Investigation of the efficacy of identified Acetolactate synthase inhibitors, Peptidyl cysteine protease inhibitors, Thiolactomycins, and Thiosemicarbazone compounds against *Mycobacterium tuberculosis*.

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DECLARATION

I, Boipelo Felicity Sebesho, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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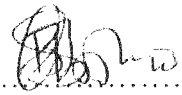
Signature: 

Date: 15 March 2006

Dedication

To my family for support, encouragement, and endurance.

Mom, you left us before you could see greater things.

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Figures and Tables

Table of contents

CHAPTER 2: Material and Methods

CHAPTER 3: Comparative *in vitro* efficacy and cytotoxic effects of identified synthetic derivatives against *M. tuberculosis*

Abstract

Tuberculosis remains an important public health problem worldwide. There has been increase in the development of drug resistance towards INH and RIF, two of the frontline antimycobacterial drugs currently used in therapeutic regimes. As an attempt to address drug resistance, the World Health Organization has implemented the DOTS strategy in 182 countries. Moreover, new chemical libraries of potential antituberculosis drugs have been designed and synthesised. We therefore assessed 121 derivatives from the acetolactate synthase inhibitors, cysteine protease inhibitors, thiosemicarbazones, and thiolactomycins classes of compounds for *in vitro* efficacy against *M. tuberculosis* using the resazurin microtitre plate assay afterwhich active compounds were assessed for cytotoxicity *in vitro* against elicited peritoneal macrophages using the MTT assay. Of the 38 acetolactate synthase inhibitors tested, 2 derivatives namely RKG162A and RKG1541 were bactericidal against *M. tuberculosis*. Both derivatives were mildly cytotoxic against macrophages. For cysteine protease inhibitors, 35 derivatives were tested. Four derivatives namely AXE1, AXE4, AXE5, and AXE29 were bactericidal whereas AXE2, AXE3, AXE35, and NAT47 where bacteriostatic. All the cysteine proteases that showed activity against *M. tuberculosis* were highly toxic against macrophages except for AXE29 and NAT47 who were mildly toxic. FM19 and FM48 were the only derivatives of 25 thiosemicarbazones that completely inhibited *M. tuberculosis* growth. FM04 and FM102 were bacteriostatic. All the thiosemicarbazones were highly toxic when tested for cytotoxicity in a cell based culture system. In contrast, none of the 13 thiolactomycins exhibited antibacterial activity against *M. tuberculosis*. Of all the mildly cytotoxic derivatives, RKG162A and AXE29 were the least toxic and were therefore investigated for intracellular killing of *M. tuberculosis* using peritoneal macrophage based system. Intracellular killing was concentration dependent with increased killing observed with a higher concentration of both AXE29 and RKG162A. AXE29 and RKG162A were further investigated for therapeutic efficacy in an experimental tuberculosis murine model. However, no therapeutic efficacy was observed when mice were treated with AXE29 or RKG162A at the selected concentration. These findings suggest the compounds that had antitubercular properties constitute lead structures around which a combinatorial library could be constructed.

CHAPTER ONE

Introduction and Literature Review

1.1. Tuberculosis

1.1.1. Epidemiology

Tuberculosis (TB) is a granulomatous disease caused by the acid-fast *Mycobacterium tuberculosis*. Tuberculosis remains an important public health problem worldwide, with a third of the world's total population infected with the disease. Of all the individuals infected with *M. tuberculosis*, only 10% develops active TB. In 2003, 8.8 million new TB cases were reported, with 80% of the cases occurring in 22 countries with high TB burden. Moreover, 2 million deaths occurring every year are due to TB. Even though TB is curable, it kills 5000 people every day. The global TB incidence is still growing at 1% a year primarily confined to a rapid increase in Africa (Fig. 1.1), with the disease affecting the most vulnerable such as the poorest and atrophied (WHO 2005).

Human Immuno-deficiency Virus (HIV) infection is a potent risk factor for tuberculosis. In countries with a rapid growth of HIV incidence, including countries that have been successful in implementing the WHO strategy, there has been a dramatic increase in the estimated number of new TB cases. Among people infected with HIV, tuberculosis is a leading cause of death accounting for 13% of TB deaths worldwide (WHO 2005). Most of HIV-associated TB deaths occur in Africa especially in the sub-Saharan Africa (Fig. 1.2). For the past 10 years, HIV has been the most important factor determining the increased TB incidence. In South Africa 55% of the TB patients were reported to have a dual infection with HIV in 2002 (WHO 2005). To control the high incidence of TB among the HIV patients, WHO and its international partners have formed the TB/HIV Working Group. This group focuses in developing global policy on the control of HIV-related TB and advices on how those fighting against TB and HIV can work together to deal with this dual infection (WHO 2005).

Until the early 1950s, there was no cure for TB. Subsequently drugs that kill *M. tuberculosis* were discovered even though today this organism has developed resistance against some of the current available TB drugs, ascribed primarily to inconsistent or partial treatment. High-risk forms of MDR-TB are those resistant to isoniazid and rifampicin, two of the frontline drugs against TB. MDR-TB is present in almost all 109 countries recently surveyed by WHO and partners and 425 000 new MDR-TB cases occur every year. The high rates of MDR-TB occur in the former USSR and China, where up to 14% of new cases are not responding to the current

standard therapy. In South Africa, 7500 MDR-TB cases were estimated by the survey conducted from 2000-2002 with 450 new MDR-TB cases being reported every year. This data correspond to MDR-TB levels of 1.7% of new cases and 6.6% re-treatment cases. To control MDR-TB in South Africa, treatment facilities have been established in eight provinces and the MRC is currently developing a national policy on MDR-TB management. On a global scale, WHO and its international partners have formed the DOTS-plus Working Group, which develops global policy on management of MDR-TB (WHO report 2005).

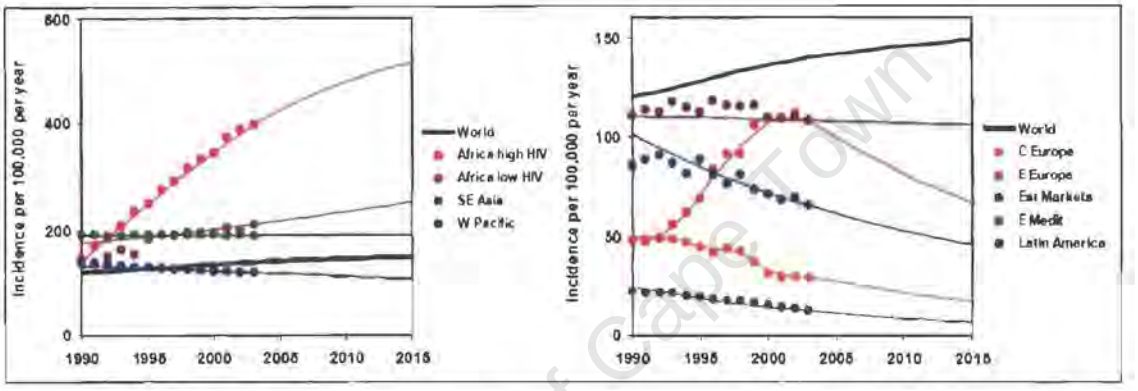


Figure 1.1. Trends in TB incidence in 9 regions of the world as estimated by WHO in 2003 (WHO 2005)

1.1.2. Transmission and Pathogenesis

Tuberculosis is a disease that is transmitted by aerosolized proteinaceous pulmonary secretion containing infectious *M. tuberculosis*. The droplets are generated when the person with infectious TB disease coughs and sneezes. The main route of infection for TB is the respiratory tract, where the bacteria are inhaled and droplets proceeds distally to the lung to establish an infection. It is not everyone who, when exposed to an infectious TB patient, becomes infected with the bacteria. Whether TB will be transmitted depends on how contagious the patient is, how long the exposure lasted, and in what kind of environment the exposure occurred. The inhaled bacilli lodge in the terminal air spaces of the lung where they are ingested by resident alveolar macrophages and conveyed to the interstitium. In addition, the inhaled bacteria are to invade the alveolar epithelial cells, which would create a niche for the bacterium to replicate and establish infection, avoiding the potentially aggressive environment of the macrophage. For disease to occur, *M. tuberculosis* must be able to invade the host

system. This is achieved by inhibition of phagosome-lysosome fusion and resistance to lysosomal enzymes as part of the pathogenic strategy (Fratti, Chua et al. 2003). Once the bacteria are inhaled, the innate response is triggered. If the eradication of the mycobacteria by the innate response is not effective, cell-mediated immunity develops within 2 to 6 weeks. Activated macrophages and lymphocytes are recruited into the lesion resulting in an increased local inflammatory reaction and granuloma formation. If the bacterial load is small, bacteria are destroyed with minimal tissue damage. However, a high bacterial load lead to production of inflammatory cytokines, activation of the complement pathway, and production of reactive species by macrophages leading to substantial tissue damage. Formed granulomas are usually intact and are able to contain the infection. However, when granuloma is larger and formed in an area with more tissue necrosis, the bacilli becomes enclosed by fibrin and therefore protected from the killing by macrophages. Fibrin enclosed *M. tuberculosis* bacilli can remain dormant for years and become reactivated when the immune defence system is weakened.

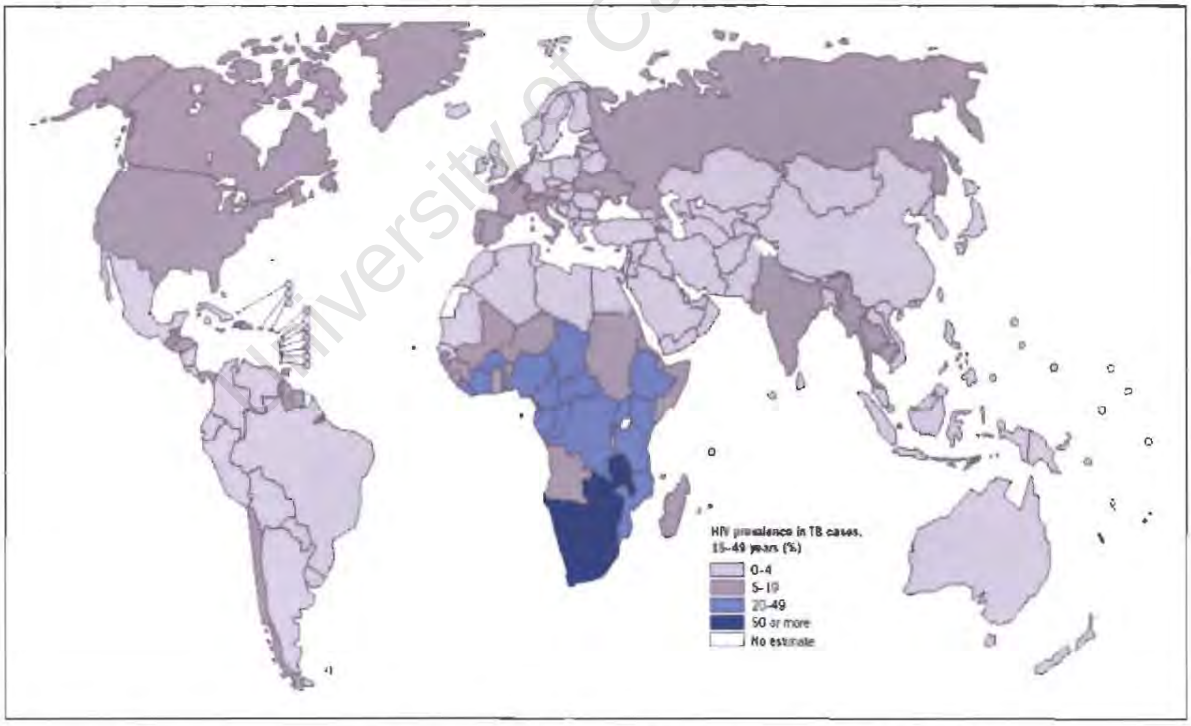


Figure 1.2. HIV prevalence in TB cases in 2003 as estimated by WHO (WHO 2005)

1.1.3. Intracellular lifestyle of M. tuberculosis

The establishment of a successful infection by pathogens depends on the initial pathogen-host interaction. Intracellular pathogens choose host cells to interact with and in the case of *M. tuberculosis* macrophages are the target cells. Macrophages have on their surface receptors that facilitate adherence and entry of pathogen, the latter that requires a complex dialogue of signalling events between pathogen and host (Ehlers and Daffe 1998). The host cell receptors include complement receptors (CR) types 1, 3, and 4 (Schlesinger, Bellinger-Kawahara et al. 1990; Stokes, Haidl et al. 1993), Fc receptors (Ehlers and Daffe 1998), surfactant protein receptors (Schlesinger 1993; Stokes, Haidl et al. 1993), CD14 on microglia (Peterson, Gekker et al. 1995), mannose receptors (Peterson, Gekker et al. 1995), and macrophage scavenger receptors (Peterson, Gekker et al. 1995). However, not enough evidence is available on whether the above receptors provide an equally favourable route of entry and what the preferred receptors are *in vivo* (Ehlers and Daffe 1998). Moreover, it is proposed that the cellular response is influenced by the receptor choice. Armstrong *et al.* 1975 reported that when the immunoglobulin G-opsoninzed mycobacteria was internalized via Fc receptors, production of reactive oxygen and nitrogen intermediates was induced permitting phagosome-lysosome fusion (Armstrong and Hart 1975), whereas entry via CR3 prevents activation of respiratory burst (Le Cabec, Cols et al. 2000) and results in arrest of phagosome maturation at early endosome (Sturgill-Koszycki, Schaible et al. 1996), thereby evading the anti-microbial effects of the lysosome. In addition, *M. tuberculosis* maintains an intraphagosomal environment advantageous to its persistence by excluding the vacuolar H^+-ATPases from bacilli-containing phagosomes to avoid the acidic environment, which destroys the *M. tuberculosis* within the phagosome (Sturgill-Koszycki, Schaible et al. 1996). CR3 was shown to be the most preferred receptor suggesting that it constitute a safe entry of pathogens (Falkow, Isberg et al. 1992; Isberg and Tran Van Nhieu 1994). Moreover, entry via CR3 reduces production of IL-12 by macrophages therefore curbing macrophage response and Th1-driven cell mediated immunity (Marth and Kelsall 1997).

M. tuberculosis bind host cell receptors via surface molecules. The outcome of an infection by this pathogen is modulated by the both host-cell and mycobacterial lipids (Nguyen and Pieters 2005). The lipid-rich cell of *M. tuberculosis* has been distinguished as providing the bacilli with an effective innate immune defence against

lysosomal degradation. The mycobacterial cell wall (Fig. 1.3) is extremely complex and confers resistance to chemical injury, dehydration and antibiotics. The protection has been attributed to the unique low permeability of the cell wall to hydrophilic molecules (Jarlier and Nikaido 1990). The understanding of the mycobacterial cell wall is still basic. The nucleus of the cell wall consists of covalently connected mycolic acids forming the principal hydrophobic wall by associating into a tight, closely packed lipid bilayer (Barry, Lee et al. 1998). Disruption of the basic structure may result in dramatic changes in surface appearance, hydrophobicity and permeability to various substances (Barry, Lee et al. 1998). Different components of the cell wall have different contributions towards *M. tuberculosis* virulence. Glycosylated phosphotidylinositol lipoarabinomannan (ManLAM) (the PI3P analogue) has been reported to block phagosome maturation (Fratti, Chua et al. 2003). ManLAM is thought to block a PI3P-dependent pathway, which is involved in transport of cargo between the *trans*-Golgi network and phagosome (Sturgill-Koszycki, Schaible et al. 1996; Fratti, Chua et al. 2003). This transport step is required for phagosome biogenesis. Moreover, ManLAM inhibit the rise in Ca^{2+} concentration within the cytosol of macrophages required for accumulation of PI3P on the phagosomal membrane (Vergne, Chua et al. 2003). Inhibition of these actions by ManLAM might be by competing for binding to proteins containing FYVE and PX domains, thereby preventing their recruitment to the phagosome. Alternatively, ManLAM might interfere with the waves of PI3P on the phagosomal membrane (Chua and Deretic 2004). Different components of *M. tuberculosis* have different contributions to virulence and together ensure a safe intracellular haven while providing nutrients to the pathogen.

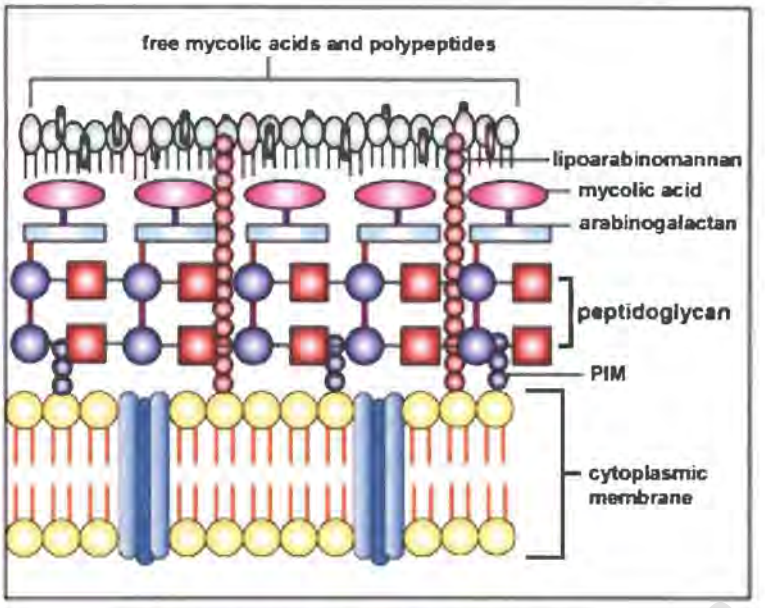


Figure 1.3. The structure of mycobacterial cell wall (Kaiser 2005)

After entry into the host cells, *M. tuberculosis* has to establish habitation. One of major problems of *M. tuberculosis* once inside the host cell, is acquiring nutrients from the intracellular environment. Acquisition of iron, which is a cofactor of macrophages in the induction of microbicidal effector mechanisms, is critical for metabolism and growth of *M. tuberculosis* (Olakanmi, Schlesinger et al. 2004). Thus, *M. tuberculosis* competes with macrophages for iron, which is acquired via the tranferrin receptor (TFR). TFR internalized iron binds to both tranferrin and lactoferrin. Iron becomes available to *M. tuberculosis* when extracellular transferrin cycles to *M. tuberculosis*-containing phagosome through plasma membrane TFR trafficking to early endosomes (Brightbill, Libraty et al. 1999; Porcelli and Modlin 1999). It has been proposed that this provides *M. tuberculosis* with iron via phagosome-endosome fusion (Olakanmi, Schlesinger et al. 2004). *M. tuberculosis* has developed specialized high affinity iron chelators (siderophores) to compete with environmental iron binding molecules for the available iron. The sidosphores transfer iron from the host proteins to specialized mycobactin molecules in the mycobacterial cell wall (Gobin and Horwitz 1996). Acquisition of iron by *M. tuberculosis* results in inhibition of the induction of macrophage antimicrobicidal processes, contributing to the microbial evasion of *M. tuberculosis* (Olakanmi, Schlesinger et al. 2004).

Recent demonstrations have described the role of cholesterol in entry of mycobacteria into macrophages. Depletion of cholesterol resulted in inhibition of mycobacterial uptake which is specific for mycobacteria as other microorganisms could enter cholesterol-depleted macrophages (Gatfield and Pieters 2000). When macrophages were depleted of cholesterol, the function of the CR3 was not compromised suggesting that cholesterol may function as a docking site for mycobacteria to stabilize their interaction with the macrophage membrane, after which, the mycobacteria can be internalized (Gatfield and Pieters 2000). Moreover, cholesterol mediates recruitment of TACO, which prevents the maturation of the phagosome to a phagolysosome (Gatfield and Pieters 2000). TACO is a 43kDa tryptophan-aspartic acid repeat actin binding protein of the host (Henderson, Watkins et al. 1997). This molecule was identified when analyzing different proteins present in the phagosomes containing live mycobacteria and was not found in phagosomes containing destroyed mycobacteria (Pieters 2001) or in endosomal/lysosomal organelles purified from uninfected cells (Ferrari, Langen et al. 1999). The inhibition by phagolysosome fusion is only possible when TACO is preserved at the surface of mycobacteria containing phagosomes. This became evident when the TACO-negative melanoma cell line transfected with TACO resulted in enhanced phagolysosomal fusion and increased killing of BCG, compared to the non-transfected melanocytes (Flynn and Chan 2003). The absence of TACO in the liver, a major site for mycobacterial clearance suggested a role for TACO in mycobacterial survival *in vivo* (Pieters 2001) and thus supporting its role in immune evasion.

