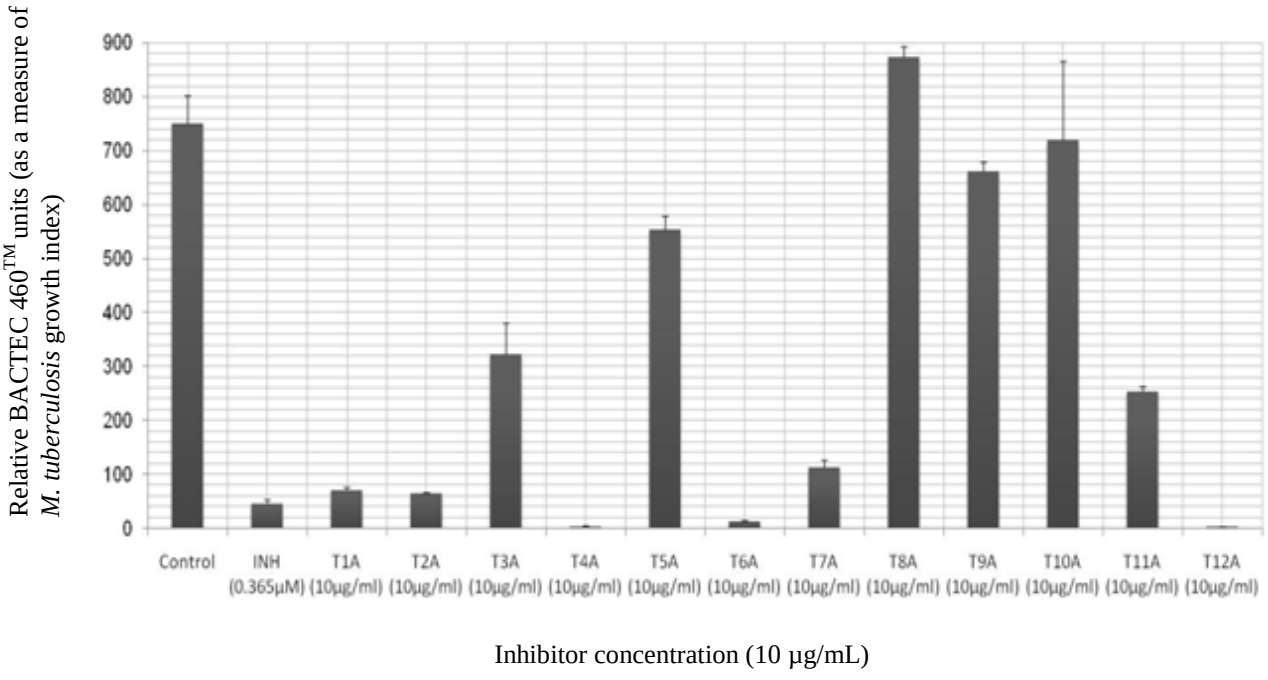


Figure 2.13: Extracellular drug testing at 1 x 10^7 *M. tuberculosis* per BACTEC 460™ vial containing 4 mL Middlebrook 7H12 medium. The relative BACTEC 460^{TM} values were used as a measure of *M. tuberculosis* growth index in Middlebrook medium.

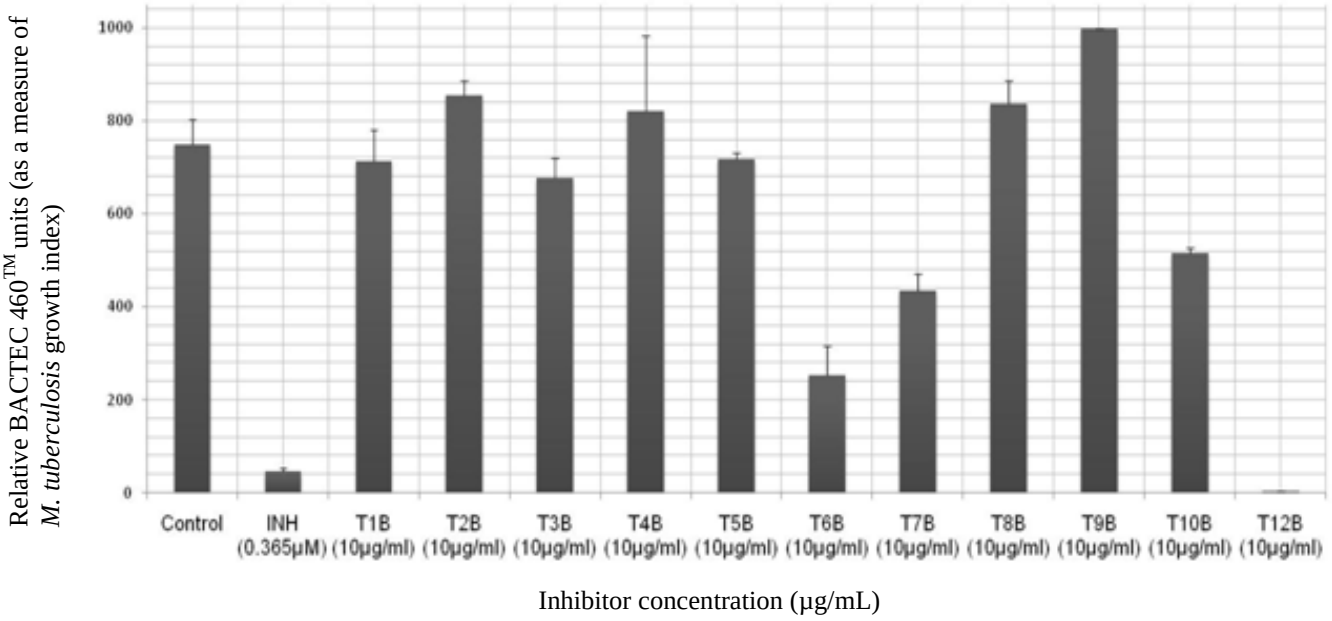


Figure 2.14: Extracellular drug testing at 1 x 10^7 *M. tuberculosis* per BACTEC 460™ vial containing 4 mL Middlebrook 7H12 medium.

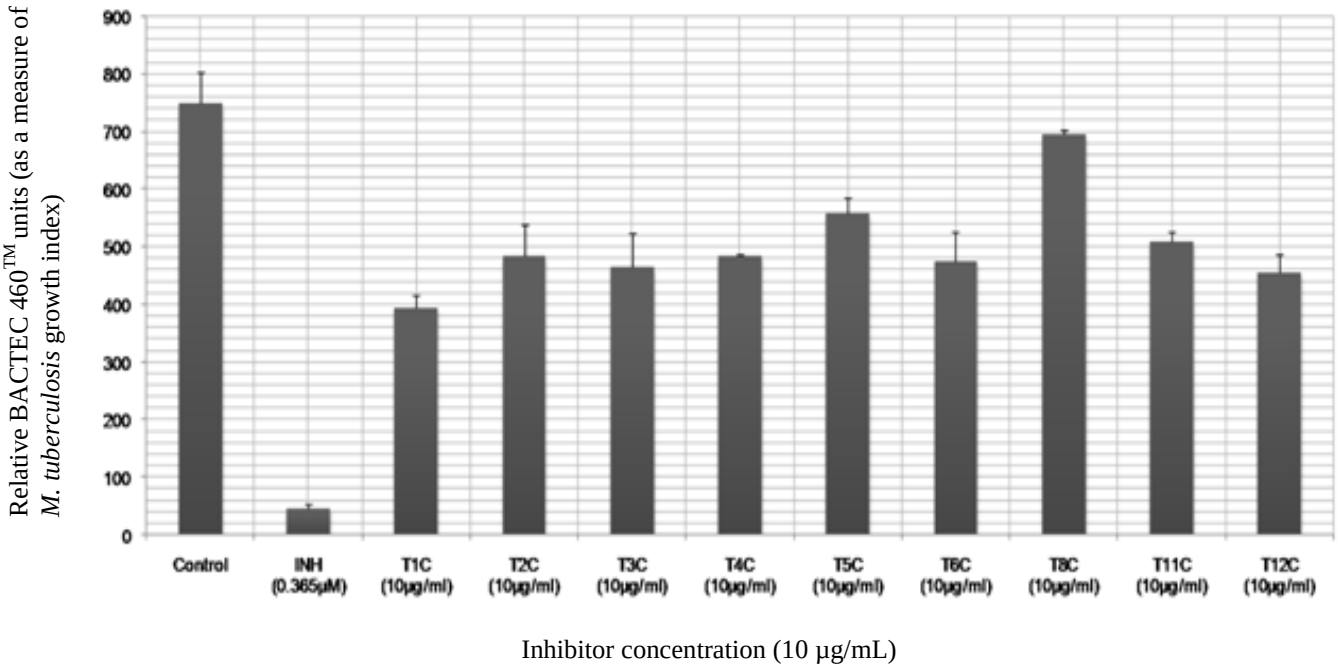


Figure 2.15: Extracellular drug testing at 1 x 10^7 *M. tuberculosis* per BACTEC 460™ vial containing 4 mL Middlebrook 7H12 medium.

The mycobacteriocidal activities of the three sets of libraries (T1A-T12A, T1B-T12B and T1C-T12C) were done extracellularly on *in vitro* grown *M. tuberculosis*. Mycobacterial survival and death were monitored using the BACTEC 460^{TM} assay (see results in *figure 2.13, figure 2.14* and *figure 2.15*). The T1A-T12A series of compounds showed increased activity relative to the T1B – T12B and T1C – T12C series. Increased inhibition of the T1A – T12A series was due to the non-selective nature in the reactivity of these compounds with any molecule containing amide moieties (Jardine, personal communication). The T1C – T12C library was derivatised from T1B – T12B library through longer reaction times. However, from this library, only T1C gave highest inhibition of 50% at 10 µg/mL. Consequently, no further tests were done in the macrophage assay with this library of compounds.

CHAPTER 3: INTRACELLULAR DRUG TESTING

3.0 INTRODUCTION

The aim of this study was to analyse the inhibitory activities of candidate inhibitors designed against enzymes of the mycothiol pathway, specifically *mshA* (a glycosyltransferase), by comparing the fate of *M. tuberculosis* in treated and untreated macrophages. In *Chapter 2* a wide variety of drugs that affect enzymes of the mycothiol pathway were tested for their ability to prevent *in vitro* growth of *M. tuberculosis*. The most potent drugs in the *in vitro* studies were now used to test their ability to inhibit growth of *M. tuberculosis* within host macrophages. BACTEC 460^{TM} measurements were used to analyse bacterial survival within the macrophages. After extracellular screening of the most active compounds, intracellular testing was performed on THP1 cell line. Human macrophage-like cell line THP1 (ATCC TIB-202) was chosen for drug screening because these cells were much easier to obtain and culture.

3.1 MATERIALS AND METHODS

3.1.1 Culture conditions for the THP1 cell line

The THP-1 human macrophage cell line were grown at 37°C in 5% CO_2 in RPMI 1640 (Sigma, USA) supplemented with 10% heat-inactivated FCS (Gibco^{TM}, Invitrogen, Germany) with penicillin-streptomycin, 1% final concentration (Invitrogen). Cells were placed in a 50 ml tube + 8 ml (+/- 10 ml) medium and pelleted by spinning at 1 300 rpm for 5 minutes at 21^{o}C. The supernatant was removed and the tube was dragged over round-bottom 96 well plate to re-suspend the pellet. A 10 ml aliquot of complete RPMI medium was added to the tube to re-suspend cells. All of the liquid was aliquoted into a flask (small filter cap TC flask), and incubated at 37^{o}C for three days. Cells were checked everyday and fresh medium added every 2nd or 3rd day. Cells were harvested at 1 week, pelleted and re-suspend in fresh complete RPMI, lacking antibiotics and counted using a haemocytometer.

THP-1 cells were differentiated into macrophages by using phorbol myristic acetate (PMA, Sigma, USA). Once differentiated THP1 cells become adherent monolayers and stop dividing and to acquire typical characteristics of macrophages such as the capacity to phagocytize bacteria and to express bactericidal

activities **(**Tsuchiya *et al*, 1982). PMA was added to a final concentration of 100 nM and 1 mL of cells added per well in 24 well plates at a concentration of 5 x 10^5 cells/mL (all experiments were done in triplicate). Cells were incubated overnight, and non-adherent cells were removed by washing 3 times with 1 ml ice-cold phosphate buffered saline. At selected time points after infection with *M. tuberculosis*, infected macrophages were exposed to inhibitors of the mycothiol pathway, and intracellular survival was assessed.

3.1.2 Macrophage infection

1 mL aliquots of *M. tuberculosis* were removed from the -80^{o}C freezer and thawed. Mycobacteria were passaged 30 times through a with 1 ml insulin syringe to remove clumps. The required amount of mycobacteria was centrifuged at 13 000 rpm for 20 minutes. The supernatant was removed and bacilli were re-suspended in 1 mL medium (RPMI, 10% FCS). The required amount was centrifuged at 13 000 rpm for 20 minutes, and the supernatant removed. A 1 mL aliquot of medium (RPMI, 10% FBS) was added and passaged 10 times through a 1 ml insulin syringe. Macrophages were infected at an MOI of 5:1 and incubated for 4 hours at 37^{o}C, in a 5% CO_2 incubator. Non-ingested *M. tuberculosis* was then washed off with RPMI containing 10% FCS. Infected macrophages were incubated at 37^{o}C in fresh medium that was renewed every 2 days thereafter.

3.1.3 Evaluating inhibitory effects of novel compounds designed against the mycothiol pathway

At day 1 or day 2 post infection, *M. tuberculosis*-infected macrophages were treated with candidate inhibitors of mycothiol enzymes to determine the optimal concentrations and treatment time for inhibitory effect on mycobacterial growth. At selected time-points during the drug treatments, infected macrophages were lysed with 0.1% Triton X-100 (Sigma-Aldrich, USA) to isolate *M. tuberculosis.* Mycobacteria were pelleted at 13 000 rpm for 20 minutes, and re-suspended in 1 ml 7H12 Middlebrook medium. The samples were analysed for *M. tuberculosis* growth in a BACTEC 460™ instrument. Inhibitory effects of each test compound were evaluated in relation to infected treated controls.