The nitric oxide synthase 2 (NOS2)-dependent pathway remains the most studied antimycobacterial mechanism of activated macrophages. *M. tuberculosis* infected macrophages are activated in several stages by macrophage activating factors such as IFN-γ released from stimulated lymphokine producing T cells, generating ROI and RNI (Dalton, Pitts-Meek et al. 1993) (Fig.1.4).

M. tuberculosis has developed mechanisms to prevent the effects ROI. *M. tuberculosis* produces a tetrameric iron-co-factored superoxide dismutase encoded by the *sodC* gene (Dussurget, Stewart et al. 2001). Superoxide dismutases are ubiquitous metalloenzymes present in aerobic organisms, including the pathogenic and the non-pathogenic species of mycobacteria. Superoxide dismutases are among the major

extracellular proteins released by *M. tuberculosis* during growth. They detoxify superoxide anion reducing the extent of intracellular pathogens killing by ROIs.

Major Histocompatibility Complex (MHC) class II presentation of *M. tuberculosis* antigens by macrophages to $CD4^+$ T cells is crucial in controlling the infection and is upregulated by interferon (IFN)-γ. *M. tuberculosis* manipulates this pathway by inhibition of the expression of MHC-class II proteins and MHC-class II presentation of bacterial antigens in macrophages after infection (Flynn and Chan 2003). The mechanism through which antigen presentation is reduced involves the secretion of a lipoprotein with a molecular weight of 19kDa. Studies showed that this 19kDa lipoprotein is involved in inhibition of antigen processing and presentation (Noss, Harding et al. 2000). Inhibition of cell-surface expression of MHC class II correlated with the decreased mRNA for MHC class II (Noss, Harding et al. 2000). Furthermore it is the 19 kD that is thought to act through TLR2 during the early phase of entry into macrophages (Smith 2003) as TLR2 but not TLR4 was required for the inhibition of antigen presentation by mycobacterial lysate and the 19kDa lipoprotein (Noss, Pai et al. 2001). TLR2 is present not only on the surface of macrophages but within the phagosome (Underhill, Ozinsky et al. 1999) suggesting that in chronic infection, *M. tuberculosis* is continually downregulating MHC class II expression. Moreover, since successful inhibition requires large amount of 19kDa lipoprotein, it can be suggested that this immune evasion is better when macrophages are infected with higher burden of *M. tuberculosis*.

1.1.4. Host immune response in tuberculosis

The host immune response to *M. tuberculosis* infection has been studied in both human and murine models. During the infection, the initial interaction in the lungs is with alveolar macrophages. The pathogen is taken up in the lung parenchyma to replicate in the tissue macrophages and macrophages that differentiate from monocytes recruited to the infection site.

Macrophages, which are important effectors of the immune system, provide an important niche for *M. tuberculosis*. *M. tuberculosis* enters alveolar macrophages, resist killing and disseminates via the lymphatic and bloodstream to distant sites, usually asymptomatically (Ehlers and Daffe 1998). Unlike resting macrophages which are unsuccessful in destroying *M. tuberculosis*, activated macrophages are able to

control the pathogen although sterile abolition is rarely achieved (Kaufmann 2001). Macrophages can be activated in several stages including innate inflammation but the ability to kill obligate intracellular microbes is only attained after stimulation by macrophage activating factors such as IFN-γ released from stimulated lymphokine producing T cells, generating NO and related ROI via NOS2 using L-arginine as the substrate (Dalton, Pitts-Meek et al. 1993) (Fig. 1.4). It has been reported that NO plays a significant role against *M. tuberculosis* both *in vivo* and *in vitro* (MacMicking, Xie et al. 1997; Shiloh and Nathan 2000). In the absence of NO, such as in a mouse lacking the NO synthase gene, disease progression is rapid and lethal (Cooper, Adams et al. 2002). When NO inhibitors were used in mice infected with *M. tuberculosis*, the mice had increased pathology therefore supporting the role of NO in reduction of inflammation (Chan, Tanaka et al. 1995; Rhoades, Frank et al. 1997). In the mouse, RNI play a protective role in both acute and chronic infection (MacMicking, North et al. 1997; Flynn, Scanga et al. 1998) and the evidence for the protective role in humans is accumulating (Nicholson, Bonecini-Almeida Mda et al. 1996; Wang, Liu et al. 1998).

The release of the cytokines and chemokines from the infected alveolar and tissue macrophages form part of the cascade of the inflammatory molecules that are set off at the initiation of the *M. tuberculosis* infection. Not only macrophages but resident dendritic cells are infected with *M. tuberculosis* (Gonzalez-Juarrero and Orme 2001), which migrate to the lymph node to prime naïve T cells, inducing bacterial containment in granulomatous lesions (Fig. 1.4). *M. tuberculosis* that persists might progress to a stage with reduced metabolic activity that allows the pathogen to survive under the conditions of low nutrients and oxygen deprivation, known as dormancy (Kaufmann 2001).

Adaptive immunity is principally a function of a cellular immune system response. The initiation and maintenance of adaptive immunity to tuberculosis require a high frequency of specific cellular interactions, which in turn require regulated and efficient mechanisms in order for them to occur (Salgame 2005). Cell-mediated Th1 immune responses rather than the humoral immune response is required for the successful control of the *M. tuberculosis* since the serum components may not gain access to the pathogen which is intracellular. This immune response is at least in part dependent on the cytokines, which regulate all cells of the immune system. Since the

habitation of *M. tuberculosis* after infection is the phagosome within macrophages, presentation of mycobacterial antigens is by MHC class II (Raja 2004).

Acquired immunity to tuberculosis is generated when CD4 T cells recognise the mycobacterial antigens presented by the APC in the context of MHC class II molecules (Orme and Cooper 1999). The cytokine IL-1 increases surface expression of class II proteins and of various adhesion molecules and thereby strengthening the binding of CD4 T cells and enhancing antigen presentation (Fulton, Johnsen et al. 1996). IL-1 promotes IL-2 secretion by CD4 T cells, IL-2 receptor expression and subsequent clonal expression of the $CD4^+$ T cells (Orme and Cooper 1999).

Interleukin (IL)-12 is induced after *M. tuberculosis* has been phagocytosed by the macrophages and dendritic cells, driving the development of Th1 responses by promoting IFN-γ secretion by $CD4^+$ T cells (Dalton, Pitts-Meek et al. 1993; Orme and Cooper 1999) and antagonizing IL-4 and IL-10, thereby down-regulating Th2 responses (Fulton, Johnsen et al. 1996). IL-12 was shown to be a crucial cytokine in controlling *M. tuberculosis* infection. IL-12p40-gene deficient mice were susceptible to infection and had a significantly increased bacterial burden, with a decreased survival time (Cooper, Magram et al. 1997).

IFN-γ is produced by the CD4 T-cells (Fig. 1.4) and plays a significant role in controlling *M. tuberculosis* infection by inducing expression of the antimicrobial enzyme NOS2 which is largely responsible for restricting *M. tuberculosis* replication via NO generation (MacMicking, Taylor et al. 2003). NOS2 was considered to be the key player in the anti-microbial activity induced by IFN-γ (Salgame 2005). Recent work has led to the discovery of an anti-microbial molecule LRG-47, a GTPase that works independent of NOS2 to protect against the disease (MacMicking, Taylor et al. 2003). LRG-47 induces autophagy causing the mycobacterial phagosomes to mature into phagolysome therefore suppressing intracellular replication of mycobacteria (Gutierrez, Master et al. 2004). IFN-γ is also produced by CD8 T-cells (Fig. 1.4) and natural killer cells. Reports have shown that IFN-γ production by the alveolar macrophages is IL-12 dependent (Fenton, Vermeulen et al. 1997; Wang, Liu et al. 1998). IFN-γ effectively opposes the survival of *M. tuberculosis* in the modified phagosomal niche by promoting phagosome acidification and phagolysosomal fusion (Salgame 2005). IFN-γ knockout mice are highly susceptible to virulent

M. tuberculosis (Cooper, Dalton et al. 1993; Flynn, Chan et al. 1993). These mice were found to have an increased bacilli burden and although granulomas do form, they quickly become necrotic. Macrophage activation of these mice is defective and NOS2 expression is low when compared to the control wild-type mice. IFN-γ production only is insufficient but is required to control the infection. TNF-α is a cytokine that plays multiple roles in immune and pathologic responses in tuberculosis. TNF-α synergises with IFN-γ to induce NOS2 expression (Fig. 1.4) (Ting, Kim et al. 1999). An infection with *M. tuberculosis* induces secretion of TNF-α by macrophages, dendritic cells and T cells and is required for control of acute infection (Dalton, Pitts-Meek et al. 1993).

B cells are present in large numbers in both human and murine granulomas even though B cells and antibodies are generally not considered important in protection against *M. tuberculosis* infection (Algood, Chan et al. 2003). B lymphocytes-deficient mice had different lung pathology to the wild-type suggesting that B lymphocytes may contribute in granuloma formation and lung pathology by releasing cytokines or chemokines (Bosio, Gardner et al. 2000; Turner, Frank et al. 2001) as well as play a role as APC (Vordermeier, Venkataprasad et al. 1996). Moreover, mice lacking B cells had distinctly less severe granuloma formation of *M. tuberculosis* highlighting the contribution of B cells in granuloma formation.

The immune response to tuberculosis is intricate and involves various components. The studies of immune and pathological responses of the lung after an infection with *M. tuberculosis* are of main interest since they play a major role in controlling infection and it is these studies that are important in determining which of the components to target in development/improvement of vaccines against tuberculosis.

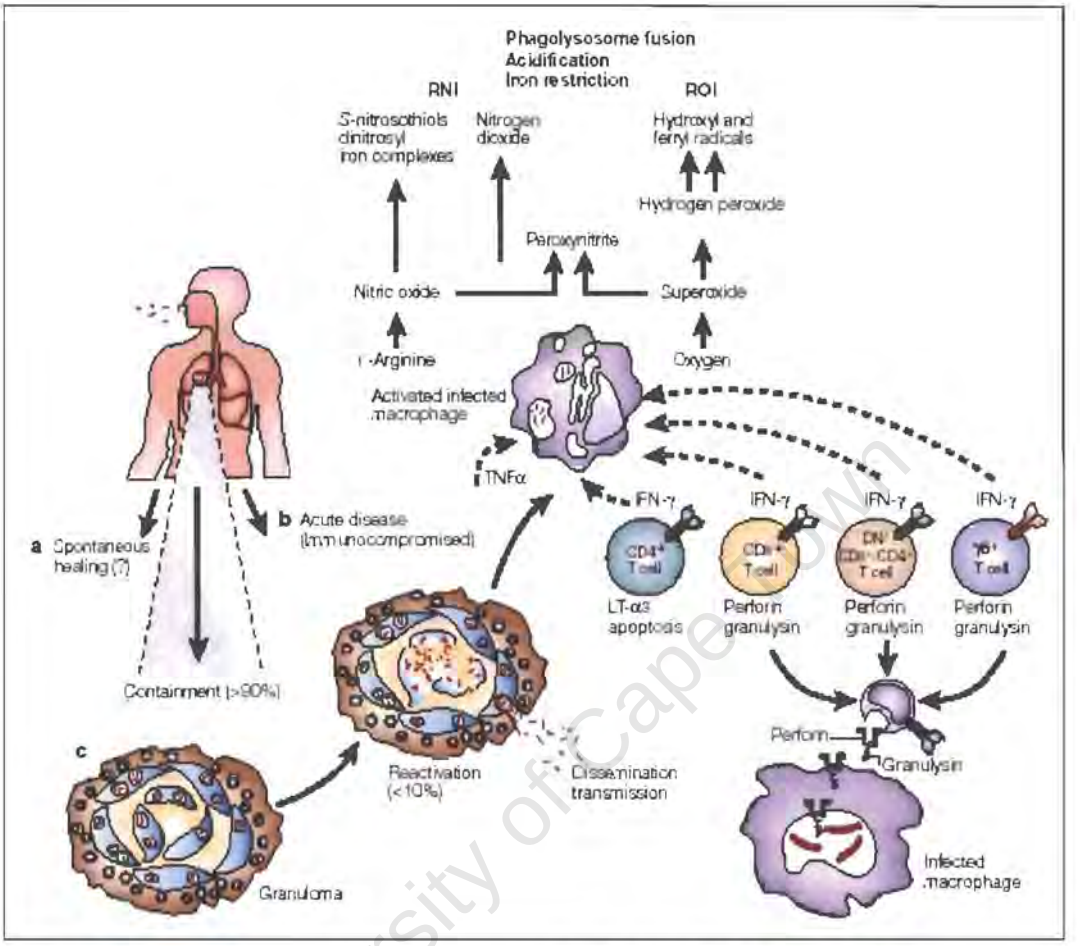


Figure 1.4. Three different potential outcomes of infection with *M. tuberculosis*.

a) Spontaneous healing following infection

b) Development of active tuberculosis in an immunocompromised individual

c) Anti-mycobacterial effector mechanisms of activated macrophage in healthy individuals. In >90% of infected individuals, infection is contained by granuloma formation. Reactivation might occur at a later stage. If reactivation occurs, T cells recognize the antigen and produce interferon gamma (INF-γ) and tumor-necrosis factor-α (TNF-α) to activate macrophages. Activated macrophage allows phagosomal maturation and produce reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) to destroy *M. tuberculosis* (Kaufmann 2001).

1.1.5. Chemotherapy of Tuberculosis

Tuberculosis chemotherapy has improved since the discovery of streptomycin, the first TB drug to be discovered. Subsequently, the treatment period has significantly been reduced from the 18 months - 2-year therapy (O'Brien 2001) to the current 6-month standard treatment with a low relapse rate. However, the current treatment is still considered lengthy with an increased rate of MDR-TB development as people fail to comply. The standard regimen was developed in which an initial 2 months (intensive phase) of isoniazid, rifampicin, pyrazinamide, and ethambutol (Fig. 1.5) was followed by 4 months of isoniazid and rifampicin. These drugs are the four first-line agents with streptomycin (Fig. 1.5) being used widely as second-line therapy. Current chemotherapy of tuberculosis relies largely on mycobacteria-specific drugs that inhibit bacterial metabolism.

1.1.5.1. Isoniazid

Isoniazid (INH) (Fig. 1.5), a hydrazide of isonicotinic acid was discovered as an intermediate when Winder and others (Winder and Collins 1970) were synthesizing the pyridine ring derivatives of thiosemicarbazone, a compound that had shown antituberculous effects (Winder and Collins 1970). INH is a product, which becomes oxidatively activated by the mycobacterial catalase-peroxidase *katG*. The active form of isoniazid inhibits the synthesis of mycolic acids, an essential component of the cell envelope of mycobacteria (Winder and Collins 1970). Mutations in *katG* affect the activation process of isoniazid, and is a mechanism in which *M. tuberculosis* gain resistance to INH (Slayden and Barry 2000).

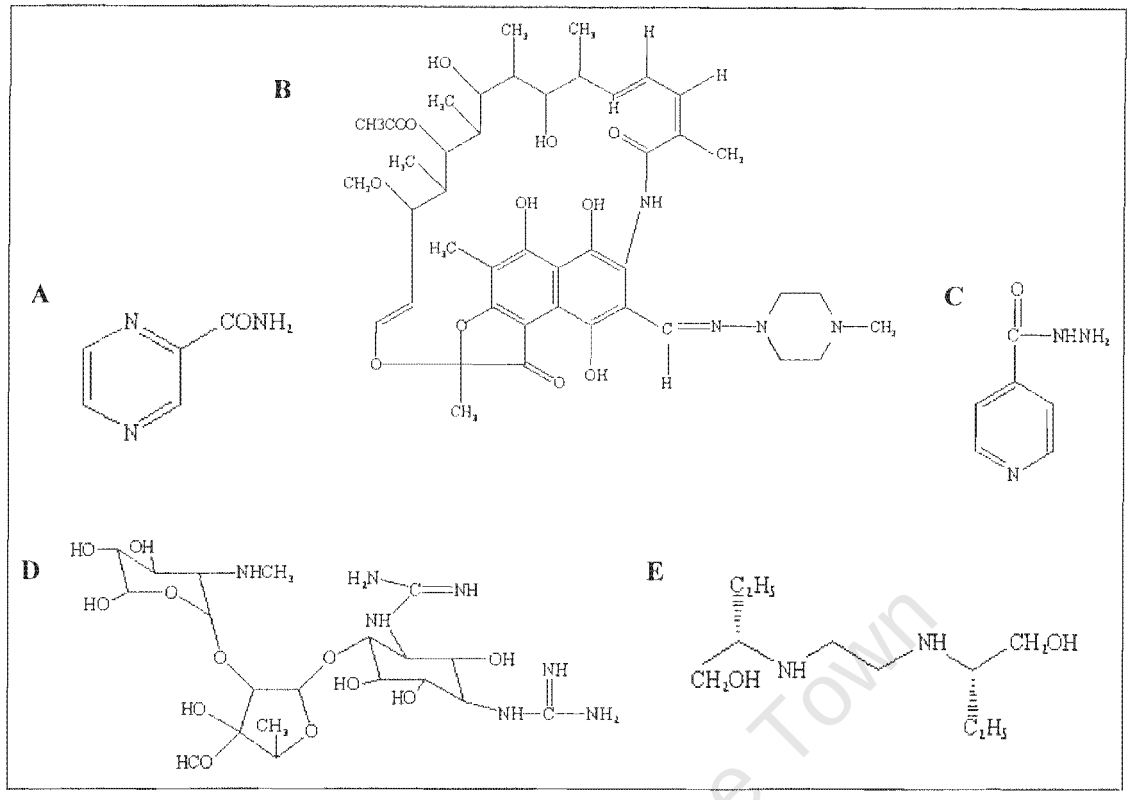


Figure 1.5. The chemical structures of the first- and second-line TB drugs. Pyrazinamide (A), Rifampicin (B), Isoniazid (C), Streptomycin (D) and Ethambutol (E) (Heritage 2003)

1.1.5.2. Rifampicin

Rifampicin (Fig. 1.5) is a broad-spectrum antibiotic that has had an impact on the duration and dose interval of treatment. Several clinical trials demonstrated that rifampicin reduced the length of tuberculosis therapy from the former 18 to 24 months to the current 6 months treatment period (Garay and Rom 1996). This semi-synthetic antibiotic binds to bacterial RNA polymerase interfering with transcription of bacterial DNA. The affinity of rifampicin for the equivalent eukaryotic enzyme is much lower. Patient isolates resistant to rifampicin had mutations within the beta-subunits of the RNA polymerase gene (*rpoB*) (Cole 1996).

1.1.5.3. Pyrazinamide

Like INH, pyrazinamide (Fig. 1.5), a structural analog of nicotinamide is also a prodrug. Activation of this drug is through conversion to pyrazinoic acid by a hydrolytic pyrazinamidase. Patient isolates resistant to this drug were found to have mutations within the gene encoding the pyrazinamidase *pncA* (Scorpio and Zhang 1996).

1.1.5.4. Streptomycin

Streptomycin (Fig. 1.5) was the first clinically effective drug to be available for tuberculosis chemotherapy. It was originally isolated from two strains of actinomycetes by Selman Waksman and coworkers in 1943(Schatz and Waksman 1944; Schatz, Bugie et al. 2005) and is one of the family of aminoglycoside antibiotics that act by inhibiting protein synthesis. Patient isolates resistant to streptomycin showed mutations in the gene encoding the 16S rRNA and in the ribosomal protein S12 (Heym, Philipp et al. 1996; Ramaswamy and Musser 1998).

1.1.5.5. Ethambutol

Ethambutol (EMB) (Fig. 1.5) is an established front-line agent for the treatment of tuberculosis. Ethambutol interferes with the construction of the arabinogalactan layer as well an incorporation of mycolic acids into the cell wall. It appears that EMB inhibits the enzyme that incorporates arabinose into the branching structures of the arabinogalactan (Belanger, Besra et al. 1996). Resistance to this drug is achieved by mutation in the arabinosyl tranferase (*embB*) gene which catalyses cell wall synthesis.

1.1.6. Control of Tuberculosis

The growing importance of tuberculosis was recognized by the forty-fourth World Health Assembly. Here the ongoing TB control efforts were reassessed and led to the development of the DOTS (directly observed treatment, short course) strategy. Persistence of tuberculosis had been due to neglect of TB control by governments, poorly managed TB control programmes, poverty, population growth and migration, and a significant rise of TB cases in HIV endemic areas. The DOTS strategy was developed as a control strategy to address the situation and was introduced in early 1990's by WHO in response to the global TB epidemic. This strategy was developed based on the revolutionary work of the British Medical Research Council, and the International Union Against Tuberculosis and Lung Disease. The strategy can prevent millions of TB cases and deaths over the coming decades. The DOTS strategy consists of five essential elements:

- Strong government commitment
- Detection of TB cases by smear microscopy
- 6-8 months of regularly supervised treatment

- Secure supply of safe, high quality drugs
- Reporting systems to monitor treatment progress and programme performance

Patients diagnosed with infectious TB are allocated health and community workers or trained volunteers to observe patients on TB therapy. Sputum smear test is repeated after two months and at the end of therapy, recorded results are assessed to monitor progress of the patients and quality of the programme.