3.2 RESULTS AND DISCUSSION FOR INTRACELLULAR DRUG TESTING

3.2.1 Drug testing in the macrophage assay

THP-1 macrophages were infected with *M. tuberculosis* at an MOI 5:1 for up to 2 days. On day 3 post-infection, *M. tuberculosis*-infected macrophages were treated with inhibitors of the mycothiol pathway, and incubated for different time-points (typically, 4, 8, 12 and 24 hrs) post-drug exposure.

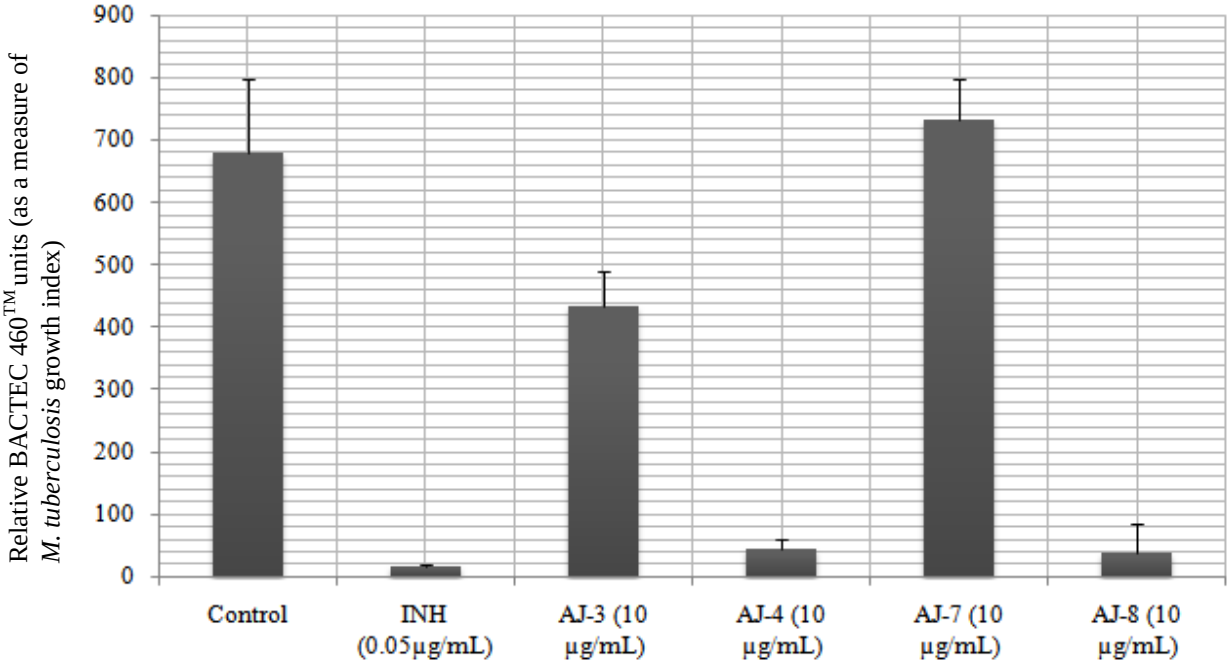


Figure 3.1: Intracellular drug testing on THP1 cells infected with *M. tuberculosis* at MOI 5:1. *M. tuberculosis* was inoculated into BACTEC 460™ vial containing Middlebrook 7H12 medium. In this figure, AJ-3 = substrate mimic for mycothiol enzymes, AJ-4 = phenothiazine, AJ-7 = nikkomycin, AJ-8 = tunicamycin. INH was used as a positive control at 0.05µg/mL concentration, while the negative control *M. tuberculosis*-infected macrophages without any drug added.

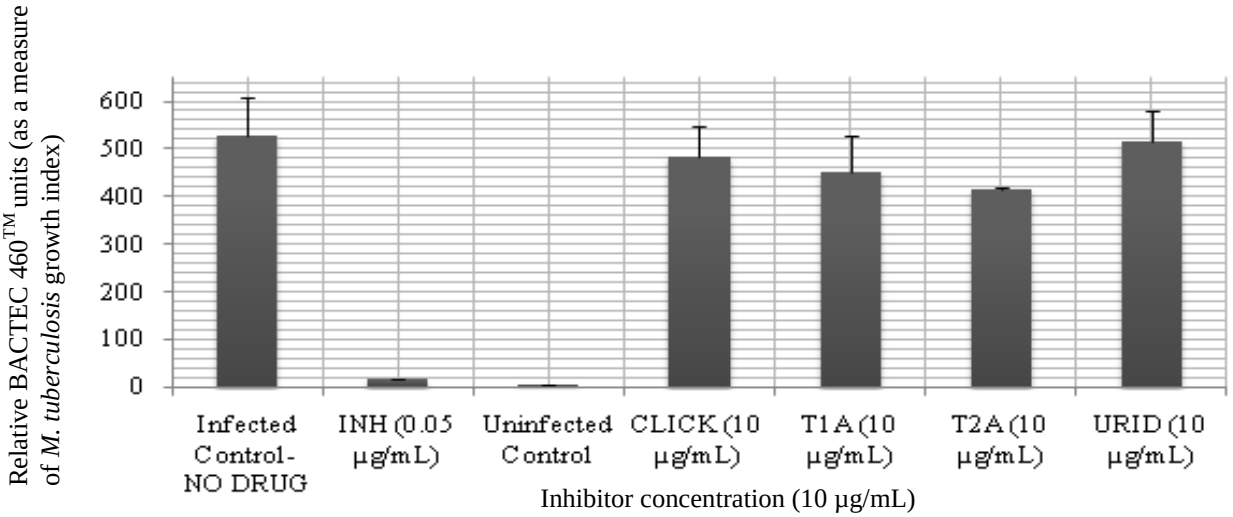


Figure 3.2: Intracellular drug testing on THP1 cells infected with *M. tuberculosis* at MOI 5:1. *M. tuberculosis* was inoculated into BACTEC 460™ vials containing 4 mL Middlebrook 7H12 medium.

As shown in *figure 3.1*, tunicamycin (AJ-8) was found to exhibit 95% inhibition on intracellular *M. tuberculosis*. In subsequent experiments, AJ-8 was used in order to determine its MIC in the macrophage environment. From the T1A-T12A series of compounds, none of these was found to display significant inhibition in the macrophage model, *figure 3.2*. Despite having shown good activity in the extracellular assay, *figure 2.8*, these compounds showed limited inhibition on the macrophage assay. The compound with highest potency in *figure 3.2* was T2A which had 19% inhibition in the intracellular assay. We suggested that this library of inhibitors lost biological activity over time due to instability since they were reaction intermediates. The other two series of libraries (T1B-T12B and T1C-T12C) did not show any inhibition, suggesting that these compounds required structural modifications if they could be used in future studies.

The CLICK and URID were tested for inhibition separately, *figure 3.2* before coupling to the 1A-12A series of compounds so as to ascertain any contribution that these compounds would contribute towards biological activity of the T1A-T12A series. However URID and CLICK displayed very little difference when compared with the infected control (with no drug added), suggesting that the coupled groups would be responsible for biological activity in the macrophage system. Thus the activity observed in T1A and T2A was due, mainly to the substituent group.

CHAPTER 4: Assessment of morphological alterations of mycobacteria in macrophages after treatment with inhibitors of enzymes of the mycothiol pathway: and the resultant consequences on phagosome maturation

4.1 INTRODUCTION

Electron microscopy approaches were used to gain more insight into the fate of *M. tuberculosis* after treatment of infected macrophages with drugs designed against enzymes of the mycothiol pathway. The aims of this study were: (i) to determine if the inhibitors had any deleterious effects on the host macrophages, (ii) to characterise the ultra-structural modifications of mycobacteria, and (iii) to determine if the phagosome maturation block which is a major survival strategy of pathogenic mycobacteria was affected by inhibitors of the mycothiol pathway.

It is well established that *M. tuberculosis* prevents phagosome maturation and therefore, fusion of phagosomes with lysosomes (*reviewed e.g. in* de Chastellier, 2008). This is considered to be a major survival strategy of pathogenic mycobacteria (de Chastellier *et al*, 1997; de Chastellier and Thilo, 2006, de Chastellier *et al*, 2009). Conclusive evidence by de Chastellier and Thilo (2006) shows that whenever phagosomes remain immature, the phagosome membrane is closely apposed all around to the mycobacterial surface. The authors proposed that whatever the molecular mechanisms involved in prevention of phagosome maturation, the establishment and maintenance of a close apposition around the mycobacterial surface is a requirement if phagosomes are to remain immature. This will occur when phagosomes contain a single bacterium (loner phagosomes).

In contrast, when phagosomes contain several bacteria or clumps (social phagosomes), the phagosome membrane is no longer closely apposed to the bacterium in regions that span two adjacent bacteria. Such phagosomes systematically mature and fuse with lysosomes (Clements and Horwitz, 1995; de Chastellier

and Thilo, 1997). Immature and matured phagosomes cannot be distinguished directly under the electron microscope, and as a result, implicit parameters are used to determine whether phagosome maturation has occurred (de Chastellier and Thilo 1997). For this purpose, endocytic tracers such as HRP are added to cells before or after infection in order to locate and distinguish different compartments of the endocytic pathway.

The study was initiated with THP-1 macrophages infected with *M. tuberculosis*. However THP-1 macrophages often contained abnormal vesicles with dense material as well as autophagic vacuoles. In addition, THP-1 cells were found to lift off the dishes and die within 2-4 days post-infection, depending on the MOI used. THP-1 cells could therefore not be used for experiments which required longer periods of drug exposure. Furthermore, THP-1 cells were found to have a small lysosomal compartment and we thought that this might jeopardise studies on fusion of *M. tuberculosis* containg phagosomes with lysosomes. Mouse BMDMs were therefore used in subsequent experiments as these have been successfully used in several laboratories studying phagosome processing (e.g. de Chastellier *et al* 1995).

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains and culture conditions

M. tuberculosis and *M. avium* were used to study the effects of different inhibitors of the mycothiol pathway on intracellular survival. Mycobacterial culture conditions were similar to those described previously in *Chapters 2* and *3*.

4.2.2 Culturing of mouse bone marrow derived macrophages (BMDM)

Bone marrow cells were obtained from femurs of 6-8 week-old C57BL/6 female mice and seeded into tissue culture dishes of 35 mm diameter or in 6-well tissue culture plates. The culture medium was Dulbecco's Modified Eagles Medium (DMEM, Invitrogen, USA) with high glucose (1g/L) and high carbonate (3.7g/L) concentrations supplemented with 10% heat-inactivated FCS (Gibco, USA), 10% L-929 cell conditioned medium (a source of colony stimulating factor-1), and 1% glutamine (Sigma, USA). At 5 days after seeding, adherent cells were washed twice with DMEM and re-fed with complete medium.

Medium was then renewed on day 6 and no antibiotics were added at any one point during the culturing process.

4.2.3 Infection of macrophages with mycobacteria and treatment with selected drugs

BMDMs were infected with *M. avium* or *M. tuberculosis.* Inhibitors of enzymes of the mycothiol pathway were added to infected macrophages at selected time points post-infection and different concentrations. Infected macrophages without treatment were used as experimental controls. At selected time points of treatment, cells were fixed and processed for transmission electron microscopy via a collaborative study with Dr. Chantal de Chastellier at the *Centre d'Immunologie de Marseille-Luminy*, France.

4.2.4 HRP uptake and staining for HRP

At selected time-points during drug treatment, infected cells were incubated for 1hr at 37^{o}C in complete medium containing 25 µg/mL or 1 mg/mL HRP. HRP was stained with an enzyme cytochemistry method (de Chastellier *et al*, 1995; de Chastellier, 2009). Cells were fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, containing 0.1M sucrose, 5mM $CaCl_2$ and 5mM $MgCl_2$ for 1 hr at room temperature. After 2 washes for 15 minutes with cacodylate buffer with sucrose.

4.2.5 Processing of samples for transmission electron microscopy

Cells were fixed for 1 hour at room temperature with OsO_4, 1% in cacodylate buffer devoid of sucrose. After 3 brief washes with sodium cacodylate buffer devoid of sucrose cells were scraped off the tissue culture dishes with a rubber policeman and then concentrated in 2% agar prepared in 0.1 M cacodylate buffer devoid of sucrose. Samples were dehydrated with a graded series of ethanol (25%, 50%, 75%, 90% each for 15 min, and 100% ethanol dried on molecular sieves, 3times for 30 min). Samples were embedded in Spurr resin. For this purpose ethanol was progressively replaced by Spurr resin by incubating samples in increasingly higher concentrations of Spurr (Spurr: ethanol ratios of 1:3, 1:1, 3:1) for 1 hour each at room temperature. Samples were immersed in pure Spurr resin at room temperature for 3 hours at room temperature, followed by 3 hours at 37^{o}C. Samples were then placed in moulds to which Spurr resin was added and incubated at 60^{o}C for polymerisation of resin. Blocks of embedded cells were sectioned and

observed using a Zeiss EM912 transmission electron microscope. Embedded cells were randomly sectioned in all directions to ensure a correct representation and phagosome count. 100–200 phagosomes per sample were examined.

4.3 RESULTS AND DISCUSSION

Macrophages were infected with *M. avium* and *M. tuberculosis* and then treated with two drugs designed against enzymes of the mycothiol pathway, i.e. tunicamycin (AJ-8) and phenothiazine (AJ-4). These drugs were chosen for treatment of infected macrophages because they seemed to be potent inhibitors of *M. tuberculosis* growth in the extracellular and intracellular assays (reported in *Chapters 2* and *3*).