Implementation of DOTS started in 1991 and to date more than 17 million patients have received treatment under this strategy. All countries with high burden of tuberculosis implemented DOTS by the end of 2002 with a total of 148 countries that had adopted the DOTS strategy for TB control and 27% of the global TB cases were treated under DOTS (WHO report 2005). By 2003, 182 countries had adopted the DOTS strategy (WHO report 2005). The DOTS strategy have shown high success rate. In 22 countries with high TB burden, 82 % of patients under DOTS had successful treatment in 1998 (Netto, Dye et al. 1999). Although there has been considerable progress over the last few years since the introduction of DOTS, much still needs to be done to inflate the services to the whole population.

1.2. Combinatorial synthesis of derivatives

In antituberculosis drug discovery, targets are selected based on the understanding of the physiology, metabolism, intracellular lifestyle of *M. tuberculosis* and the immunology of tuberculosis. Following the selection of the targets, compounds are designed and synthesised. Moreover, lead compounds could be obtained from natural products such as the plant kingdom, microbiological world, marine world, and animal sources. Lead compounds are identified through high-throughput screening and surveys of chemical diversity for compounds that inhibit the target selected. In most cases, the binding capacity of identified lead compounds is not sufficient suggesting the need for lead compound optimization. The process of lead compound optimization has been greatly enhanced by the use of combinatorial chemistry. Combinatorial chemistry approach creates compound diversity giving rise to chemical libraries for the high-throughput screening, one of which may prove to be a useful lead compound. However, this chemistry can give rise to compounds with limited structural diversity. The structures are modified without compromising the essential features of the original structure to improve binding of compounds to selected target and other

characteristics. This process is facilitated by knowledge of structure of the target. Lead optimization gives rise to thousands of new classes of compounds and therefore increasing the chances of discovering potential antituberculosis drugs.

1.3. Potential inhibitors of *M. tuberculosis*

1.3.1. Acetolactate Synthase inhibitors

The first step of the branched chain amino acids pathways such as Leucine, Isoleucine, and Valine pathways are catalysed by acetolactate synthase (ALS) (also called acetohydroxy acid synthase (AHAS)), an enzyme found in microbes and plants but absent in humans (Grandoni, Marta et al. 1998). ALS catalyzes the decarboxylation of pyruvate and its condensation either with a second molecule of pyruvate, to produce acetolactate (a precursor of valine, leucine, and co-enzyme A), or with 2-ketobutyrate, to produce acetohydroxybutyrate (a precursor of isoleucine) (Epelbaum, LaRossa et al. 1998). ALS is a target for a large number of herbicides used in the agrochemical industry to control weeds. There are three major classes of commercial herbicides that are known to inhibit ALS including sulphonyl ureas, imidazolinones, and sulphonalides (LaRossa and Schloss 1984). Sulphometuron methyl, a major commercial herbicide, inhibited ALS activity in bacteria and subsequent studies showed that inhibition of plant ALS was the basis for the phytotoxicity of other sulphonyl urea herbicides (LaRossa and Schloss 1984). *M. tuberculosis* has to synthesize the branched-chain amino acids in order to survive within the host. Leucine auxotrophs of *M. bovis* (BCG) were found to have a reduced ability to survive in spleens and lungs of mice (Grandoni, Marta et al. 1998), suggesting the possible use of inhibitors of branched-chain amino acids as anti-tuberculosis agents.

1.3.2. Thiosemicarbazones

Thiosemicarbazones are a class of small molecules that have been evaluated for years as anticancer therapeutics (Finch, Liu et al. 1999) and as antivirals (Condit, Easterly et al. 1991; Mishra, Pandeya et al. 2002). Thiosermicarbazones inhibit the growth of microorganisms by withholding iron from the organism including *M. tuberculosis* (Kochan 1973). Kochan (1973) demonstrated inhibition of *M. tuberculosis* growth in human serum through its ability to withhold iron from the tubercle bacillus (Kochan

1973) suggesting that iron status determines the occurrence and outcome of tuberculosis.

M tuberculosis requires iron to multiply and cause disease and survive within the host. *M. tuberculosis* must compete against the host to fulfil its own iron requirement. *M. tuberculosis* requires iron as an obligate co-factor for 40 different enzymes encoded by its genome (Cole, Brosch et al. 1998) including enzymes involved in oxygen metabolism and DNA synthesis (D'Orazio, Folcarelli et al. 2001). These different enzymes are all controlled by the iron-dependent regulator (IdeR) (Rodriguez and Smith 2003). Under the conditions of starvation, *M. tuberculosis* secretes hydrophilic mycobactin-T, salicylic acid, and citric acid (De Voss, Rutter et al. 1999) to compete with the extract iron from the host proteins such as transferrin, lactoferrin and ferritin. Acquisition of iron may also occur through eleven P-type adenosine triphosphatase (ATPases) (De Voss, Rutter et al. 2000) and cell wall associated Nramp, which encourages iron uptake in an acidified environment (Dussurget and Smith 1998). Iron withhold as a therapeutic approach have been studied for different organisms including *Plasmodium falciparum* (Mabeza, Loyevsky et al. 1999), hepatitis C (Sartori, Andorno et al. 2001), HIV (Georgiou, van der Bruggen et al. 2000), *M. avium* (Gomes, Dom et al. 1999). Although *M. tuberculosis* has a different pathogenic strategy to these organisms, they serve as a model where iron starvation demonstrates beneficial effects. Iron removal during infection may be beneficial by reducing pathogen replication while increasing the immunocompetence of the host.

1.3.3. Cysteine protease inhibitors

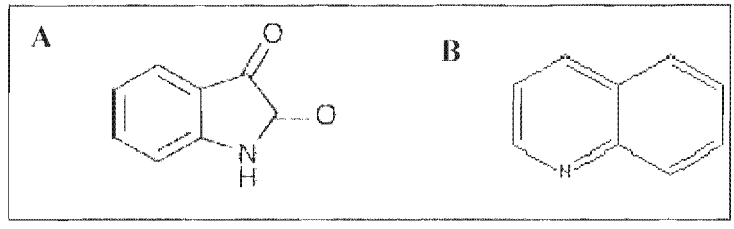


Figure 1.6. chemical structure of Isatin (A) (LAND21 2000) and Quinoline (B) (LAND21 2000)

Cysteine proteases are a class of proteins that mediates protein hydrolyses via nucleophillic attack on the carbonyl carbon of a susceptible peptide bond by function of a catalytic cysteine protein hence their name. Studies with protease inhibitors

provided important information regarding the function of cysteine proteases (Rosenthal 2001). Cysteine proteases are involved in haemoglobin hydrolysis, erythrocyte invasion by merozoites (Rosenthal 2001), and hydrolysis of the gametocyte surface protein Pfs230 (Williamson, Fujioka et al. 1996) during the life cycle of malaria parasite. Recently, another function of cysteine proteases was elucidated. The knockout of falcipain-1 showed to have a decreased oocyst production (Eksi, Czesny et al. 2004). This suggested a role of cysteine proteases in sexual stage of parasite. Haemoglobin hydrolysis is crucial for survival of *plasmodium falciparum*. Haemoglobin hydrolysis provides amino acids for parasite protein synthesis (McKerrow, Sun et al. 1993; Francis, Sullivan et al. 1997) to maintain osmotic stability of malaria parasite (Lew, Tiffert et al. 2003).

Different subclasses of cysteine proteases including double drugs (bi-therapeutic strategy) in which either quinoline inhibitors of hemozoin formation are combined with a cysteine protease inhibiting moiety in the same molecule have been designed and synthesized as an effort to decrease the potential quinoline-based anti-malarial drug resistance. Isatin-based synthetic thiosemicarbazones with cysteine protease inhibition properties were also designed and synthesized.

Quinoline is an aromatic nitrogen compound characterized by a double-ring structure containing benzene ring fused to a pyridine ring at two adjacent carbon atoms (Fig. 1.6B). Quinoline-containing drugs such as chloroquine and quinine have had a long and successful history in antimalarial chemotherapy (Slater 1993). This prompted the design of quinoline-containing double drugs to overcome the issue of drug resistance.

Isatin is a natural product found in a number of plants including those of the genus Isatis (Fig. 1.6A) (Chiyanzu, Hansell et al. 2003). Pharmacological properties of Isatin including antiprotozoal activities have been reported (Imam and Varma 1975; Varma and Khan 1977). Moreover, isatin has been reported to inhibit cysteine and serine protease (Webber, Tikhe et al. 1996; Chiyanzu, Hansell et al. 2003) recommending isatin as a potential scaffold for designing and synthesising chemical libraries. Due to the aforementioned cross species activity of isatin-based derivatives including the cysteine protease inhibition of these derivatives, we tested these derivatives for potential antitubercular activity on *M. tuberculosis*. Moreover, none isatin-based derivatives with the potential to inhibit cysteine proteases were evaluated for antitubercular properties.

1.3.4. Thiolactomycins

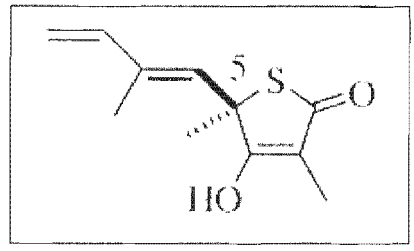


Figure 1.7. Structure of thiolactomycin (Sakya, Suarez-Contreras et al. 2001)

Thiolactomycin (TLM) (Fig. 1.7), the first thiolactone to demonstrate antibiotic activity was initially isolated from a soil bacterium, *Norcadia* spp (ATCC 31319) (Oishi, Noto et al. 1982). TLM is effective against a broad spectrum of pathogenic bacteria, including Gram-negative, Gram-positive, and *M. tuberculosis* with low toxicity (Miyakawa, Suzuki et al. 1982; Noto, Miyakawa et al. 1982). TLM acts as an inhibitor of the beta-ketoacyl carrier protein synthase condensing enzymes (FAS-II), mtFabH and KasA, the enzymes of the type II fatty acid biosynthesis, leading to inhibition of the cell wall mycolic acid biosynthesis and cell death (Choi, Kremer et al. 2000; Kremer, Douglas et al. 2000; Schaeffer, Agnihotri et al. 2001). TLM inhibits bacterial and plant FAS-II but not mammalian or yeast type I fatty acid synthases (FAS-I) (Hayashi, Yamamoto et al. 1983). Due to the essential role of the FAS-II, inhibition of this system have attracted attention as a lead for development of potential antibacterial agents (Heath, White et al. 2001; Payne, Warren et al. 2001). When β-ketoacyl synthase (FabB) was overexpressed or missense mutations introduced, *Escherichia coli* was resistant to TLM, demonstrating FabB as a target (Tsay, Rock et al. 1992; Jackowski, Zhang et al. 2002). Previous studies have shown inhibition of the FAS-II system by TLM derivatives. For instance, the aliphatic substituents at the 5-position of the thiolactone ring, had significant activity against mycolate synthesis (Kremer, Douglas et al. 2000; Douglas, Senior et al. 2002). The use of 10-carbon isoprenoid-based side-chains conferred enhanced inhibition. Analogues of thiolactomycin inhibited the FAS-II of *Plasmodium falciparum*, two of which inhibited FAS-II with six-fold efficacy than thiolactomycin (Waller, Ralph et al. 2003). INH have been reported to disrupt the synthesis of mycolic acids but there is no cross resistance between the two molecules. TLM showed to be active *in vitro* against a wide range of *M. tuberculosis*, including the INH resistant strains (Slayden,

Lee et al. 1996). The reported data make thiolactamycin and its derivatives an attractive lead due to its novel mechanism of action, which minimizes the cross-resistance to human therapeutics.

1.4. Aim and Objectives

1.4.1. Aim

To investigate the *in vitro* and *in vivo* efficacy of identified Acetolactate Synthase inhibitors, Cysteine Protease synthase inhibitors, Thiosemicarbazones, and Thiolactomycin against *M. tuberculosis*.

1.4.2. Objectives

1. To identify the hit compounds for *in vivo* therapeutic study by using:
    i. Directing *M. tuberculosis* killing assay
    ii. Cell culture based cytotoxicity assay
    iii. Intracellular killing assay using elicited macrophages
2. To identify the lead compounds using an experimental tuberculosis murine model.

CHAPTER TWO

Materials and Methods

2.1. Compounds

Synthetic derivatives used in this study were synthesized by the Prof. Kelly Chibale, Department of Chemistry, University of Cape Town. Rifampicin (RIF) (Sigma, SA) and Isoniazid (INH) (Sigma, SA) were purchased from Sigma. Dimethyl Sulfoxide (DMSO) (BDH, AnalaR, England) was used as a solvent for synthetic derivatives and Rifampicin, and sterile distilled water for Isoniazid.

2.2. Bacterial strain

Mycobacterium tuberculosis strain H37Rv was obtained from the Tradeau Mycobacterial culture. Culture was grown at 37°C until mid log phase in Middlebrook 7H9 broth (Difco, USA) containing 0.5% glycerol and supplemented with 10% oleic acid, albumin, dextrose, and catalase (OADC) enrichment medium (Life Technologies, Gaithersburg, MD). Sample aliquots were frozen and stored at -70°C. To determine the mycobacterium concentration of frozen stocks, an aliquot was thawed and passed through a 29G needle (B:Braun, Switzerland) to disband clumps and 100µl of sample plated in duplicate on Difco Middlebrook 7H10 agar plates in 10 fold serial dilutions. Plates were incubated for 21 days at 37°C after which colonies were counted and the concentration of the batch calculated.

2.3. Resazurin microtitire plate assay (REMA)

2.3.1. Primary screening of identified synthetic derivatives for antituberculosis activity

In vitro evaluation of antitubercular activity was performed as previously described by Collins *et al.,* 1997 with some modifications. Primary screening was conducted at 10µg/ml of compound against *Mycobacterium tuberculosis* in Middlebrook 7H9 broth (Difco, USA) supplemented with 10% OADC enrichment medium (Life Technologies, Gaithersburg, MD). Antimycobacterial susceptibility assay was performed in flat bottomed 96-well microtitre plate (Greiner bio-one, Germany). Outer wells were filled with sterile water in order to minimize evaporation in experimental wells. The compound was initially prepared in either DMSO (BDH, AnalaR, England) or distilled water and diluted further in 7H9 broth to give final concentration of 20µg/ml. One hundred micro litres of each DMSO-dissolved compound (20µg/ml) was added to experimental wells.

The passaged inocula were diluted to give a concentration of $4x10^5$CFU/ml. One hundred micro litres of inoculum ($4x10^5$CFU/ml) was added to wells containing drugs to give a final mycobacterial concentration of $2x10^5$CFU/ml and compound concentration of 10µg/ml. Plates were sealed with parafilm and incubated at 37°C. After day 7 of incubation, 50µl of freshly prepared Resazurin (7-hydroxy-3H-Phenoxazin-3-1-10 oxide) (BDH, Germany) was added to one well containing *M. tuberculosis* and medium only and incubated for 24h. The sample was observed for colour change from blue (oxidized state) to pink (reduced form) as an indication of reduction of resazurin to resorufin (Fig. 2.1). If the sample turned pink, dye was added to the entire plate, incubated at 37°C for 24h, after which plates were read using the microplate reader ($VERSA_{max}$) at 570nm and 600nm. If wells remained blue, plates were incubated further and those that were still blue 5 days post reagent addition were recorded as active against the *M. tuberculosis*. The percentage of resazurin reduced when samples read at λ_1 = 570nm and λ_2 = 600nm was calculated using the following equation:

$$\% \text{ Reduced} = \frac{(\varepsilon_{ox}\,\lambda_2)\,(A\,\lambda_1) - (\varepsilon_{ox}\,\lambda_1)\,(A\,\lambda_2)}{(\varepsilon_{red}\,\lambda_1)\,(A'\,\lambda_2) - (\varepsilon_{red}\,\lambda_2)\,(A'\,\lambda_1)} \times 100$$

Where:
$(\varepsilon_{red}\,\lambda_1)$ = 155.677
$(\varepsilon_{red}\,\lambda_2)$ = 14.652
$(\varepsilon_{ox}\,\lambda_1)$ = 80.586
$(\varepsilon_{ox}\,\lambda_2)$ = 117.216
$(A\,\lambda_1)$ = Absorbance of test wells at 570nm
$(A\,\lambda_2)$ = Absorbance of test wells at 600nm
$(A'\,\lambda_1)$ = Absorbance of negative control wells which contain medium plus resazurin but to which no cells have been added at 570nm
$(A'\lambda_2)$ =Absorbance of negative control wells which contain medium plus resasurin but to which no cells have been added at 600nm (BioSource International)

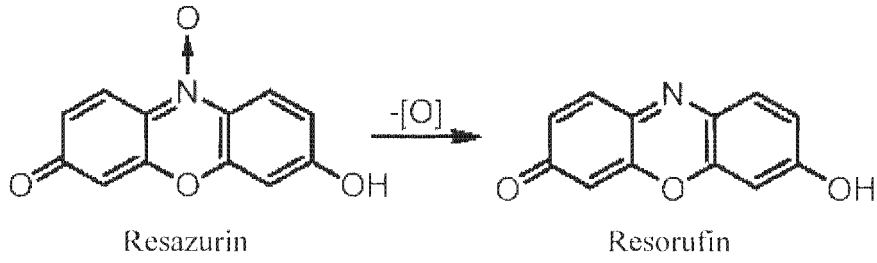


Figure 2.1. Reduction of resazurin to resorufin by metabolically active cells (Tratnyek, Reilkoff et al. 2001)

2.3.2. Determination of the Minimum Inhibitory Concentration

In vitro evaluation of antituberculor activity was performed as previously described by Collins *et al.*, 1997 with some modifications. The antimycobacterial susceptibility assay was performed in transparent flat bottomed 96-well microtitre plates (Greiner bio-one, Germany). Outer wells were filled with 200µl sterile water to minimize evaporation in experimental wells (Fig. 2.2). One hundred microlitres of Middlebrook 7H9 broth (Difco, USA) was added to wells in column 2 to 11, row B to E. The initial concentration was prepared in either DMSO (BDH, AnalaR, England) or distilled water and diluted further in Difco Middlebrook 7H9 broth (Difco, USA) to give final concentration of 40µg/ml. One hundred microlitres of compound containing Middlebrook 7H9 broth (Difco, USA) was added to column 2 (row B to E). Subsequent 1:2 dilutions until column 11 were performed in 100µl of Middlebrook 7H9 broth (Difco, USA) in microplates (Greiner bio-one, Germany) (Fig. 2.2). One hundred microlitres of compound from the column 11 (row B to E) was discarded after dilution. The inoculum was passaged using a 29G needle (B:Braun, Switzerland). The passaged inoculum was diluted in Middlebrook 7H9 broth (Difco, USA) to give a concentration of $4x10^5$CFU/ml. One hundred microlitres of $4x10^5$CFU/ml was added to wells to give a final concentration of $2x10^5$CFU/ml. For growth control wells, 100µl compound was replaced with 100µl of broth and 100µl of inoculum with 100µl of broth in sterile control wells. Wells containing drugs only were used to detect absorption by compounds and medium. Additional wells containing Isoniazid (Sigma, SA) and Rifampicin (Sigma, SA) were incorporated as positive controls. The MIC of drugs were assessed by the REMA assay as described (section 2.3.1)

and was defined as the lowest concentration that prevented colour change at day 5 of resazurin (BDH, Germany) addition to the experimental wells.

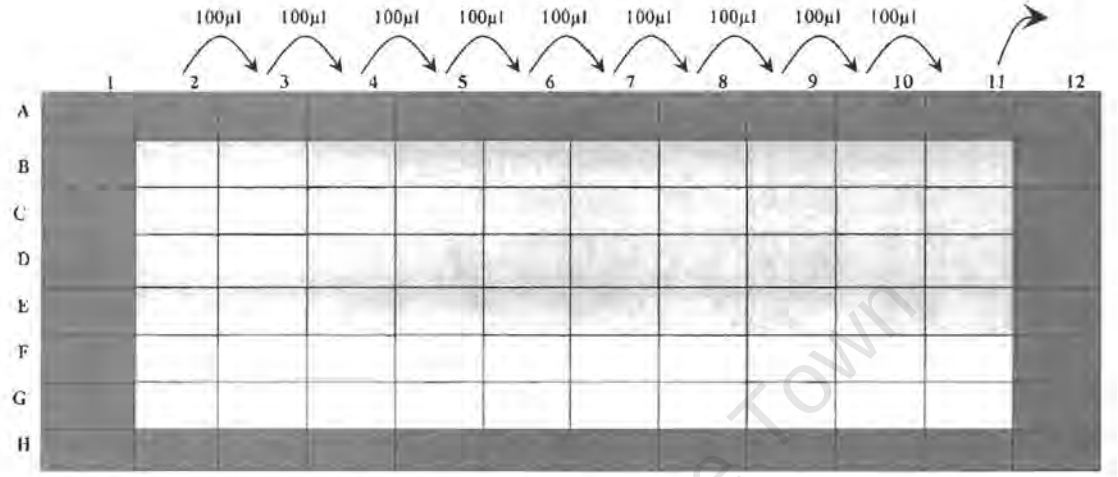


Figure 2.2. Resazurin Microtire plate for minimum inhibitory concentration determination using REMA.