4.3.1 Morphological appearance of infected macrophages treated with selected inhibitors

4.3.1.1 Treatment with tunicamycin (AJ-8)

Macrophages infected with *M. tuberculosis* or *M. avium* were treated with tunicamycin (AJ-8) at different concentrations and time points. Ultra-structural changes in *M. tuberculosis* or *M. avium*-infected and drug treated macrophages were compared to the control experiments, *figure 4.1*. Infected cells were incubated with HRP at either 25 µg/mL or 1 mg/mL to locate lysosomal compartments which appear as dark and dense vacuoles within the cytoplasm. After treatment with tunicamycin (AJ-8) at 5µg/mL for 24 hours, the endoplasmic reticulum of cells was found to be swollen, but ribosomes were still attached. The cytoplasm was less dense than usual due to extraction of protein or cell components from the cytoplasm, thereby indicating that cells were undergoing permeabilisation and cell lysis. Cholesterol crystals were observed to be accumulating in lysosomes. Cisternae of Golgi apparatus were swollen, suggesting that the observed effects were due to tunicamycin. With lower concentrations of tunicamycin, cells were less frequently lysed but they still displayed the above alterations and a large proportion were permeabilised. Tunicamycin being cytotoxic to macrophages, was abandoned as a potential drug candidate.

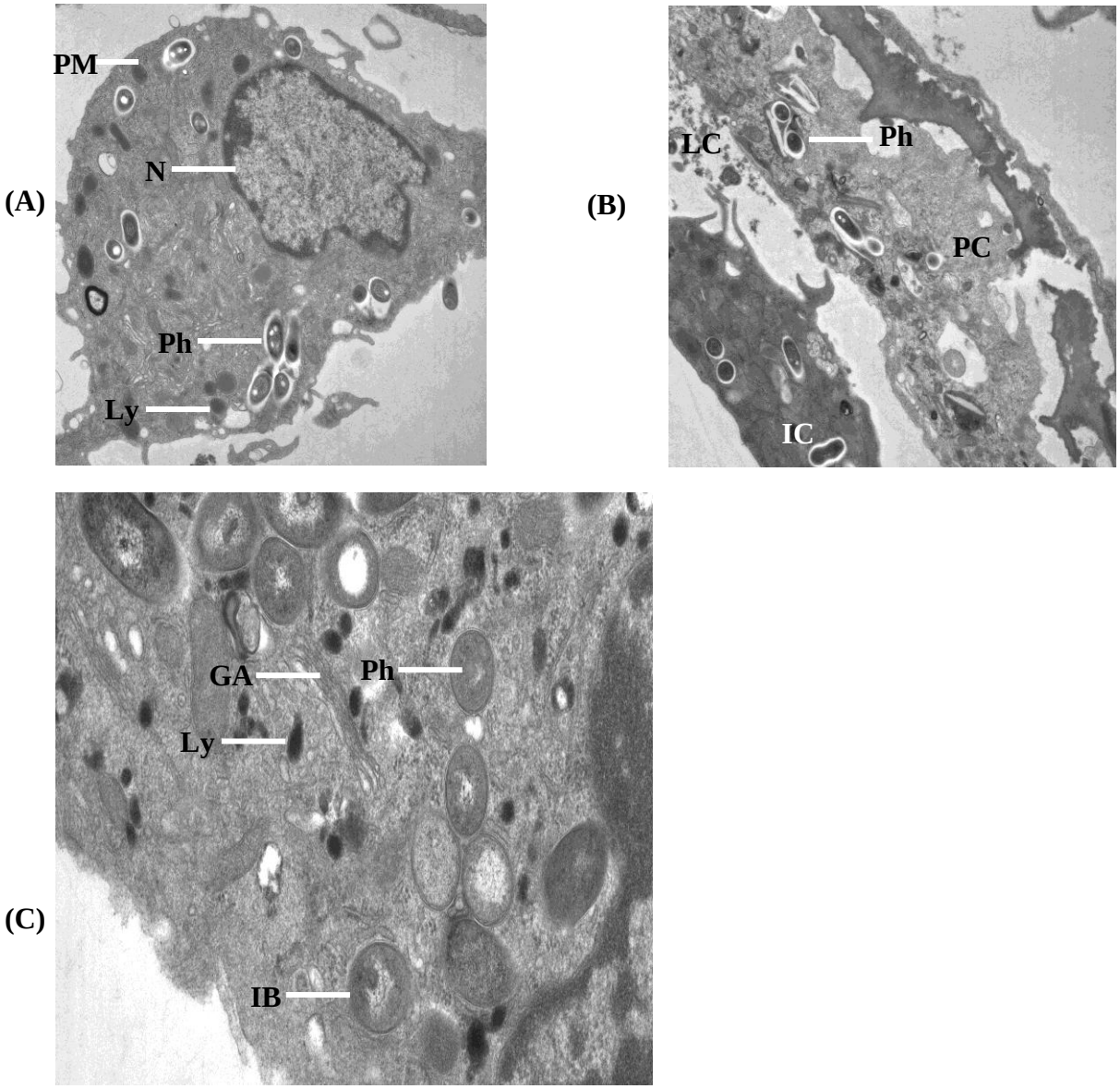


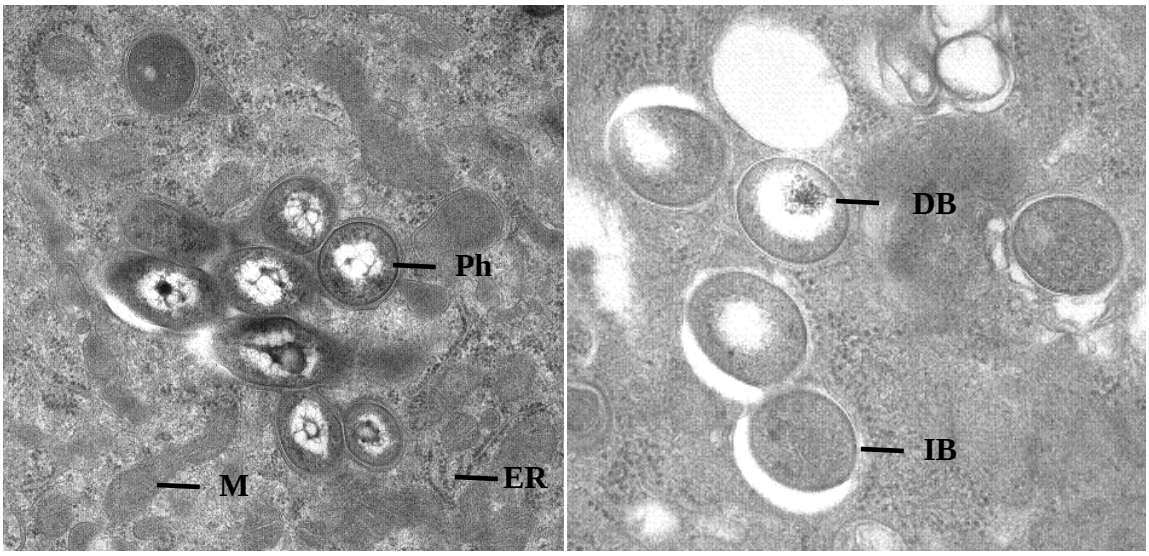
Figure 4.1: Electron micrographs showing macrophages infected with *M. tuberculosis* and then exposed to inhibitors of enzymes of the mycothiol pathway: (A) = control macrophages infected with *M. tuberculosis*, (B) = macrophages infected with *M. tuberculosis* and exposed to AJ-8, and (C) = macrophages infected with *M. tuberculosis* and exposed to AJ-4. In these electron micrographs, Ly = lysosome, GA = Golgi apparatus, Ph = phagosome, IB = intact bacteria, N = nucleus, LC = lysed cell, PC = permeabilised cell, IC = intact cell.