2.4. *In vitro* cytotoxicity assay

2.4.1. Isolation and culture of macrophages

Peritoneal macrophages were obtained from 6-8 weeks old specific-pathogen free C57Bl/6 female mice as described (Coligan, Kruisbeek et al. 1995). Cells were pelleted, washed and assessed for their density by counting using a haemocytometer (Neubauer, Germany) and viability by 0.4% Trypan blue (Sigma, SA). Cells were then diluted and cultured in 48-well tissue culture plates (Corning International Costar, USA) at $1x10^5$cell/ml in pre-warmed complete medium (2Mm L-Glutamine, 5%FCS, 10% L929 medium, RPMI) overnight at 37°C in 5% CO_2 incubator (Forma Scientific, USA, Model: S/N 28192-484) for 8 days. Non-adhering cells were removed, and complete medium (2Mm L-Glutamine, 5%FCS, 10% L929 medium, RPMI) was replaced with pre-warmed complete medium containing compound at a final concentration of 0-32µg/ml. Plates were re-incubated at 37°C in 5% CO_2 incubator (Forma Scientific, USA, Model: S/N 28192-484) for 8 days.

2.4.2. MTT assay

The 3-(4, 5-dmethylthiazol-2yl)-2, 5-diphenyl-tetrazonium bromide (MTT) yellow dye (Sigma, SA) was used to assess the viability of cells following treatment with compounds as described (Mosmann 1983) with modifications (O'Brien, Wilson et al. 2000). Stock MTT (Sigma, SA) was prepared by dissolving MTT (Sigma, SA) in 1x phosphate-buffered saline (PBS) to a final concentration of 5mg/ml. Volume (20µl) of stock MTT solution were added to each well containing 200µl of complete medium to give a 10% (v/v) final concentration and incubated with cells for 4h at 37°C in 5% CO_2 incubator (Forma scientific, USA, model S/N 28192484. Medium was removed and violet precipitate (formazan) solubilized with 100µl of DMSO (BDH, AnalaR, England). Formazan crystals (MTT reduced form) were read on the microplate reader ($VERSA_{max}$) at 550nm and 690nm. Subtraction analysis of the dual wavelength was performed to increase accuracy of the measurement and cell viability calculated. Cytotoxicity was expressed as the concentration at which 50% of macrophages were metabolically active.

2.5. Macrophage assay

2.5.1. Isolation and culture of macrophages

Peritoneal macrophages were obtained from 6-8 weeks-old specific-pathogen free C57Bl/6 female mice (Coligan, Kruisbeek et al. 1995). Cells were pelleted, washed and assessed for their density counting using a haemocytometer (Neubauer, Germany), and viability by 0.4% Trypan blue (Sigma, SA). Cells were then diluted and cultured in 48-well tissue culture plates (Corning International Costar,USA) at $1x10^5$cell/ml in pre-warmed complete medium overnight at 37°C in 5% CO_2 incubator (Forma Scientific, USA, Model: S/N 28192-484) for 8 days. Non-adhering cells were removed, and the medium (2Mm L-Glutamine, 5%FCS, 10% L929 medium, RPMI) was replaced with a compound to be assayed for intracellular drug activity.

2.5.2. Intracellular drug Activity

Macrophages were infected with *M. tuberculosis* at a multiplicity of infection (MOI) of 2:1 (2 bacteria: 1 macrophage) and allowed to phagocytose the bacteria overnight at 37°C in a 5% CO_2 incubator (Forma Scientific, USA, Model: S/N 28192-484). Extracellular bacteria were thoroughly washed away with 1X PBS. The number of effectively phagocytized bacteria was determined by lysing the macrophages using 0.02% (w/v) SDS (Saarchem, SA), and plating the 10 fold serial dilutions of lysate on Difco Middlebrook 7H10 agar plates for viable count determination. After phagocytosis, fresh complete medium (2Mm L-Glutamine, 5%FCS, 10% L929 medium, RPMI) containing the compound of interest was added to macrophages and reincubated for 8 days. Each compound was tested in quadruplicate. After 8 days of incubation, macrophages were washed gently with 1X PBS to remove traces of compound. Macrophages were lysed using 0.02% (w/v) Sodium Dodecyl Sulphate (SDS) (Saarchem, SA), plated on Difco Middlebrook 7H10 agar plates and bacteria enumerated after incubation for 21 days at 37°C. The results were compared with the growth of the control culture (untreated macrophages). Data was expressed as a percentage of the untreated samples.

2.6. Experimental animals

Female C57Bl/6 wild-type mice were bred and maintained under specific pathogen free (SPF) conditions in the animal unit at the University of Cape Town (Cape Town, South Africa) and used at 6-8 weeks. Mice were housed in filter-top cages and supplied with sterile water and food. After infections, mice were housed in filter top cages in a biohazard level 3 containment facility.

2.7. Inoculum preparation

A frozen stock culture aliquot of *M. tuberculosis* was thawed at room temperature and the clumps disseminated by aspirating 30 times through a 29G needle (B:Braun, Switzerland). Six millilitres of inoculum was prepared by diluting stock *M. tuberculosis* in saline to a final concentration of 2 x 10^6CFU/ml. The inoculum concentration was confirmed by plating 10 fold serial dilutions in duplicate on Difco Middlebrook 7H10 agar plates.

Plates were incubated at 37°C for 21 days, colonies counted and concentration of the inoculum calculated.

2.8. Aerosol procedure

Mice were challenged by aerosol exposure at a dose of 100cfu/lung. The aerosol chamber (Glas-Col inhalation exposure system, model A4224) was prepared by wiping once with 3% virkon and twice with 70% ethanol. The footplate was placed into the chamber and the drum carefully placed on top of the footplate. Mice were transferred into the different divisions of the basket. The chamber was firmly sealed with a Perspex lid. The glass nebuliser (Nebuliser-Venturi) was securely attached to the chamber and 5ml of the inoculum gently transferred into the nebuliser. The following program was used:

Preheating	15 min
Nebulising	40min
Cloud decay	40 min
Decontamination	15 min

The air flow was adjusted to 60CFH and the preheat cycle was allowed to complete. The compressor valve was then adjusted to 10CFH at the start of the nebulising cycle. At completion of infection, mice were transferred to their allocated cages.

2.9. Treatment of infected mice

Compounds and RIF (Sigma, SA) were dissolved in DMSO (BDH, AnalaR, England) and INH in distilled water. Mice were treated with 50µl of AXE29 (100mg/kg), RKG162A (100mg/kg), RIF (20mg/kg) (Sigma, SA), DMSO (BDH, AnalaR, England) (100%) and INH (25mg/kg) (Sigma, SA). Compounds were administered intraperitoneally with a 29G needle (B:Braun, Switzerland) everyday for 2 weeks starting from day 1 of infection (n=4). Control groups were sacrificed at day 1 of infection to determine the bacterial load at the onset of treatment. The dynamics of body weight were monitored and treatment was stopped after 14 days of treatment.

2.10. Therapeutic efficacy

Therapeutic efficacy is defined as the reduction in bacterial colony formation in the organs of infected mice. Mice were sacrificed by carbon dioxide. The spleen, lung, and liver were aseptically removed and weighed. The organs were homogenized in 2ml of sterile saline. Homogenate samples were diluted 10-fold in 0.04% Tween-80 saline and 100µl plated in duplicate on Difco Middlebrook 7H10 agar plates. Plates were incubated for 21 days at 37°C after which colonies were counted and the bacilli organ load calculated.

2.11. Microscopic analysis of mouse organs

2.11.3. Paraffin wax embedding

Mice were sacrificed by carbon dioxide inhalation, organs aseptically removed and fixed in 10% buffered formalin (pH 7.00). Since paraffin wax does not penetrate tissues in the presence of water, tissues were dehydrated once in 70% ethanol, twice in 96% ethanol and four times in absolute ethanol. Tissues were transferred to Zylol to allow mixing of alcohol and paraffin wax during embedding. Wax with a melting temperature of 55-60°C was used to embed tissues. Paraffin blocks were first trimmed and 1-2µm sections were cut using a microtome (Leica, model RM-2125). Sections were floated onto 35-45% alcohol, waterbathed at 40-45°C, and transferred onto clean glass slides and placed at 37°C incubator overnight for fixing. Before staining, slides were left at 60°C for 2hrs to overnight to dewax.

2.11.2. Haematoxylin and eosin stain

Slides were dehydrated for 1minute in xylol (2X), 100% ethanol (2X), 96% ethanol (2X), followed by 70% ethanol (1X) and water. Sections were stained with Haemotoxylin for 8 minutes, rinsed in water and immersed in 1% acid alcohol for 10 seconds. After rinsing in running water for 30 minutes, sections were counterstained with 1% eosin for 2 minutes. The counterstain was removed by rinsing in water and sections dehydrated for 10 seconds in 70% ethanol, 96% ethanol and xylol. Sections were then mounted using Canada Balsam.

2.11.3. Ziehl-Neelson stain

Slides were rehydrated for 1 minute in xylol, 100% ethanol (2X), 96% ethanol (2X), 70% ethanol (2X) and water. Six percent carbol fuchsin was added to tissue sections, flamed and left to cool. Excess carbol fuchsin was removed by rinsing with water, 1% acid alcohol until excess colour is removed and again with water. Tissue sections were then placed in 25% H_2SO_4 for 20minutes, washed with running water for 10 minutes to remove acid, counterstained in Loeffers' methylene blue for 0.5 to 1 minute, and again rapidly rinsed in water. Slides were dehydrated sections dehydrated for 10 seconds in 70% ethanol, 96% ethanol and xylol and mounted in synthetic resin (Depex mounting medium).

2.12. Imaging and Software

Images of stained sections were captured using a Nikon DMX 1200 digital still camera attached to a Nikon Eclipse E40 microscope and the ACT-1 software application programme.

2.13. Statistical analysis

The data is expressed as the mean ±SD. The significance of differences between groups was analyzed by the student's t-test and a p-value of <0.05 was considered significant.

REAGENTS

A. Bacteriological reagents

1. Difco Middlebrook 7H9 Broth

Nine hundred millilitre of distilled H_2O containing 2 ml glycerol was used to dissolve 4.7g Difco 7H9 broth. The broth solution was autoclaved for 10min at 121°C. One 100ml OADC (Life Technologies, Gaithersburg, MD) was added to the broth solution.

2. Difco Middlebrook 7H10 Agar

Nineteen grams 7H10 agar was added 900ml distilled H_2O containing 5ml glycerol, stirred and autoclaved at 121°C for 10 minutes. The agar solution was cooled at 56°C and

100ml OADC (Life Technologies, Gaithersburg, MD) (100ml) added to agar solution. Seven milliliters was poured in either side of two-compartmented Petri dishes (Biby sterilin, Staffordshire, England).

B. General reagents

1. Brewer Thioglycollate

Three grams of brewer thioglycollate was added to 90ml distilled H_2O. The volume of the solution was adjusted to 100ml by adding distilled H_2O. The solution was autoclaved for 15 minutes at 121°C.

2. Homogenising buffer for organs

Nine grams NaCl was dissolved in 900ml distilled H_2O containing 400µl Tween-80. The volume of saline solution was adjusted to 1000ml using distilled H_2O. The solution was sterilized by autoclaving for 30 minutes at 121°C and stored at room temperature.

3. Phosphate Buffered Saline (PBS)

Eight grams NaCl, 0.2g KCl, 1.44g Na_2HPO_4, and 0.24g KH_2PO_4 were dissolved in 900ml distilled H_2O. The pH of PBS was adjusted to 7.4 with HCl. The volume of solution was adjusted to 100ml using distilled H_2O. PBS was sterilized by autoclaving for 30min at 121°C and stored at room temperature.

4. Saline

Nine grams NaCl was dissolved in 900ml distilled H_2O. The volume of saline solution was adjusted to 1000ml using distilled H_2O. The solution was sterilized by autoclaving for 30 minutes at 121°C and stored at room temperature.

C. *M. tuberculosis* Susceptibility testing reagents

1. Isoniazid

One hundred milligram isoniazid was dissolved in 10ml distilled H_2O. The solution was sterilized by filtering through a 0.45µm filter and stored at 4°C.

2. MTT

Five milligram MTT was dissolved in 5ml PBS. The solution was filtered through a 0.45µm filter and stored at 4°C.

3. Resazurin

One resazurin tablet was dissolved in 50ml distilled H_2O. The solution was sterilized by filtering through a 0.45µm filter and stored at 4°C.

4. Rifampicin

One hundred milligram isoniazid was dissolved in 10ml DMSO. The solution was stored at -20°C.

D. Histology reagents

1. APES coated slides

Slides were washed in 1% Teepol for 10 minutes at room temperature. Slides were washed in a running tap water for 1 hour and dried overnight at 37°C. Slides were immersed in 3.3 APES for 10-20 seconds, 10 seconds in acetone and rinsed with distilled H_2O. Slides were dried overnight at 37°C and stored at room temperature.

2. Carbol Fuchsin

Ten millilitres of 6% Basic Fuchsin (in absolute alcohol) was added to 90ml 5% carbolic acid solution. The solution was sterilized by filtering through a Whatmann filter paper no. 1 and stored at room temperature.

3. Eosin

One hundred and fifty millilitres 1% Eosin solution was added to 75ml 1% Phloxine. The volume of solution was adjusted to 450ml with distilled H_2O. The solution was sterilized by filtering through a Whatmann filter paper no. 1 and stored at room temperature.

4. Loeffers' Methylene Blue

One millilitre of 1% KOH was added to 99ml distilled H_2O. Thirty millilitres 0.8% Methylene Blue (in absolute alcohol) was added to the KOH solution, filtered through Whatmann filter paper no. 1 and stored at room temperature.

5. Mayers haematoxylin

One gram haematoxylin was dissolved in 800ml distilled H_2O. Fifty grams aluminium ammonium sulphate was added to solution and allowed to dissolve. One gram citric acid, 0.2g sodium iodate, and 50g chloral hydrate were added to the solution ensuring that each reagent is completely dissolved before adding the next reagent. The solution was adjusted to 1000ml using distilled H_2O, filtered through a Whatmann filter paper no. 1 and stored in the dark at room temperature.

6. Wegert's haematoxylin

6.1. Solution A

One gram haematoxylin was dissolved in 100ml absolute alcohol.

6.2. Solution B

Four millilitres 30% Aqueous Ferric Chloride (anhydrate) and 1ml concentrated HCl were added to 95ml distilled H_2O.

Equal volumes of solution A and solution B were mixed before use.

CHAPTER THREE

Comparative *in vitro* efficacy and cytotoxic effects of identified synthetic derivatives against *M. tuberculosis*

Summary

There is an increase in the development of drug resistance towards the current therapeutic regimen for tuberculosis. This prompted the design and synthesis of new chemical libraries as an attempt to reduce/slow down the emergence of drug resistance. We therefore assessed derivatives from the acetolactate synthase inhibitors, cysteine protease inhibitors, thiosemicarbazones, and thiolactomycins classes of compounds for *in vitro* efficacy against *M. tuberculosis*. For the direct killing of *M. tuberculosis* and MIC determination of active derivatives, *M. tuberculosis* was incubated with derivatives for 6 days, resazurin added and incubated further. Absorbance of samples was read after 24 hours to assess the amount of resazurin that was reduced in order to determine the bacterial growth. For cytotoxicity assay, the MTT assay was used to assess the viability of elicited C57BL/6 peritoneal macrophage cells after treatment with active derivatives for 8 days. Eight derivatives (AXE1, AXE4, AXE5, AXE29, FM19, FM48, RKG162A, and RKG1541) from the above mentioned classes of compounds were bactericidal against *M. tuberculosis* and six were bacteriostatic (FM04, FM102, AXE2, AXE3, AXE35, and NAT47). All the bacteriostatic (FM04 and FM102) and bactericidal (FM19 and FM48) thiosemicarbazones where highly toxic when tested in a cell based culture using the elicited peritoneal macrophages. Cysteine protease inhibitors AXE1, AXE4, AXE5, had cytotoxic effects similar to that of thiosemicarbazones. AXE29 was the least toxic among the cysteine protease inhibitors. Both RKG162A and RKG1541 were mildly toxic to cultured macrophages. However, bactericidal thiosemicarbazone had the lowest MIC when compared to compounds from other derivatives classes. Of the compounds tested for cytotoxicity, AXE29 and RKG162A displayed the lowest cytotoxic effects and were selected for further investigation in an experimental murine model. AXE1, AXE4, AXE29, RKG162A, and RKG1541 exhibited an MIC of 10μg/ml, 5μg/ml for AXE5 and FM19, and 2.5μg/ml for FM48.

3.1. *In vitro* efficacy of identified synthetic derivatives

One hundred and twenty two synthetic derivatives from 4 different classes of compounds were tested for their antimycobacterial potential using *M. tuberculosis* H37Rv in a resazurin microtitre plate assay (REMA). The resazurin assay incorporates the reduction of resazurin (blue) by metabolically active cells to resorufin (pink) (Fig. 2.1 & 3.1.2). Briefly, to test for antimicrobial activity, *M. tuberculosis* was incubated with identified synthetic derivatives. After 6 days resazurin was added to assess the antimicrobial activity. INH and RIF were incorporated into the assay as known inhibitors of mycobacterial growth and samples receiving no drug treatment indicated positive growth (Fig. 3.1.1) In each assay the inclusion of "blank controls" i.e. samples containing resazurin and synthetic compounds or INH or RIF were used to validate that the absorbance measured was as a result of the reduction of resazurin by metabolically active *M. tuberculosis* and not due to the compound being tested. Blank control wells, containing either medium alone, or medium and compound did not show a change of colour or absorbance after the addition of resazurin indicating that change in colour or absorbance was not due to the reduction of resazurin by metabolically active *M. tuberculosis* (Fig. 3.1.2).