4.3.1.2 Treatment with phenothiazine (AJ-4)

Cells treated with different concentrations of phenothiazine, *i.e*. 25 µg/mL, 10 µg/mL and 1 µg/mL. At higher concentrations, phenothiazine resulted in cell lysis, *i.e*. 100% and >80% cell lysis at 25 µg/mL and 10 µg/mL, respectively. At 1 µg/mL phenothiazine, cells were found to be morphologically intact, and this concentration was used for further drug testing and morphological assessments. These observations indicated that phenothiazine could be used as an inhibitor since it had no deleterious effects on the macrophages. Subsequent experiments were done with phenothiazine to investigate its killing effects on *M. tuberculosis* within infected macrophages.

4.3.2 Ultra-structural appearance of M. tuberculosis within infected macrophages treated with phenothiazine (AJ-4)

Mouse BMDMs were infected with *M. tuberculosis*, MOI 2:1, treated with phenothiazine (AJ-4) at 1 µg/mL starting from day 4 post-infection and fixed at day 5 post-infection after 1 day and at day 7 after a 3-day treatment. We then determined the effect of drug treatment in terms of its morphological alterations on *M. tuberculosis, figure 4.2*. Intracellular bacteria were sorted into two distinct categories on the basis of their morphological appearance (Fréhel *et al*, 1997): (i) intact bacteria which display a regular shape, a well organized nuclear region and cytoplasm with few or no intracellular lipid inclusions, and an intact cytoplasmic membrane and cell wall with an electron translucent outer wall layer; and (ii) degraded bacteria which characteristically display breaks in the cell wall and/or cytoplasmic membrane, a disorganized cytoplasm.



(A) **(B)**

Figure 4.2: Assessment of morphological alterations of *M. tuberculosis* in macrophages treated with AJ-**4**. In this figure, (A) is the control showing intact bacteria and normal morphology for both cells and *M. tuberculosis*, (B) is AJ-4 treated macrophages infected with *M. tuberculosis*. IB= intact bacteria, DB = damaged bacteria, M = mitochondrion, ER = endoplasmic reticulum. The zone of exclusion was used as an indicator of damaged bacteria.

After a 1 day treatment, the bacilli had the same morphological appearance as bacteria residing within untreated macrophages, *i.e*. were morphologically intact as defined above. After a 3-day treatment, part of the bacilli displayed morphological alterations. The most obvious alteration was evidenced by exclusion of cytoplasm and DNA from the centre of the bacilli, suggesting that bacilli were undergoing degradation and therefore being killed. We then determined the percentage of bacilli that were intact in drug treated macrophages (*Table 4.1*). After 1 day treatment, the percentage of intact bacilli was similar to that observed for untreated cells, *i.e*. 86% *vs* 91%. In contrast, after 3-days of treatment only 55% of the bacilli were intact. These results suggested that phenothiazine was a potent inhibitor of mycothiol biosynthesis in *M. tuberculosis.*

Table 4.1: Percentages of intact bacilli: in this table mouse BMDM were infected with *M. tuberculosis* at MOI 2:1. At day 4 post-infection, AJ-4 was added at 1 µg/mL for 24 hours or 72 hours. Control experiments were mouse BMDM + *M. tuberculosis* without AJ-4.

AJ-4 conc	**Time of treatment**	**Time of fixation**	**% intact bacteria (average %)**
-	-	D5 p.i	**91.3%**
-	-	D7 p.i	**85.8%**
1 µg/mL	24 hr	D5 p.i	**85.6%**
1 µg/mL	72 hr	D5 p.i	**54.6%**

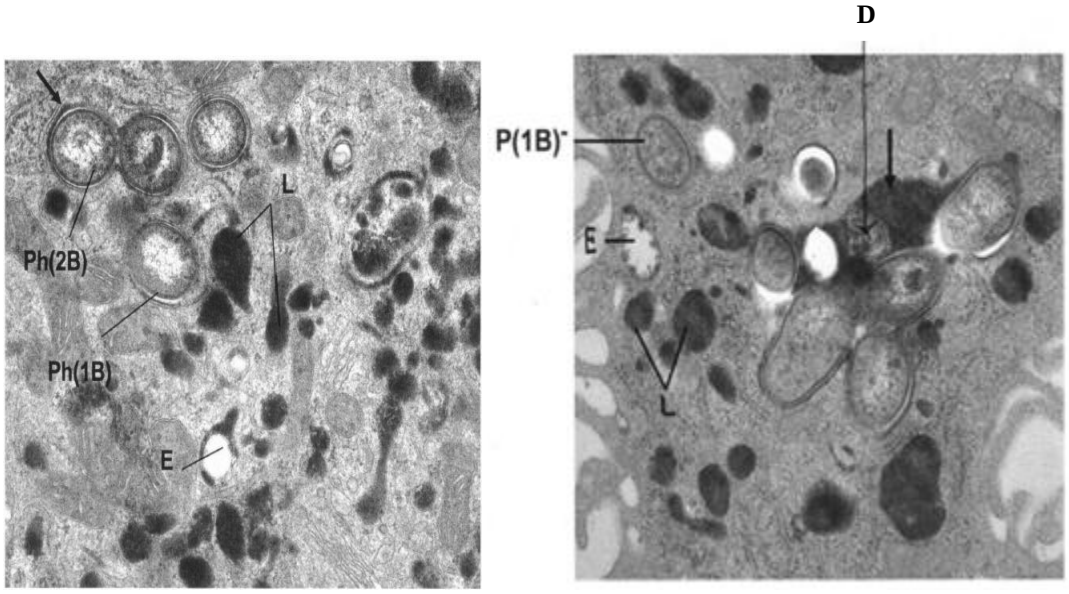
4.3.3 Phagosome processing after treatment of infected macrophages with phenothiazine (AJ-4)

BMDM were infected with *M. tuberculosis* at MOI 2:1 with 1 µg/mL AJ-4 for 3 days. As recalled in the *Introduction* of *Chapter 4*, only phagosomes containing a single live mycobacterium (loner phagosome), can remain immature whereas those containing several bacilli systematically mature and fuse with lysosomes. If for some reason bacilli are unable to block loner phagosome maturation, the later are processed into phagolysosomes that can fuse together to form phagolysosomes containing several bacilli. We therefore determined whether the percentage of social phagosomes and of bacteria enclosed in social phagosomes increased in the presence of AJ-4. We found no differences between the control and drug-treated cells since the percentage of social phagosomes was 40 % of the total in drug-treated *vs* 31 % in the control case. In both cases 70% of the bacteria were enclosed in social phagosomes.