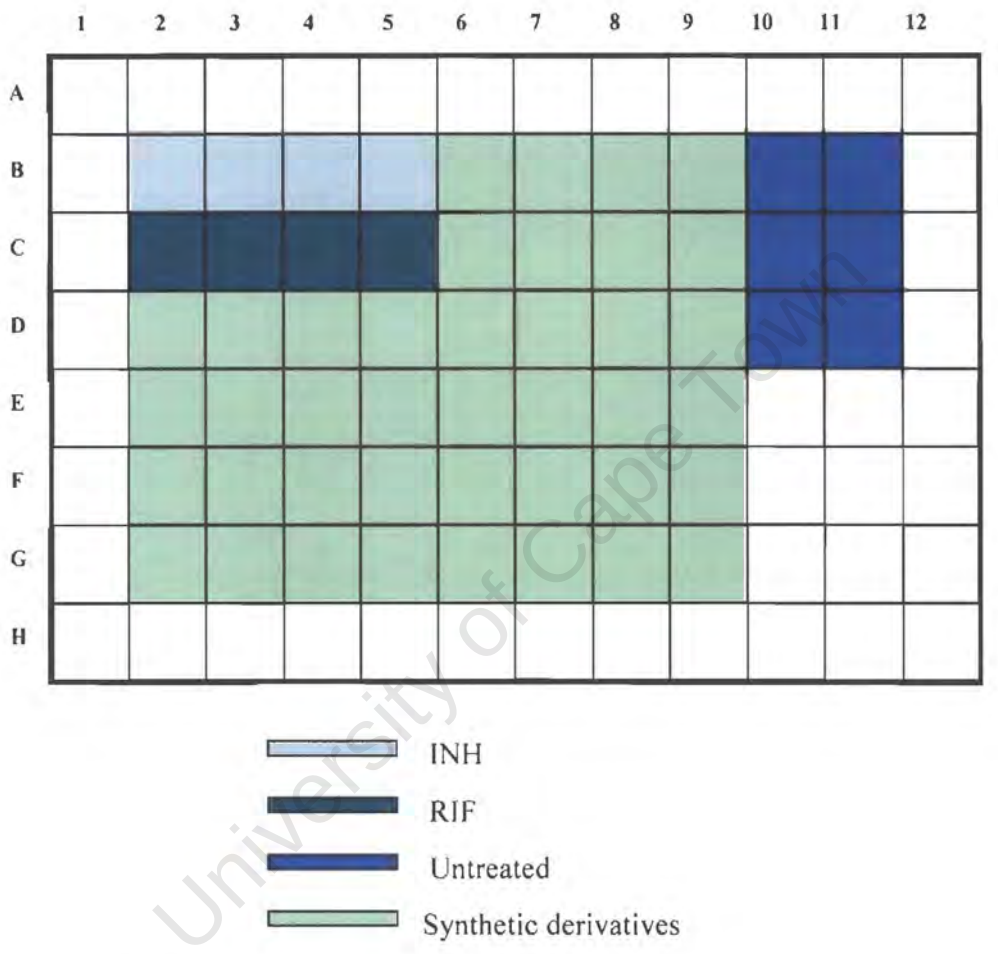


Figure 3.1.1. The layout of the Experimental and control resazurin plates in Figure 3.1.2

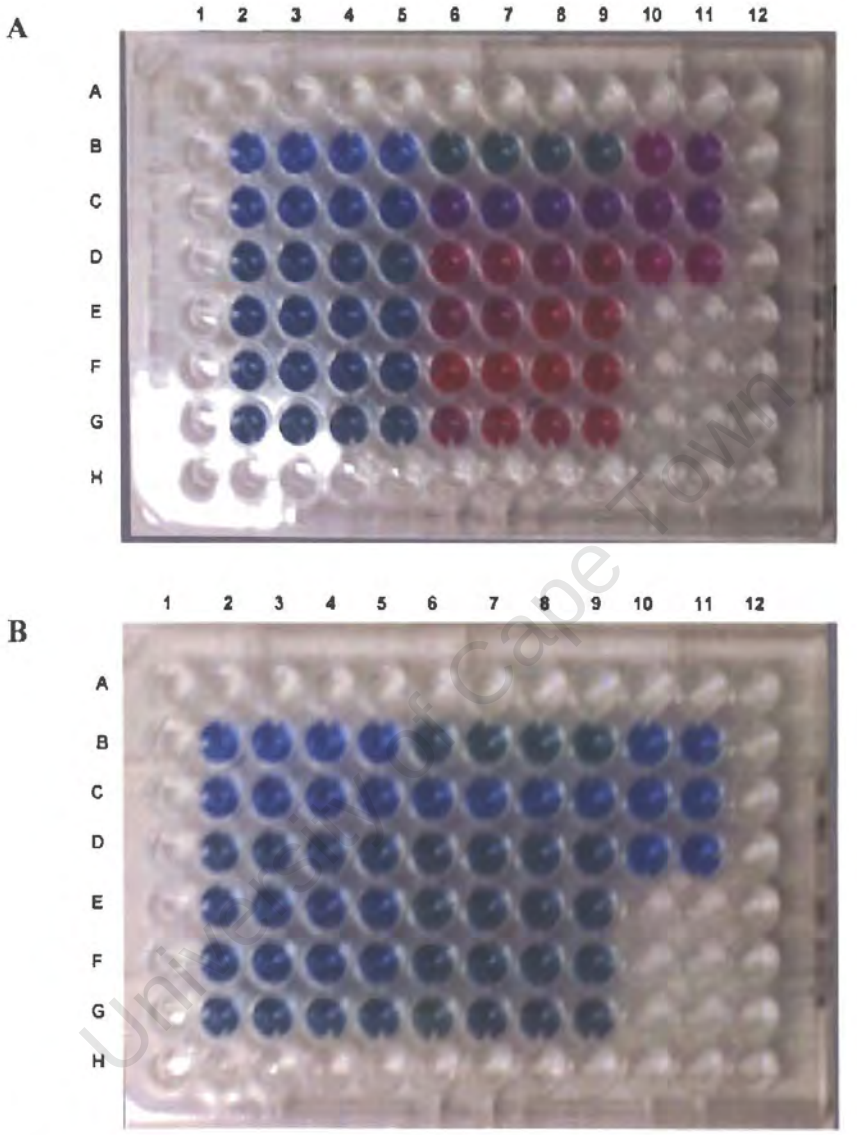
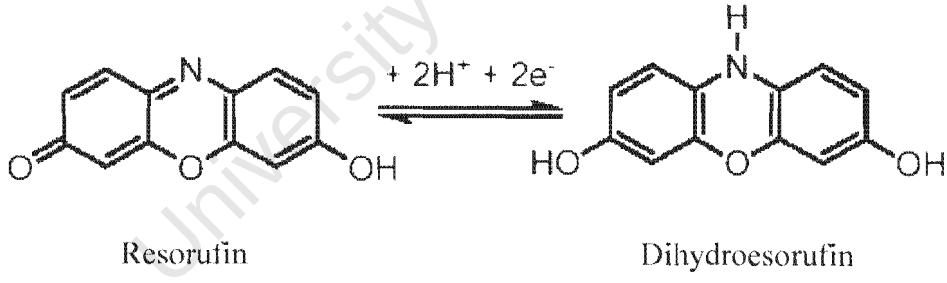


Figure 3.1.2. The reduction of resazurin to resorufin in the experimental wells (A) and blank controls (B) at 24 hours following addition o f resazurin during the reasazurin microtitre plate assay

3.2. Kinetics of the resazurin reduction in a resazurin microtitre plate assay

In this study, the kinetics of resazurin reduction on exposure to *M. tuberculosis* was investigated using the resazurin microtitre plate assay (REMA). The inclusion of untreated samples acted as indicators of positive growth, whereas treatment of *M. tuberculosis* with INH and RIF served as controls for growth inhibition. Resazurin was added to actively growing *M. tuberculosis* after 6 days and incubated further. The percentage of resazurin reduced was measured immediately after adding the redox dye and subsequently measured every 24h for 5 days. It was found that resazurin was reduced when mycobacteria was incubated with INH and RIF (fig 3.2.2). Although resazurin reduction was measured over 5 days, for INH and RIF it never exceeded 15%. This correlated with a lack of colour change in these wells. For untreated wells, reduction was maximal after 2 days subsequent to the addition of resazurin. Some of the resorufin, a product that was measured following reduction of resazurin, was further reduced to form dihydroresorufin, the colourless form of resazurin (Fig. 3.2.1). Further reduction of resazurin to dihydroresorufin increased subsequent to day 2 resulting in a decreased amount of resazurin reduced. We therefore measured activity of compounds against *M. tuberculosis* at day 2 for subsequent studies.



Resorufin Dihydroesorufin

Figure 3.2.1. Reduction of resorufin (pink) to Dihydroresorufin (colourless) (Tratnyek, Reilkoff et al. 2001)

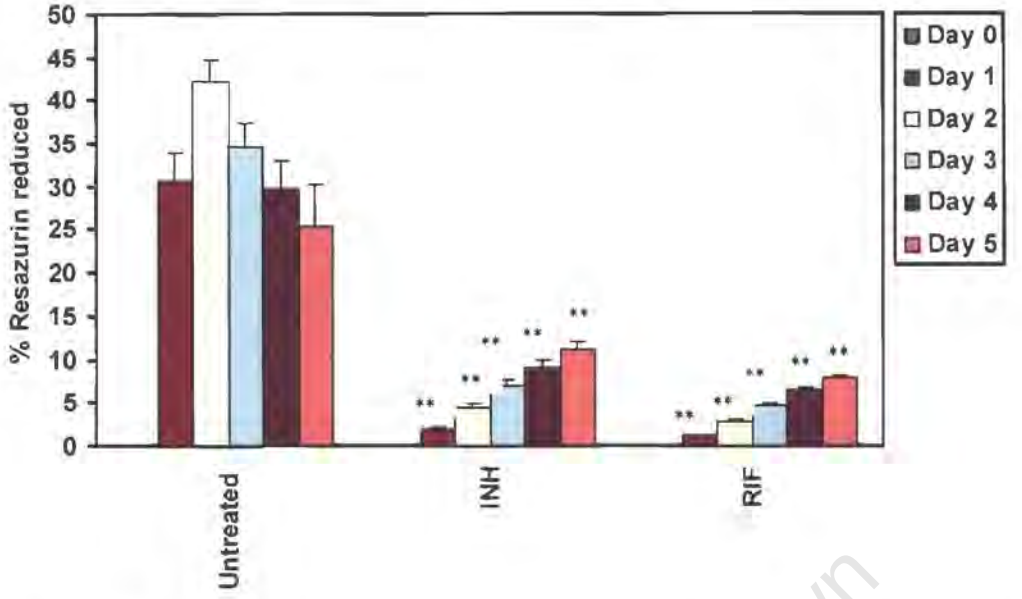


Figure 3.2.2. Kinetics of *M. tuberculosis* growth during the resazurin assay. INH and RIF were tested for antimycobacterial activity against *M. tuberculosis* using REMA. *M. tuberculosis* was incubated with 10μg/ml of INH and RIF for 6 days. Resazurin was added to samples to a final concentration of 5% (v/v) and incubated further. Plates were read at 570nm and 600nm. Percentage resazurin reduced was measured relative to controls wells containing medium only (for growth control) or medium and INH/RIF and no *M. tuberculosis*. Values are representative of the mean and standard deviation of quadruplicate measurements. ** represent $p<0.01$.

3.3. Efficacy of DMSO against *M. tuberculosis*

In this study, compounds were solubilized in 100% (v/v) DMSO. We therefore determined the lowest concentration at which DMSO inhibited *M. tuberculosis* growth prior to susceptibility testing of selected derivatives. This was to determine the maximum concentration of DMSO that could be tolerated in our assays without affecting *M. tuberculosis* growth in order to distinguish between the effect of the synthetic derivatives and the solvent. *M. tuberculosis* was incubated with an increasing concentration of DMSO for 6 days after which resazurin was added and incubated further. The DMSO concentration used ranged from 0 to 10% (v/v).

In the presence of 0-1.25 % (v/v) DMSO, colour changed and *M. tuberculosis* growth similar to that of untreated samples was observed indicating that at these concentrations, *M. tuberculosis* growth was not inhibited (Fig. 3.3.1). In contrast, decreased bacterial growth was observed with 2.5-10% (v/v) DMSO 2 days after

addition of resazurin. We therefore assessed the kinetics of inhibition at 2.5, 5, and 10% (v/v) DMSO. Bacterial growth in DMSO treated samples were expressed relative to the maximum growth observed at day 2 after the addition of resazurin in untreated samples. Complete bacterial growth inhibition was observed with 5 and 10% (v/v) DMSO (fig 3.3.2). *M. tuberculosis* growth was retarded when treated with 2.5% (v/v) DMSO with increase in percentage growth ranging from approximately 2% at day 2 to a complete restoration of growth by day 5 (fig 3.3.2).

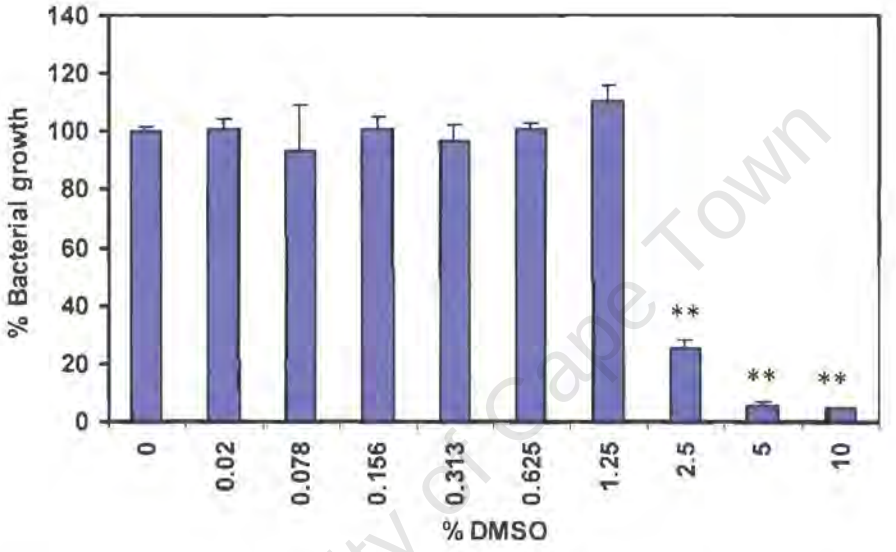


Figure 3.3.1. *In vitro* efficacy of DMSO. DMSO was tested for antimycobacterial activity against *M. tuberculosis* using REMA. *M. tuberculosis* was incubated with DMSO at a concentarion ranging from 0-10% (v/v) for 6 days. Resazurin was added to samples to a final concentration of 5% (v/v) and incubated further. Plates were read at 570nm and 600nm after 24 hours of adding resazurin. Percentage growth was calculated relative to untreated samples containing medium and *M. tuberculosis*. Values are representative of the mean and standard deviation of quadruplicate measurements ** represents $p<0.01$.

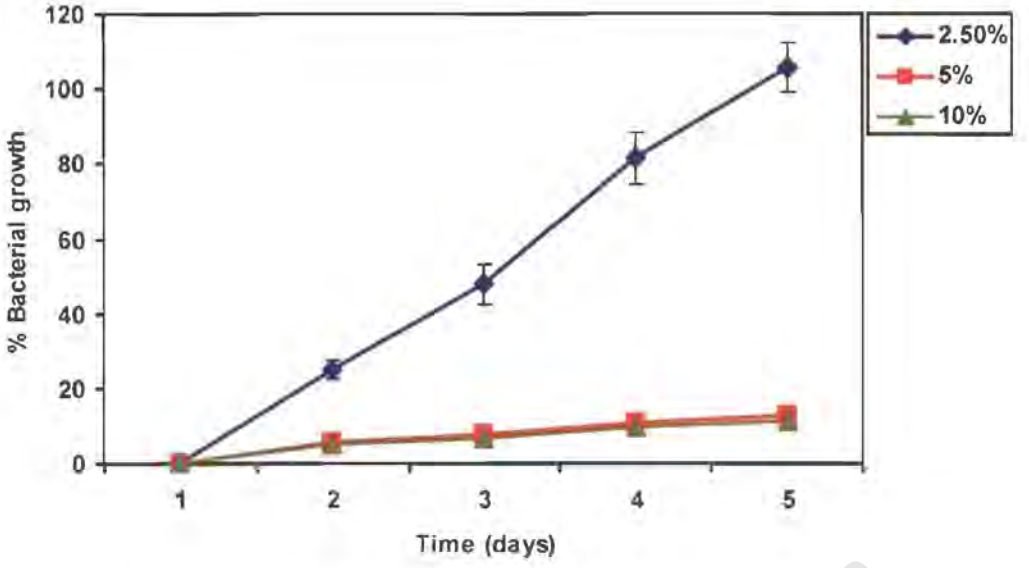


Figure 3.3.2. Kinetics of *M. tuberculosis* growth during treatment with DMSO. Kinetics of *M. tuberculosis* growth was monitored at 24 hours interval for 5 days after adding resazurin. *M. tuberculosis* was incubated with 2.5%, 5%, and 10% (v/v) DMSO for 6 days. Resazurin was added to samples to a final concentration of 5% (v/v) and incubated further. Plates were read at 570nm and 600nm. Percentage growth was calculated relative to day 2 untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements.

3.4. Efficacy of Acetolactate Synthase (ALS) inhibitors against *M. tuberculosis*

In this study we tested the antimycobacterial efficacy of 38 synthetic derivatives from 8 different subclasses of ALS inhibitors using REMA. *M. tuberculosis* was incubated with 10µg/ml of ALS inhibitors for 6 days after which resazurin was added and incubated further. All assays included INH and RIF treatment of samples to indicate growth inhibition and untreated samples to serve as controls for bacterial growth.

3.4.1. Imidazolinone

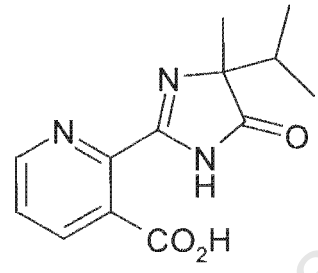


Figure 3.4.1. The chemical structure of RKG11m

RKG11m was the only compound from this ALS inhibitors subclass that was tested for efficacy against *M. tuberculosis*. RKG11m treatment resulted in a small but insignificant inhibition of *M. tuberculosis* (Fig. 3.4.2). In contrast INH and RIF treatment inhibited growth by >85%.

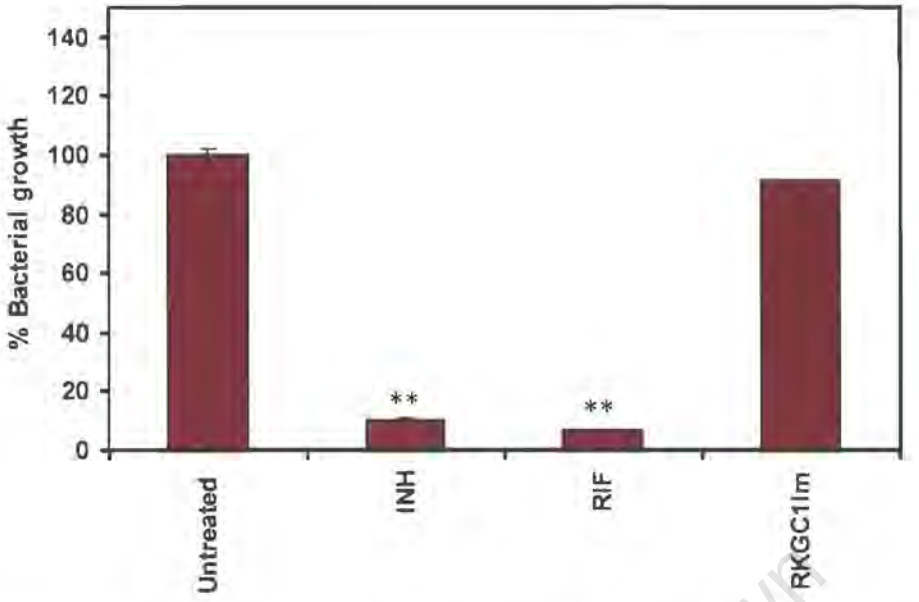


Figure 3.4.2. *In vitro* efficacy of Imidazolinone. RKGC1lm was tested for antimycobacterial activity against *M. tuberculosis* using REMA. *M. tuberculosis* was incubated with RKGC1lm at 10μg/ml for 6 days. Resazurin was added to samples to a final concentration of and incubated further. Plates were read at 570nm and 600nm after 24 hours of adding resazurin. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements. ** represents $p<0.01$.

3.4.2. Phthalamic acids

Four compounds from this ALS inhibitors subclass were tested for efficacy against *M. tuberculosis*. None of the compounds displayed any significant inhibition of *M. tuberculosis* growth (Fig. 3.4.4). In contrast INH and RIF inhibited growth by > 85%.

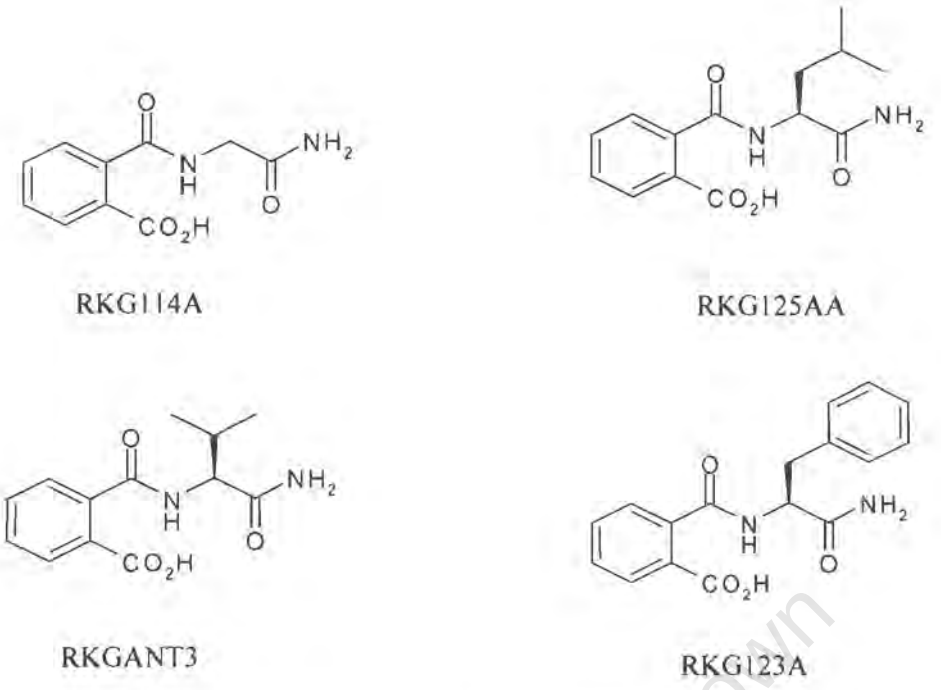


Figure 3.4.3. The chemical structures of Pthalamic acids

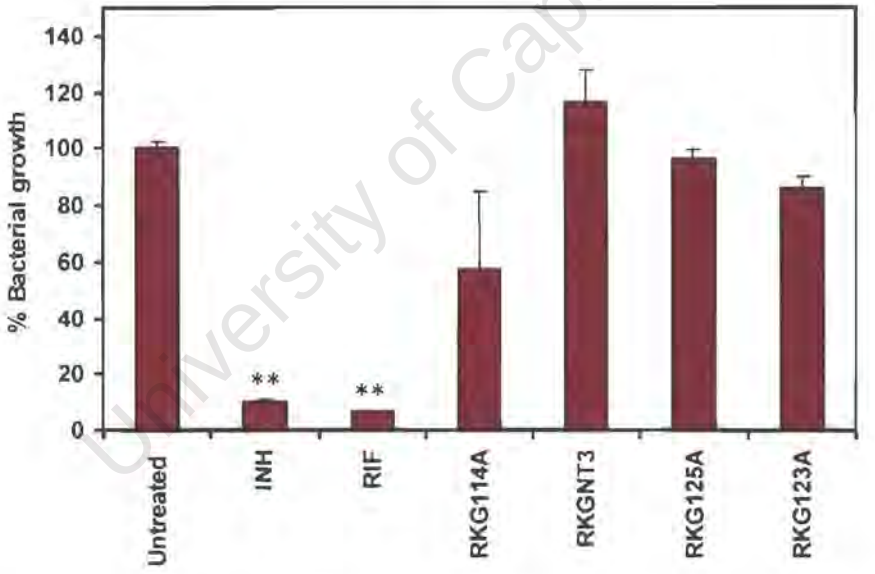


Figure 3.4.4. *In vitro* efficacy of Phthalamic acids. Four compounds from this ALS inhibitors subclass were tested for antimycobacterial activity against *M. tuberculosis* using REMA. *M. tuberculosis* was incubated with phthalamic acids at 10μg/ml for 6 days. Resazurin was added to samples to a final concentration of 5% (v/v) and incubated further. Plates were read at 570nm and 600nm after 24 hours of adding resazurin. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements. ** represents $p<0.01$.

3.4.3. 1,3-dioxo-2H-isoindole-2-acetamides

Three compounds from this ALS inhibitors subclass were tested for efficacy against *M. tuberculosis*. No bacterial growth inhibition was observed when *M. tuberculosis* was treated with RKG119A, RKG133A, and RKG145A (Fig. 3.4.6). In contrast, INH and RIF treatment inhibited growth by >85%.

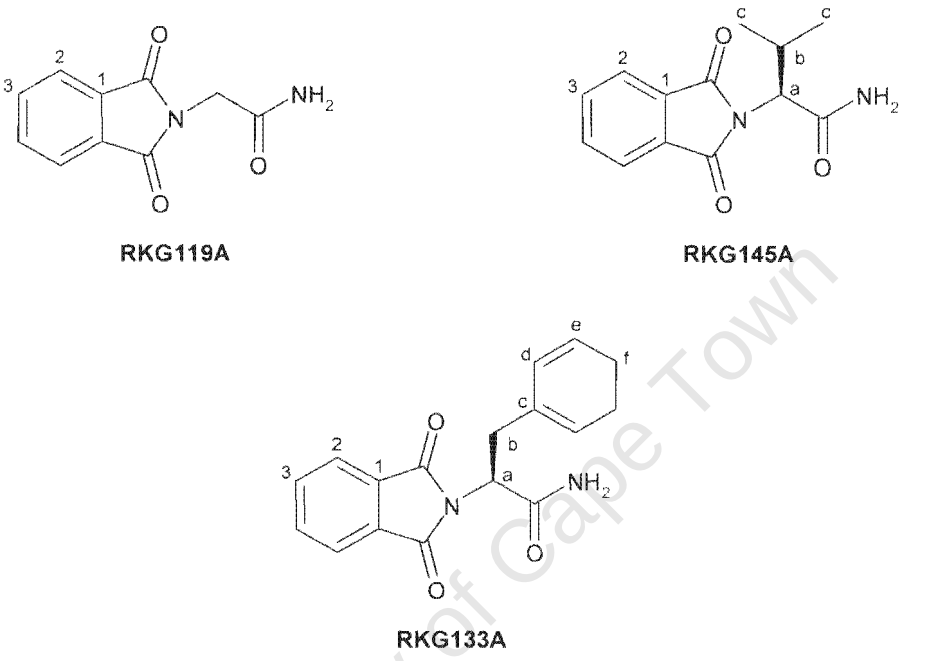


Figure 3.4.5. The chemical structures of 1,3-dioxo-2H-isoindole-2-acetamides

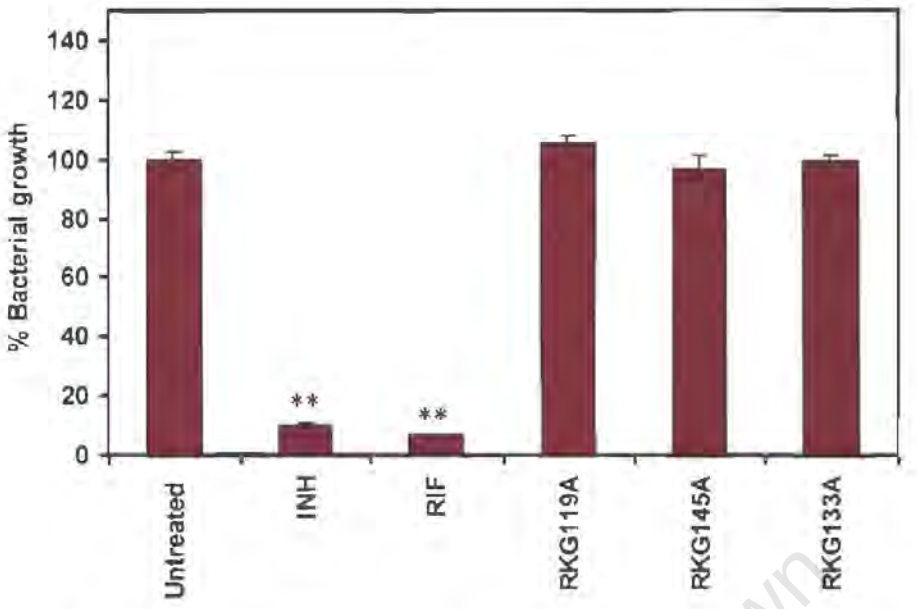


Figure 3.4.6. *In vitro* efficacy of 1,3-dioxo-2H-isoindole-2-acetamides. RKG119A, RKG145A, and RKG133A were tested for antimycobacterial activity against *M. tuberculosis* using REMA. *M. tuberculosis* was incubated with 1,3-dioxo-2H-isoindole-2-acetamides at 10μg/ml for 6 days. Resazurin was added to samples to a final concentration of 5% (v/v) and incubated further. Plates were read at 570nm and 600nm after 24 hours of adding resazurin. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements. ** represents $p<0.01$.

3.4.4. N-cyano-S-methyl-isothioureas

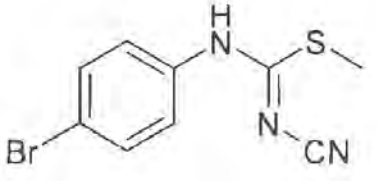


Figure 3.4.7. The chemical structure of RKG136A

RKG136A was the only compound tested from this ALS inhibitors subclass. No antimycobacterial activity was observed following treatment with RKG136A (Fig. 3.4.8). In contrast, INH and RIF treatment inhibited growth by >85%.

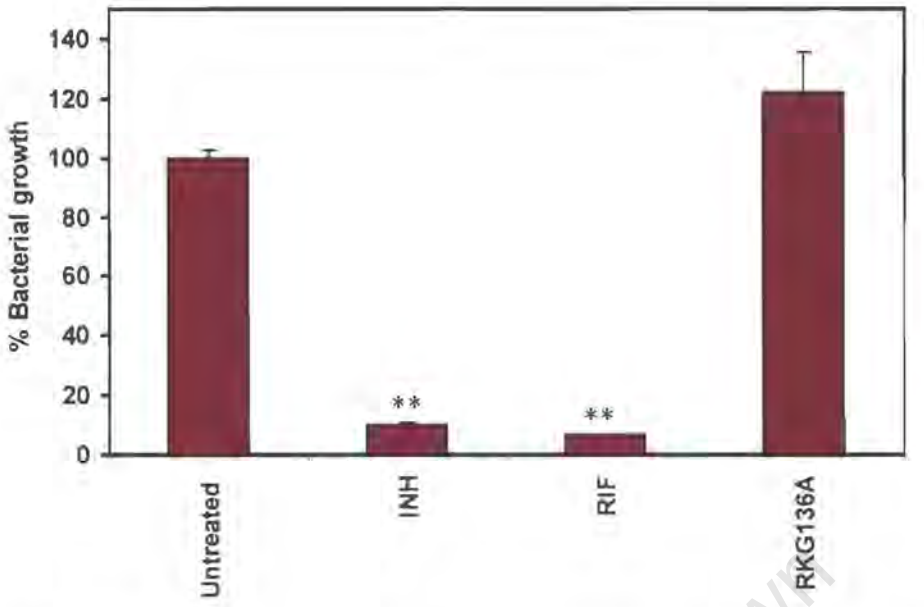
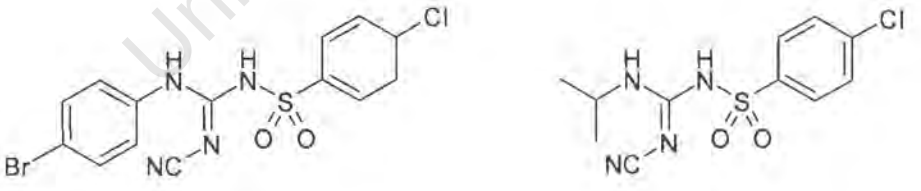


Figure 3.4.8. *In vitro* efficacy of N-cyano-S-mrthyl-isothioureas. RKG136A was tested for antimycobacterial activity against *M. tuberculosis* using REMA. *M. tuberculosis* was incubated with N-cyano-S-mrthyl-isothioureas at 10μg/ml for 6 days. Resazurin was added to samples to a final concentration of 5% (v/v) and incubated further. Plates were read at 570nm and 600nm after 24 hours of adding resazurin. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements. ** represents $p<0.01$.

3.4.5. Sulfonyl cyanoguanidines



RKG139F RKG151A

Figure 3.4.9. The chemical structures of Sulfonyl cyanoguanidines

Two compounds from this ALS inhibitors subclass were tested for efficacy against *M. tuberculosis*. Both RKG139F3 and RKG151A displayed a small but insignificant ($p>0.05$) *M. tuberculosis* growth inhibition (Fig. 3.4.10). In contrast, INH and RIF treatment inhibited growth by >85%.

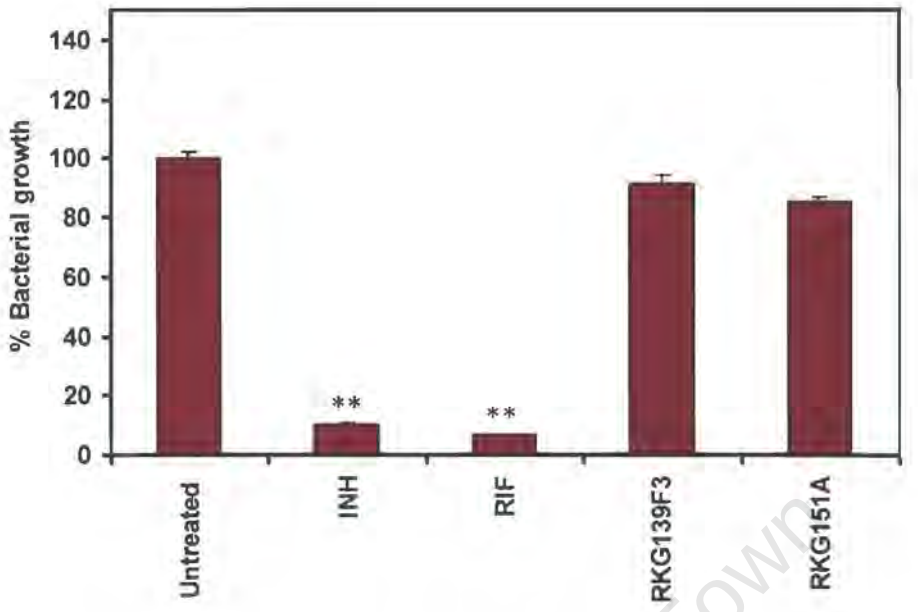


Figure 3.4.10 *In vitro* efficacy of Sulfonyl cyanoguanidines. RKG139F3 and RKG151A were tested for antimycobacterial activity against *M. tuberculosis* using REMA. *M. tuberculosis* was incubated with Sulfonyl cyanoguanidines at 10μg/ml for 6 days. Resazurin was added to samples and incubated further. Plates were read at 570nm and 600nm after 24 hous of adding resazurin. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements. ** represents $p<0.01$.

3.4.6. N-alkyl-N'-cyano-guanidines

RKG143A RKG161A

Figure 3.4.11. The chemical structures of N-alkyl-N'-cyano-guanidines

Two compounds from this ALS inhibitors subclass were tested for efficacy against *M. tuberculosis*. Treatment with RKG143A and RKG161A did not have any inhibitory

effect on *M. tuberculosis* growth (Fig. 3.4.12). In contrast, INH and RIF treatment inhibited growth by >85%.

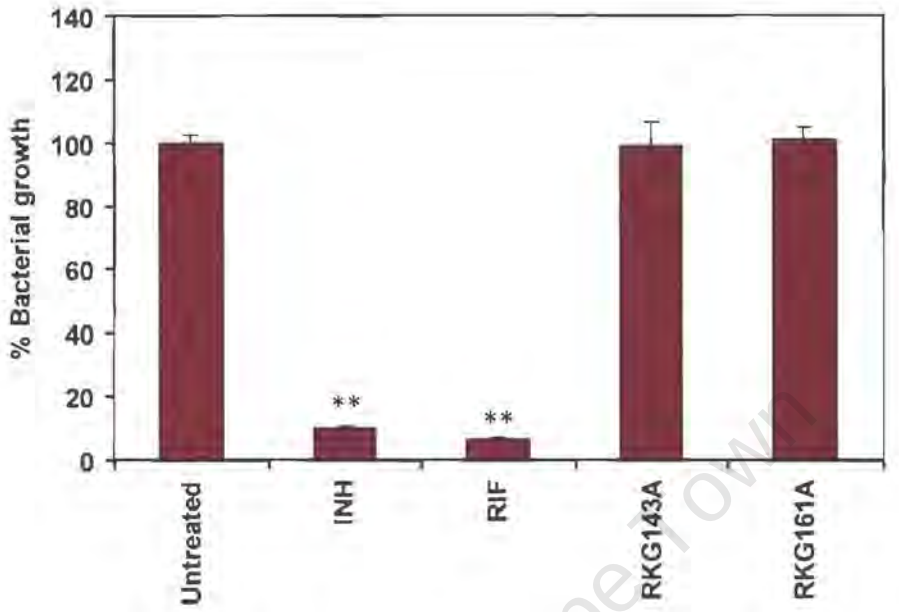
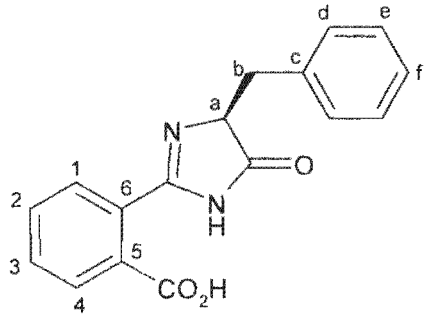


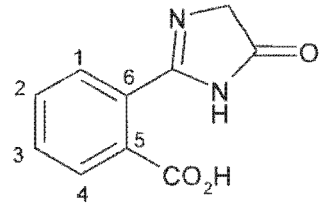
Figure 3.4.12. *In vitro* efficacy of N-alkyl-N'-cyano-guanidines. RKG143A and RKG161A were tested for antimycobacterial activity against *M. tuberculosis* using REMA. *M. tuberculosis* was incubated with N-alkyl-N'-cyano-guanidines at 10μg/ml for 6 days. Resazurin was added to samples to a final concentration and incubated further. Plates were read at 570nm and 600nm after 24 hours of adding resazurin. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements. ** represents $p<0.01$.

3.4.7. Benzene derivatives of imidazolines

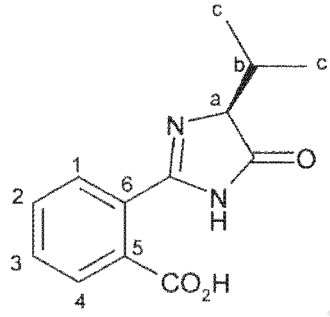
Four compounds from this subclass namely RKGAS4EGLY, RKGAS4ELEU, RKGAS4EPA, and RKGAS4EVAL were tested for efficacy against *M. tuberculosis*. There was no difference in *M. tuberculosis* growth in samples treated with Benzene derivatives of imidazolines when compared to the untreated samples (Fig. 3.4.14). In contrast, INH and RIF treatment inhibited growth by >85%.



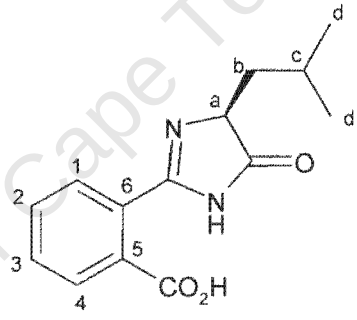
RKGAS4EPA



RKGAS4EGLY



RKGAS4EVAL



RKGAS4EVAL

Figure 3.4.13. The chemical structures of benzene derivatives of imidazolinones

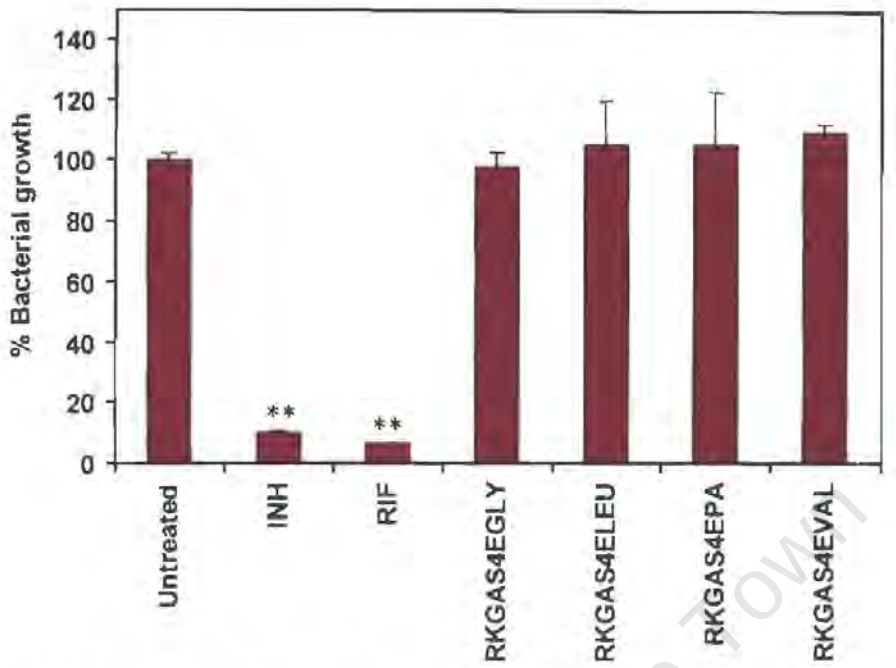
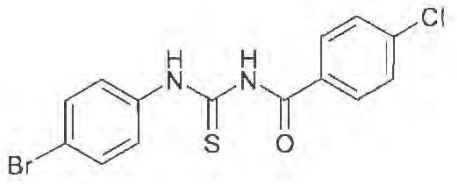


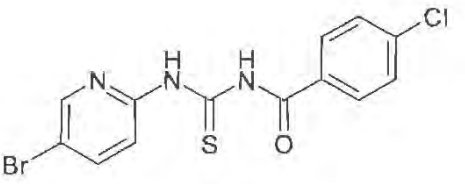
Figure 3.4.14. *In vitro* efficacy of Benzene derivatives of imidazolines. Four compounds from this ALS inhibitors subclass were tested for antimycobacterial activity against *M. tuberculosis* using REMA. *M. tuberculosis* was incubated with Benzene derivatives of imidazolines at 10µg/ml for 6 days. Resazurin was added to samples to a final concentration of 5% (v/v) and incubated further. Plates were read at 570nm and 600nm after 24 hours of adding resazurin. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements. ** represents $p<0.01$.

3.4.8. Acyl thioureas

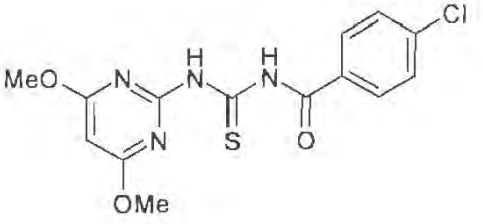
In this study, four compounds were tested from this ALS inhibitors subclass for efficacy against *M. tuberculosis*. The compounds RKG1543 and RKG1542 did not exhibit antibacterial activity against *M .tuberculosis* (Fig 3.4.16). Interestingly, compound RKG1541 inhibited growth significantly by approximately 70%. Compound RKG162A displayed a higher inhibition capacity with antibacterial activity being similar to that observed for INH and RIF.