In the second set of experiments, BMDM were infected with *M. tuberculosis* at an MOI of 2:1. Five days later cells were treated with 1 µg/mL AJ-4 for 3 days. Before processing for TEM, cells were first incubated for 1 hour with HRP at a concentration of 25 µg/mL. After fixation with glutaraldehyde HRP was stained by cytochemical methods as described in the *Materials and Methods* section. The use of HRP serves two purposes. First, it allows to distinguish early endosomes from lysosomes which differ in their cytochemical staining pattern, *figure 4.3*. Secondly, it allows to discriminate between immature

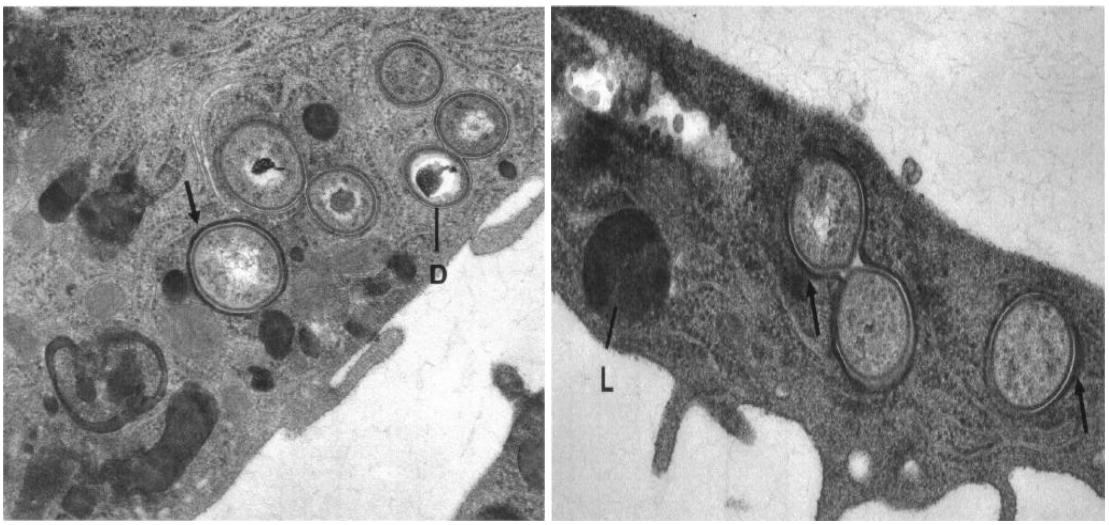
phagosomes that fuse with early endosomes and matured phagosomes that no longer fuse with early endosomes but fuse with lysosomes to become phago-lysosomes.

The morphological appearance of loner phagosomes that do not contain HRP is shown in *figures 4.3 (A)* and *4.4 (A)*, of loner phagosomes that fuse with lysosomes and therefore become HRP positive is shown in *figure 4.4 (B)*. Social phagosomes showing HRP reaction product are shown in *figure 4.3 (B)*. and *figure 4.4 (B)*.



(A) **(B)**

Figure 4.3: Fate of loner and social phagosomes on the maturation block. **(A)** Mouse BMDMs were cultured for 6 days, and infected with *M. tuberculosis* at an MOI of 2:1. No inhibitor (AJ-4) was added, and fixation was done on day 7 after HRP uptake for 1 hour. E = early endosome, and L = lysosomes, both showing a positive stain for HRP. In the electron micrograph, a single phagosome (with 2 bacteria) can be seen with a rim of HRP (arrow). All bacteria appear morphologically intact. **(B)** Mouse BMDMs cultured for 6 days, and infected with *M. tuberculosis* at an MOI 2:1. At day 4 post-infection, inhibitor AJ-4 was added at 1µg/mL for 72 hours, after which cells were fixed at day 7 after HRP uptake for 1 hour. The arrow shows HRP staining in a phagosome containing several bacteria. This phagosome contains 5 intact bacteria and perhaps 1 damaged bacterium, indicated as D. The phagosome with a single bacterium (1B) did not stain positive with HRP.



(A) **(B)**

Figure 4.4: Loner and social phagosomes are processed differently during the endocytic pathway. **(A)** In this electron micrograph, both the phagosome containing a single bacterium (1B) and the one with 2 bacteria (2B) stain positive for HRP as indicated by the arrows. All bacteria are morphologically intact. **(B)** The arrow shows a phagosome containing a single bacterium with positive HRP staining, and a damaged (D) bacterium (no cytoplasm), L = lysosome.

The percentages of HRP positive loner and social phagosomes are shown in *Tables 4.2* and *4.3*. Drug treatment did not result in an increase in the percentage of HRP positive loner phagosomes.

Table 4.2: Proportion of loner phagosomes (1B) that were HRP-positive

AJ-4 conc	**Time of treatment**	**Time of fixation**	**% HRP$^+$**
-	-	D7 p.i	52 % 30.1% (*correct literature value*)
1 µg/mL	72 hr	D7 p.i	59%

We observed that about 90% of the social phagosomes had fused with lysosmes in agreement with data from de Chastellier *et al*, 2005.

Table 4.3: Proportion of social phagosomes (≥2B) that were HRP-positive

AJ-4 conc	**Time of treatment**	**Time of fixation**	**% HRP$^+$**
-	-	D7 p.i	87 %
1 µg/mL	72 hr	D7 p.i	85%

These studies provide evidence that phenothiazine can induce morphological alterations in bacteria, however we did not observe major differences in phagosome processing in the presence of the drug. Further studies would therefore be required if this drug can be fully exploited as an inhibitor of the mycothiol pathway in *M. tuberculosis.*

CHAPTER 5: GENERAL RECOMMENDATIONS FOR FUTURE DIRECTIONS

5.1 TB Research Opportunities

This study attempted to address critical areas that require additional research and new product development in order to close the TB knowledge gap. Specifically, mycothiol was studied as a potential drug target by testing inhibitors designed against enzymes involved in its biosynthesis. The identification of novel drug targets continues to be used as an important tool in drug discovery. In this way, various genes have been knocked out in *M. tuberculosis* with the idea of establishing essentiality of genes and result phenotype.

Russell *et al* (2010) suggested that instead of investigating on gene knock-outs alone during drug discovery, it was necessary to integrate the interaction between gene products and metabolic pathways which are required for survival in the hostile internal environment of the macrophages. In this work, *M. tuberculosis* infected-macrophages were screened to establish the responses when macrophages were exposed to conditions to stress such as drugs.

5.2 Structural biology/biology (e.g. elucidations of TB/host interactions)

Genes involved in mycothiol biosynthesis have been deleted using conventional gene knock-out strategies except for the *mshC* that we attempted to knock-out using the conditional knock-out strategy. Novel MSH binding proteins; macrophage interactions have been implicated in resistance mechanisms in *M. tuberculosis*; but the question remains as to how TB bacilli remain in macrophages for such long periods of time. It would also be ideal to investigate how bacilli keep macrophages from or "activating". Persistent bacteria have been a major problem in TB treatment; hence it would be interesting to investigate what accounts for these persistent TB populations? Is it lack of drug penetrations in all cell types, or mutant/tolerant bacilli? For efficient clearances of bacilli from the granulomas, it would be desirable to have drugs that have increased killing rates and sterilisations.