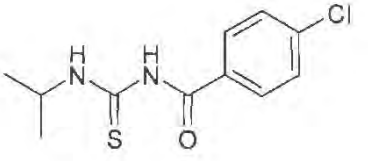
RKG1541



RKG1542



RKG1543



RKG162A

Figure 3.4.15. The chemical structures of N-alkyl-N'-cyano-guanidines

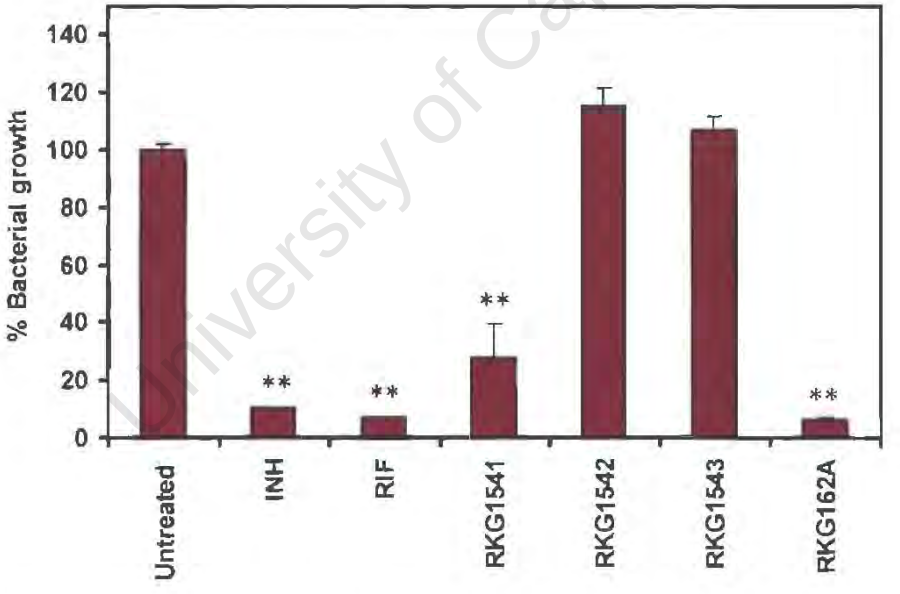
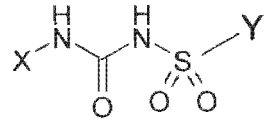


Figure 3.4.16. *In vitro* efficacy of Acyl thioureas. Four compounds from this ALS inhibitors subclass were tested for antimycobacterial activity against *M. tuberculosis* using REMA. *M. tuberculosis* was incubated with Acyl thioureas at 10μg/ml for 6 days. Resazurin was added to samples to a final volume of 5% (v/v) and incubated further. Plates were read at 570nm and 600nm after 24 hours of adding resazurin. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements. ** represents $p<0.01$.

3.4.9. Sulfonyl ureas

Thirteen commercially available sulfonyl ureas (prefix RKGC-) and 4 of their derivatives were tested for efficacy against *M. tuberculosis*. Treatment with compounds from this subclass did not result in mycobactreial growth inhibition (Fig. 3.4.17). In contrast, INH and RIF treatment inhibited growth by >85%.



Compound	X	Y
RKG1491	C_6H_4Br	C_6H_4Cl
RKG1493A	C_2H_7	C_6H_4Cl
RKG1531	$C_4H_3N_2(MeO)_2$	C_6H_4Cl
RKG1532	$C_5H_3N_2MeO$	C_6H_4Cl
RKG1533PC	$C_6H_8N_2$	C_6H_4Cl
RKGC2Be	$C_4H_3N_2(MeO)_2$	C_8H_6OMeO
RKGC3Ch	C_4H_6MeO	C_6H_4Cl
RKGC4Ci	$C_2H_3N_3(MeO)_2$	$C_8H_{10}OMeO$
RKGC5Et	$C_4H_3N_2(MeO)_2$	C_6H_4OEtO
RKGC6Me	C_4H_6MeO	C_7H_4OMeO
RKGC7Pri	$C_6H_5O_2F_4$	C_7H_4OMeO
RKGC8Pro	C_4H_6MeO	$C_9H_8F_3$
RKGC9Py	$C_4H_3N_2(MeO)_2$	$C_5H_4N_2EtO$
RKG10Ri	$C_4H_3N_2(MeO)_2$	$C_7H_9NSO_2$
RKG11Tria	C_4H_6MeO	C_8H_8ClO
RKGC12Th	C_4H_6MeO	C_7H_4OMeO
RKGC13Trib	C_4H_6MeO	C_7H_4OMeO
RKGC14Trif	$C_5H_7N_4F_3Me_2$	C_7H_4OMeO

Table 3.4.1. The chemical structures of sulfonyl ureas

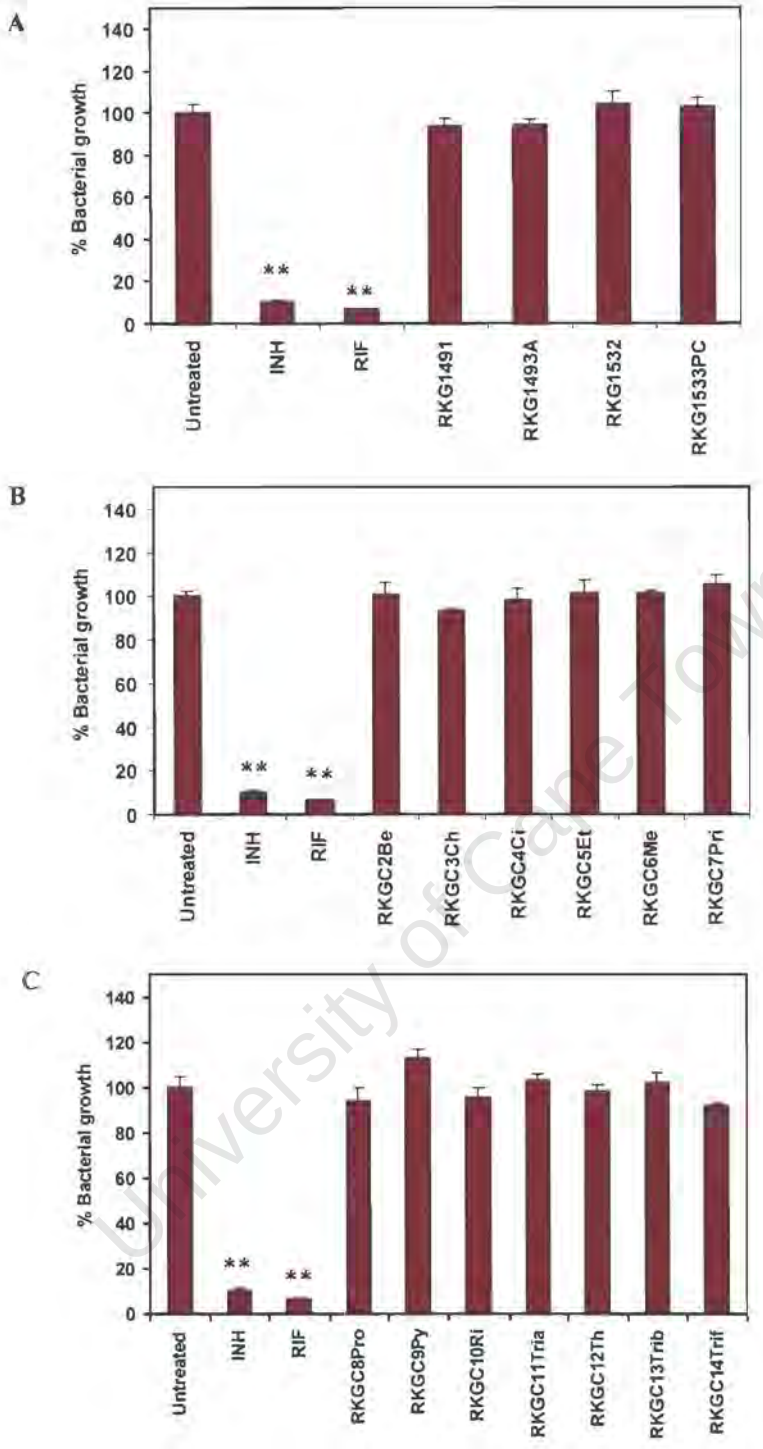


Figure 3.4.17. *In vitro* efficacy of Sulfonyl ureas. Seventeen compounds from this ALS inhibitors subclass were tested for antimycobacterial activity against *M. tuberculosis* using REMA. *M. tuberculosis* was incubated with Sulfonyl ureas at 10μg/ml for 6 days. Resazurin was added to samples to a final concentration of 5% (v/v) and incubated further. Plates were read at 570nm and 600nm after 24 hours of adding resazurin. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements. ** represent $p<0.01$)

3.5. Kinetics of *M. tuberculosis* growth following treatment with Acetolactate Synthase inhibitors

RKG162A and RKG1541 were found to exert antibacterial activity against *M. tuberculosis* (see section 3.4). To further assess the antibacterial properties of these compounds, kinetic inhibition studies against *M tuberculosis* were performed. Mycobacterial growth was monitored for 5 days and expressed relative to growth obtained on day 2 of untreated samples. There was a significant difference ($p<0.05$) between the untreated samples and samples treated with RKG162A and RKG1541 comparatively. The extent of mycobacterial growth inhibition was similar for RKG162A and RKG1541 on day 1, 3, 4, and 5. However, on day 2 RKG162A inhibited *M. tuberculosis* growth more ($p<0.05$) when compared to RKG1541 indicating a more rapid inhibitory effect of RKG162A. This effect appeared to be transient as subsequent inhibitory activity were similar. The lack of colour change confirmed the absence of actively growing *M. tuberculosis*. These compounds were thus recorded as highly active against M. *tuberculosis*.

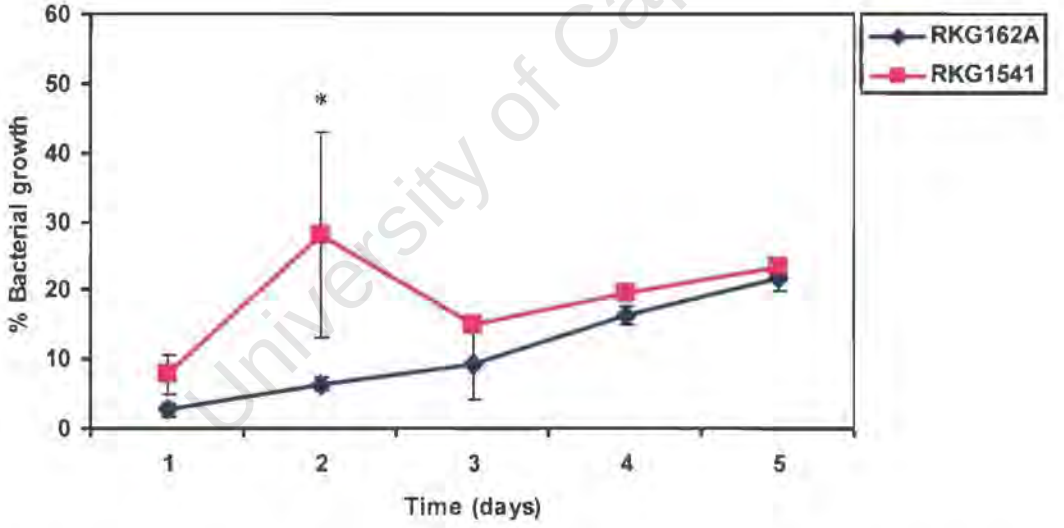


Figure 3.5. Kinetics of *M. tuberculosis* growth during treatment with Acetolactate Synthase inhibitors. Kinetics of *M. tuberculosis* growth was monitored at 24 hours interval for 5 days after adding resazurin. *M. tuberculosis* was incubated with RKG162A and RKG1541 at 10µg/ml for 6 days. Resazurin was added to samples to a final concentration of 5% (v/v) and incubated further. Plates were read at 570nm and 600nm. Percentage growth was calculated relative to day 2 untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements. * represents $p<0.05$.

3.6. Efficacy of Cysteine Protease inhibitors against *M. tuberculosis*

In this study we tested the antimycobacterial efficacy of 35 synthetic derivatives from 2 subclasses of cysteine protease inhibitors against *M. tuberculosis* using REMA. *M. tuberculosis* was incubated with 10μg/ml of cysteine protease inhibitors for 6 days after which resazurin was added and incubated further. All assays included INH and RIF treatment of samples to indicate growth inhibition and untreated samples to serve as controls for bacterial growth.

3.6.1. Isatin-derived cysteine protease inhibitors

3.6.1.1. Isatin and cyclic amine thiosemicarbazones

Eleven derivatives from this subclass were tested for efficacy against *M. tuberculosis*. AXE1, AXE3, AXE4, AXE5, and AXE29 inhibited *M. tuberculosis* growth by 90.4%, 91.1%, 90%, 94.1%, 95.2% respectively (fig 3.6.2A). The absence of actively growing bacilli was confirmed by the lack of colour change. No growth inhibition was observed with treatment using AXE9, AXE10, and AXE11 (Fig. 3.6.2B). Interestingly, treatment with AXE17 enhanced bacterial growth significantly ($p<0.05$) suggesting that it could be used by bacilli as a growth supplement (Fig. 3.6.2B). In contrast to the previous five compounds who recorded inhibition of activity of >90%, inhibition activity of AXE2 was significantly less ($p<0.05$) with 73.1%. INH and RIF treatment inhibited growth by >85%.

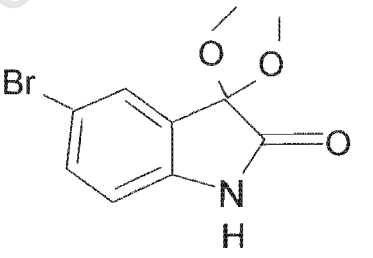
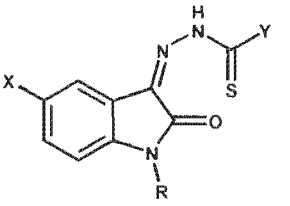


Figure 3.6.1 The chemical structure of AXE29



Compound	X	Y	R
AXE1	H	SMe	Me
AXE2	H	C_4H_9N	Me
AXE3	H	CH2(CH2)4NH	Me
AXE4	H	C_4H_9N	Me
AXE5	H	$C_5H_{12}N_2$	Me
AXE9	Br	SMe	H
AXE10	Cl	SMe	H
AXE11	F	SMe	H
AXE12	NO_2	SMe	H
AXE17	Me	SMe	H

Table 3.4.2. The chemical structures of Isatin and cyclic amine thiosermicarbazones

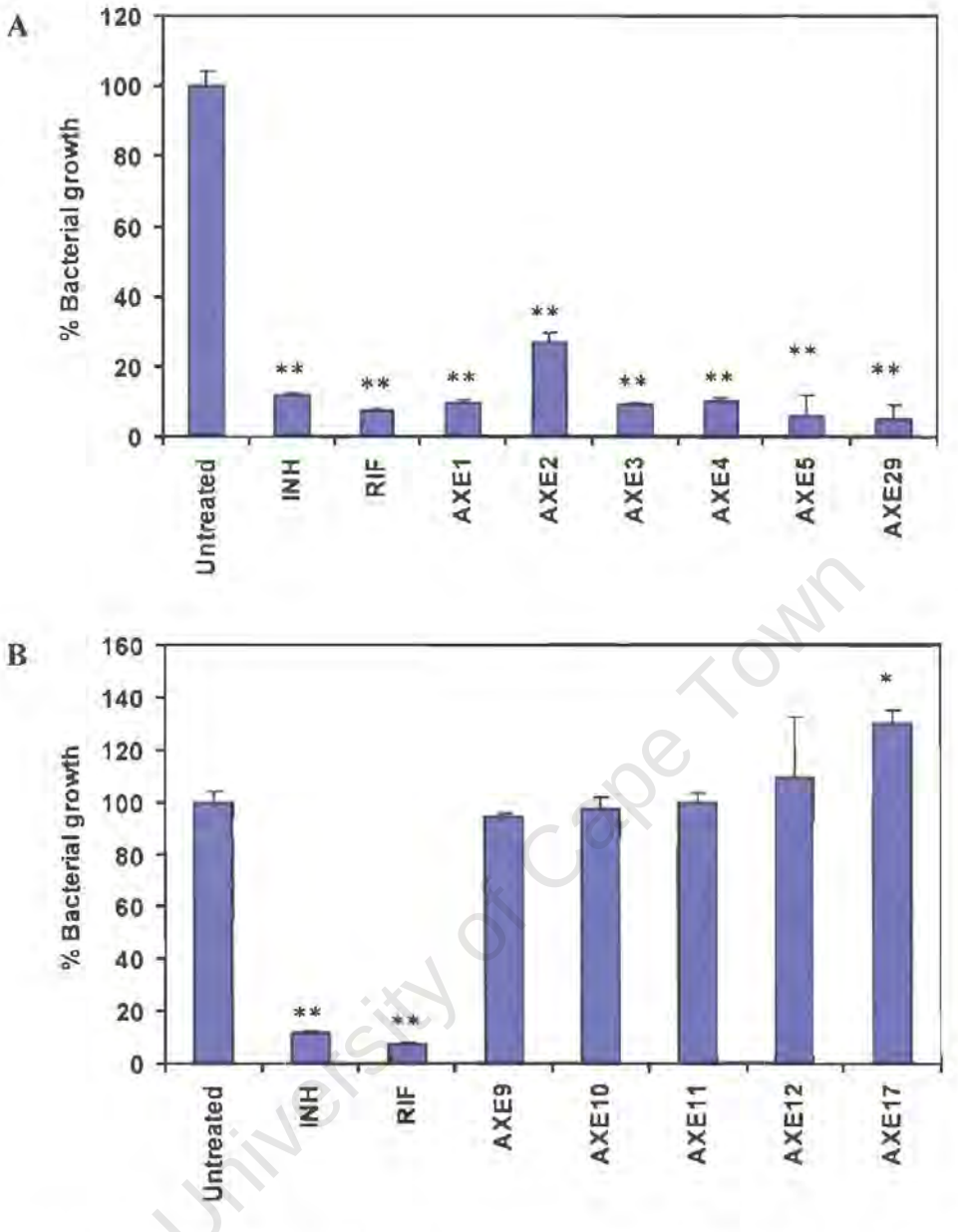
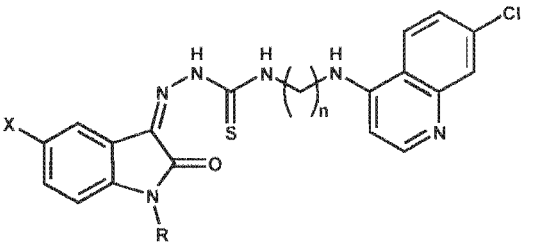


Figure 3.6.2 *In vitro* efficacy of Isatin and cyclic amine thiosermicarbazones. Eleven compounds from this Cysteine Protease inhibitors subclass were tested for antimycobacterial activity against *M. tuberculosis* using REMA. *M. tuberculosis* was incubated with Isatin and cyclic amine thiosermicarbazones at 10μg/ml for 6 days. Resazurin was added to samples to a final concentration of 5% (v/v) and incubated further. Plates were read at 570nm and 600nm after 24 hours of adding resazurin. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements.

3.6.1.2. 4-aminoquinolines

In this study twenty-four derivatives from this subclass were tested for efficacy against *M. tuberculosis*. No bacterial growth inhibition was observed when *M. tuberculosis* was treated with AXE21, AXE22, AXE25, AXE27, AXE28, AXE30, AXE32, and AXE33 (Fig. 3.6.3.B-D). Treatment with AXE35, AXE36 and AXE37 inhibited bacterial growth significantly ($p<0.05$) (Fig. 3.6.3C&D). AXE6, AXE13, AXE14, AXE15, AXE18, AXE20, AXE23, and AXE26 enhanced *M. tuberculosis* growth. However, treatment with AXE18 inhibited growth significantly (Fig. 3.6.3 A&C). INH and RIF treatment inhibited growth by >85%.