5.3 Target validations/drug efficacy (e.g. validations through chemical inhibition)

In order to increase the screening process for hit compounds it would be desirable to develop medicinal chemistry protocols and high throughput screening systems that also involve whole cell screening. A wide range of validated binding pockets have been modelled and computationally screened by the group of Dr Anwar Jardine at the University of Cape Town.

Attempts have also been made (by medicinal chemists) to investigate the range of protein/ligand co-crystallisations as well as measuring/monitoring in real-time the drug penetrations *in vitro*, *in vivo* and within macrophages. Electron microscopy was used to follow the fate of *M. tuberculosis* within macrophages, but more biochemical techniques were required to establish the physiochemical properties necessary for drugs to penetrate dormant TB bacilli. In particular, what drug properties are required to penetrate the waxy coat of bacilli and their protective granulomas?

5.4 Therapeutics (e.g. efficacious, expeditious, and inexpensive drugs or diagnostics)

In this study of mycothiol as a drug target the Phase I milestone was to demonstrate feasibility, enzyme-to-whole cell inhibition, phase II milestone would be to provide lead compound screens and optimisations. Different compounds were tested for *in vitro* inhibition of *M. tuberculosis*, and different macrophage systems were used as models to study how mycobacteria are processed within the cell.

5.5 Diagnostics (e.g. to monitor and differentiate TB strains, point of care diagnostics).

In order to address issues of TB transmission, it would be desirable if diagnostic tools to identify active and latent TB infections could be developed. In view of this objective it would be worthwhile to identify which host and pathogen biomarkers are indicative of disease progression or cessation. Are sequestered persistent persistent-bacilli detectable? Can sensitive and reliable diagnostics devices be made inexpensively? Can TB be imaged? Can spectroscopic analytical techniques help monitor disease progression? Can small numbers of “active” TB bacilli be detected in the host? Can TB physiology and clearance be monitored?

5.6 REFERENCES

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APPENDIX I

CULTURING OF L-929 FIBROBLASTS (SOURCE OF CSF-1 FOR BMD MOUSE MACROPHAGES)

I Medium

Complete medium is RPMI 1640 or DMEM + 10% FCS (heat inactivated) + 2mM L-glutamine.

Trypsin 0.05%/ EDTA 0.02% (TE) to trypsinise the cells

II Cell culturing

(Start from a new frozen stock aliquot each time. You may want to prepare L-cell conditioned medium)

a) Step 1:

Put 1mL medium in the first 6 wells of a 24 well tissue culture plate (Falcon). Thaw (rapidly) 1 cryotube of L-cells (stored in liquid nitrogen - contains 1 mL at approx 1-2 x 10^6 cells/mL). Put the contents of the cryotube in the first well, then do 5 successive two-fold dilutions in the next 5 wells. Do not wash and/or centrifuge cells before. Let them grow for 24-48 hrs at 37^{o}C in 5-7.5% CO_2. After 24 hrs add 1 mL fresh medium

b) Step 2:

48 hrs later cells have started to grow. They are still spindle-shaped and have not yet reached full confluence. Trypsinise cells of the last 3 wells as follows:

Put 1 mL trypsin-EDTA, remove immediately (this allows to remove excess FCS).

Put 250 mL TE/well. Incubate for 2-3 minutes at 37^{o}C.

Collect cells of the 3 wells into a centrifuge tube (containing 10 mL of complete medium (to stop reaction, cool on ice). Centrifuge at 1200 rpm for 3 minutes, at 4^{o}C. Do not count cells at this stage.

c) Step 3:

Re-suspend pellet first in 1 mL, then add and additional 5 mL of complete medium

Put the 5 mL in a 25 cm^2 tissue culture flask (Falcon is the best)

Let cells grow for two days at 37^oC in 7.5% (or 5%) CO_2 incubator

d) Step 4:

Cells should be close to confluence (spindle shaped, not cubic)

Trypnize cells as follows:

Put 1 mL T-EDTA, remove immediately

Put 2 mL T-EDTA, incubate cells at 37^oC for 2-3 minutes

Collect cells and put into centrifuge tube containing 10 mL of ice cold complete medium

Count cells before spinning them down

Centrifuge at 1200 rpm, 3 minutes at 4^oC

e) Step 5

Dilute cells, in order to have 5 x 10^4cells/mL, in complete medium

Put 10 mL per 25 cm^2 tissue culture dish. We prepare 3 flasks to have enough cells for final expansion

Let cells grow for 2-3 days

Trypsinise as before

Step 6: Production of L-cell conditioned medium

Dilute cells in order to have 2.5 x 10^4cells/mL

Distribute in 75cm^2 tissue culture flasks (Falcon), 40 mL/flask. We prepare 6 to 8 flasks in order to be able to work with the same batch of L-cell conditioned medium for a while. Let the cells grow. This time they must reach confluence, become tightly packed and even reach the point where they are about to (but haven't) come off the flask. This is when they produce the most CSF-1. At D + 6 collect supernatant, filter on 0.22 µm filter, aliquot and store at -20^oC. This is the L-cell conditioned medium. It can be kept for several months. One can freeze and thaw it 2 or 3 times but it would be advisable not to do it more often (so sizes of aliquots depend on how much you need each time you prepare medium).

APPENDIX II

RELEVANT WORK ASSOCIATED WITH MSC TRAINING 2009-2010

A. CONFERENCES

1. **Anwar Jardine**, Bienyameen Baker, James Mazorodze, Dirk Lapmrecht. (2009). Overcoming the crisis of TB and AIDS. Keystone Symposium, Arusha, Tanzania.
2. **James Mazorodze**, Anwar Jardine, Chantal Chastellier, Paul van Helden and Bienyameen Baker. (2010). Importance of mycothiol as a defense molecule in *Mycobacterium tuberculosis*. Capebiotech Forum Conference, Somerset West, Capetown.

B. TRAINING

1. MSc training in advanced tissue culture, cell biology and electron microscopy (under the mentorship of Dr Chantal de Chastellier) at the *Centre d'Immunologie de Marseille-Luminy* (CIML), *Aix-Marseille Universite´, Faculte´ des Sciences de Luminy*, Marseille, France under the French-South Africa Bilateral Agreement, 1st – 31st October 2009 and 8th October – 3rd November 2010.
2. Trained under the Stanford-South Africa Biomedical Informatics Program's short course in Genomics: Micro-arrays and High throughput Sequencing, 10-14th May 2010 held at the University of the Western Cape in South Africa.
3. Trained and successfully completed the Collaborative Institutional Training Initiative (awarded the CITI: Human Research Curriculum Completion Certificate) under Fogarty Global Infectious Diseases Fellowship. 2010.
4. MS Excel Postgraduate training course, sponsored by the Postgraduate International Office, Stellenbosch University, 2009.

C. AWARDS AND SCHOLARSHIPS

1. Awarded a 3 months Fellowship (July – September 2010) under the Fogarty International Scheme: Short-Term Pre-doctoral Traineeship in TB Research at the Howard Hughes Medical Institute (Dr William Jacobs Jr Laboratory), Albert Einstein College of Medicine (Yeshiva University) USA.
2. Recipient of University Bursary for MSc Medical Biochemistry studies for the period 2009/2010 (Stellenbosch University).