Compound	X	R	n
AXE6	H	Me	2
AXE13	Br	H	2
AXE14	Cl	H	2
AXE15	F	H	2
AXE16	NO_2	H	2
AXE18	Me	H	2
AXE19	Me	H	4
AXE20	Me	H	6
AXE21	Br	H	2
AXE22	Cl	H	4
AXE23	F	H	4
AXE24	NO_2	H	4
AXE25	Br	H	6
AXE26	Cl	H	6
AXE27	F	H	6
AXE28	NO_2	H	6
AXE30	Br	H	3
AXE31	Cl	H	3
AXE32	F	H	3
AXE33	CH_3	H	3
AXE34	NO_2	H	3
AXE35	H	CH_3	3
AXE36	H	CH_3	4
AXE37	H	CH_3	6

Table 3.4.3. The chemical structures of 4-aminoquinoline isatin-derived thiosermicarbazones

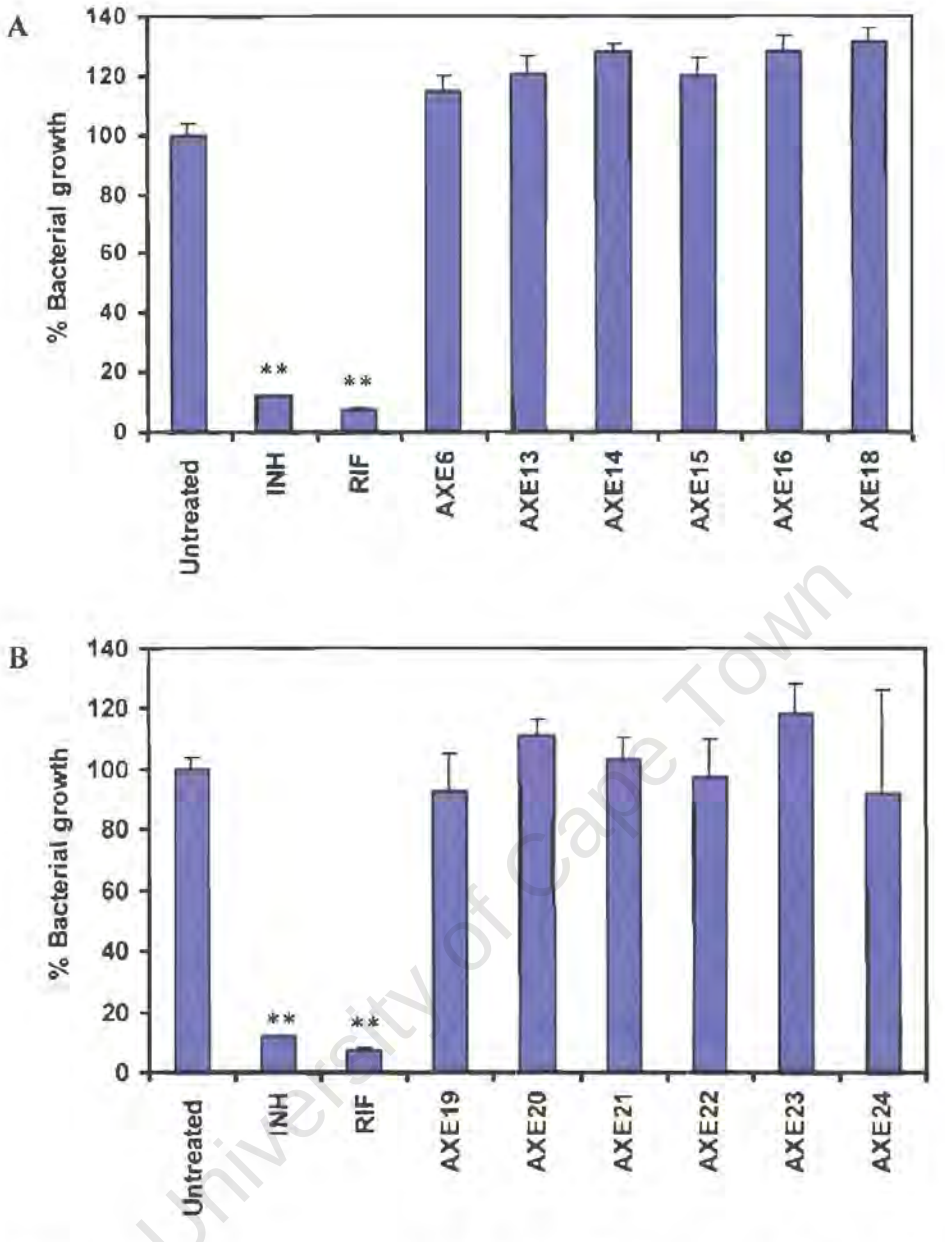


Figure 3.6.3 (...continued on next page). *In vitro* efficacy of Isatin and cyclic amine thiosemicarbazones. Twenty four compounds from this Cysteine Protease inhibitors subclass were tested for antimycobacterial activity against *M. tuberculosis* using REMA. M. tuberculosis was incubated with Isatin and cyclic amine thiosemicarbazones at 10μg/ml for 6 days. Resazurin was added to samples to a final concentration of 5% (v/v) and incubated further. Plates were read at 570nm and 600nm after 24 hours of adding resazurin. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements. ** represents $p<0.01$.

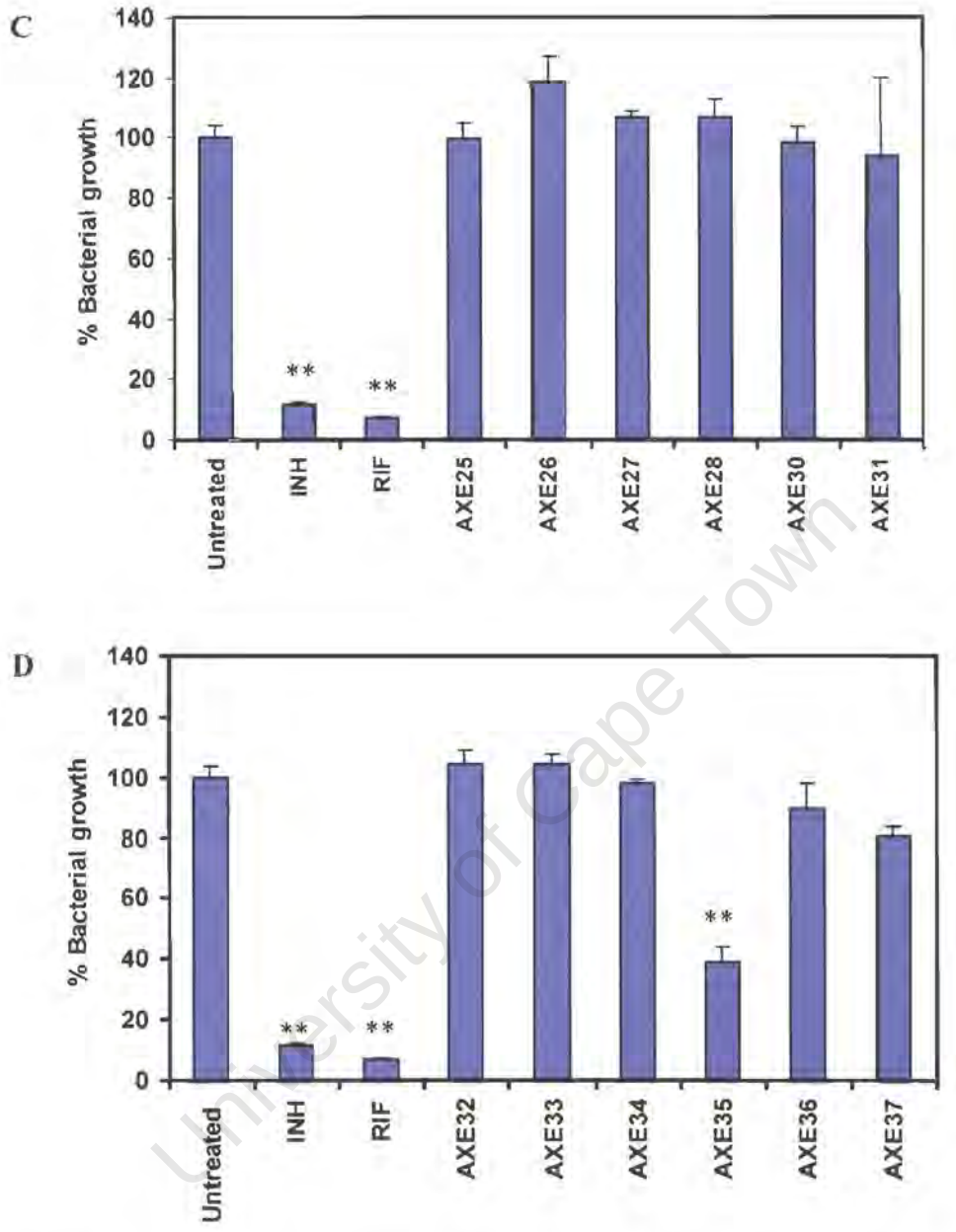


Figure 3.6.3 *In vitro* efficacy of *Isatin* and cyclic amine thiosermicarbazones. Twenty four compounds from this Cysteine Protease inhibitors subclass were tested for antimycobacterial activity against *M. tuberculosis* using REMA. *M. tuberculosis* was incubated with Isatin and cyclic amine thiosermicarbazones at 10μg/ml for 6 days. Resazurin was added to samples to a final concentration of 5% (v/v) and incubated further. Plates were read at 570nm and 600nm after 24 hours of adding resazurin. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements. ** represents $p<0.01$.

3.6.2. Peptidyl cysteine protease inhibitors

Seven derivatives from this subclass were tested for efficacy against *M. tuberculosis*. Of these compounds only NAT47 showed significant ($p<0.05$) inhibition of mycobacterial growth (Fig. 3.6.4). Relative to INH and RIF treatment which inhibited growth by >85%, NAT47 inhibited growth by approximately 50%. Therefore relative to INH and RIF, NAT47 had a significantly lower inhibitory potential.

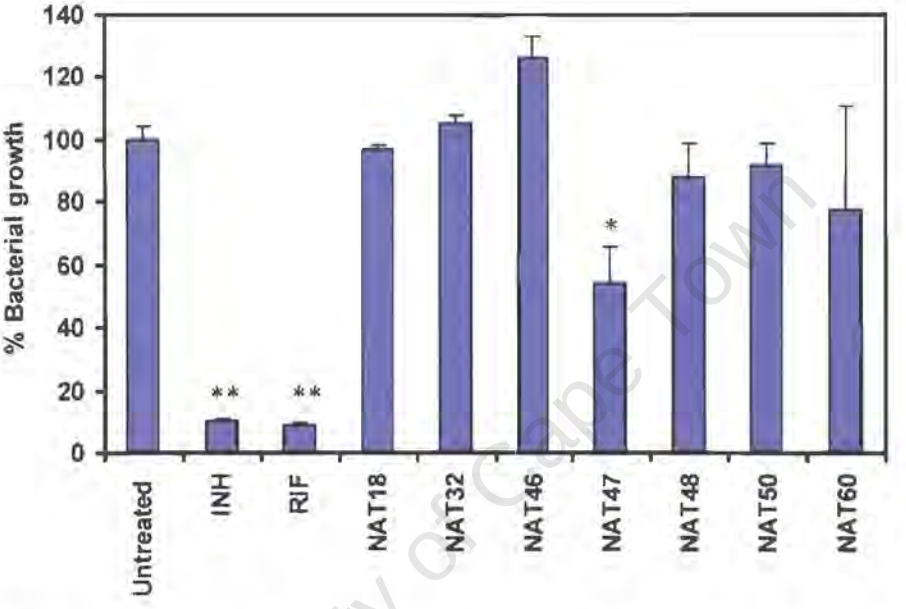
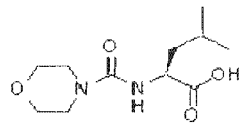
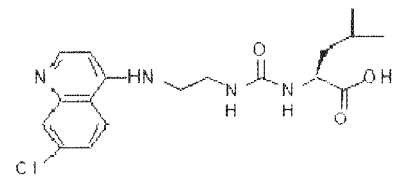


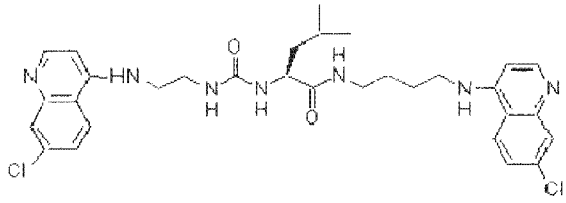
Figure 3.6.4. *In vitro* efficacy Peptidyl cysteine protease inhibitors. Seven compounds from this Cysteine Protease inhibitors subclass were tested for antimycobacterial activity against *M. tuberculosis* using REMA. *M. tuberculosis* was incubated with Peptidyl cysteine protease inhibitors at 10µg/ml for 6 days. Resazurin was added to samples to a final concentration of 5% (v/v) and incubated further. Plates were read at 570nm and 600nm after 24 hours of adding resazurin. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements. ** represents $p<0.01$ and * represents $p<0.05$.



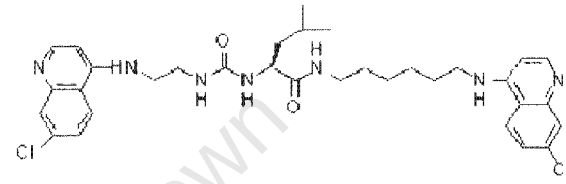
NAT 18



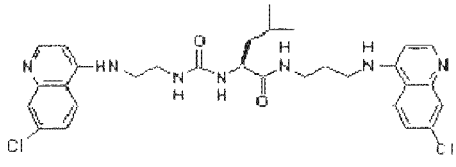
NAT 32



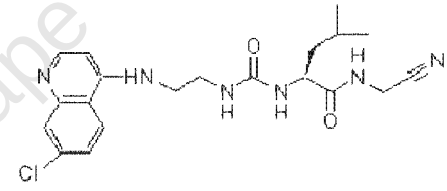
NAT 46



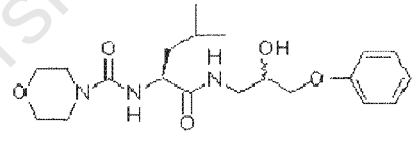
NAT 47



NAT 48



NAT 50



NAT 60

Figure 3.6.5. The chemical structures of peptidyl cysteine protease inhibitors

3.7. Kinetics of *M. tuberculosis* growth following treatment with Cysteine Protease inhibitors.

Seven cysteine protease inhibitors were found to exert antibacterial activity against *M. tuberculosis* (see section 3.6). To further assess the antibacterial properties of these compounds, kinetic inhibition studies against *M tuberculosis* were performed. Mycobacterial growth was monitored for 5 days and expressed relative to growth obtained on day 2 of untreated samples.

3.7.1. Isatin-derived cysteine protease inhibitors

3.7.1.1. Isatin and cyclic amine thiosermicarbazones

In this study 6 derivatives were further assesses for kinetic inhibition of *M tuberculosis* growth. All derivatives inhibited mycobacterial growth on Day 1 (>70%) confirming our previous observation (section 3.6) (Fig. 3.7.1). The kinetics of inhibition over 5 days was found to be different for the respective compounds. Growth inhibition by bacilli was maintained for the duration of the experiment when treated with AXE4, AXE5 and AXE 29 and can be regarded as bactericidal. AXE1 and AXE3 showed growth inhibition of <10% at day 1 and bacterial growth resumed from day 2. At day 5, the *M. tuberculosis* growth was between 30-60% forAXE1 and AXE3 and can therefore be regarded as bacteriostatic. Although AXE2 inhibited growth significantly ($p<0.05$) on day 1, the inhibitory effect was not maintained and by day 4 bacilli growth was fully restored. The bacteriostatic potential of AXE2 was therefore reduced when compared to AXE1, AXE3, and AXE4.

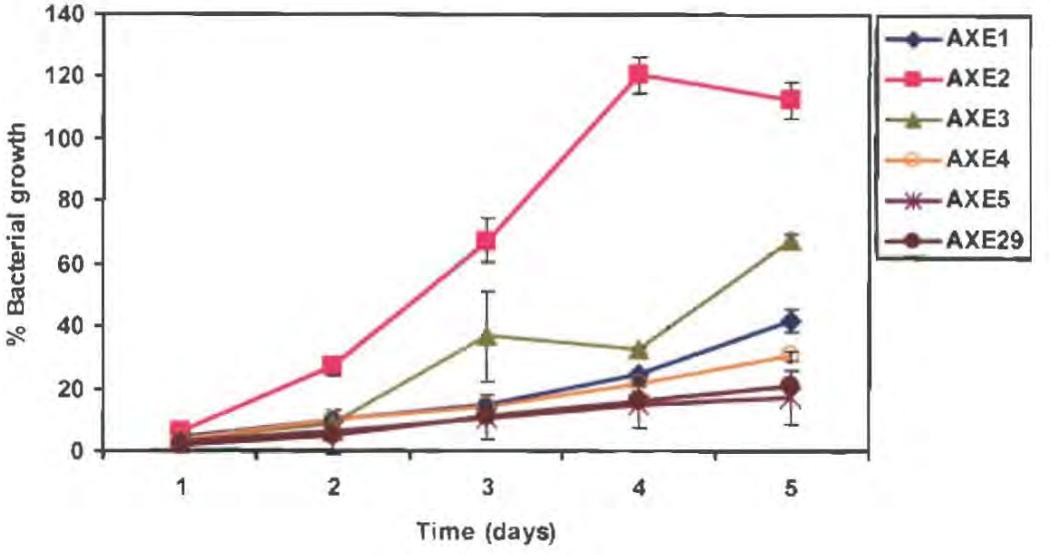


Figure 3.7.1. Kinetics of *M. tuberculosis* growth during treatment with Cysteine Protease inhibitors. Kinetics of *M. tuberculosis* growth was monitored at 24 hours interval for 5 days after adding resazurin. *M. tuberculosis* was incubated with AXE1, AXE2, AXE3, AXE4, AXE5, and AXE29 at 10μg/ml for 6 days. Resazurin was added to samplesto a final concentration of 5% (v/v) and incubated further. Plates were read at 570nm and 600nm. Percentage growth was calculated relative to day 2 untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements.

3.7.1.2. 4-aminoquinolines

In this study one derivative namely AXE35 was further assessed for kinetic inhibition of *M tuberculosis* growth. There was already a significant ($p<0.01$) inhibitory effect noted on day 1 during treatment with AXE35 (Fig. 3.7.2) but the inhibitory effect was not maintained. Bacterial growth after treatment with AXE35 was completely restored at day 4. This compound has therefore bacteriostatic rather than bacteriocidal properties.

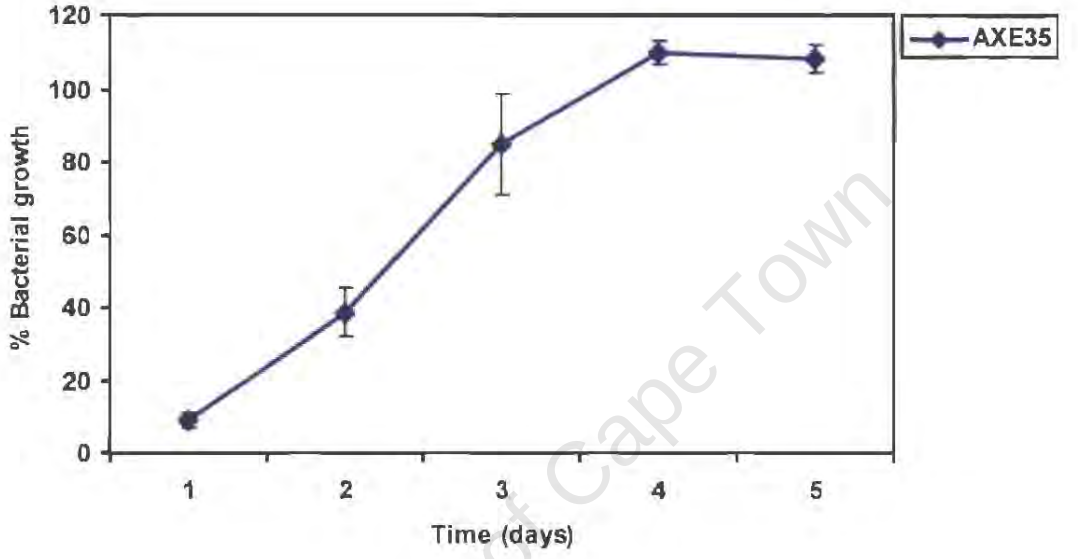


Figure 3.7.2 Kinetics of *M. tuberculosis* growth during treatment with Cysteine Protease inhibitors. Kinetics of *M. tuberculosis* growth was monitored at 24 hours interval for 5 days after adding resazurin. *M. tuberculosis* was incubated with AXE35 at 10µg/ml for 6 days. Resazurin was added to samples to a final concentration of 5% (v/v) and incubated further. Plates were read at 570nm and 600nm. Percentage growth was calculated relative to day 2 untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements.

3.7.2. Peptidyl cysteine protease inhibitors

In this study one derivative was further assessed for kinetic inhibition of *M. tuberculosis* growth based on the findings in section 3.6. Treatment with NAT 47 resulted in retarded bacterial growth, indicative of the bacteriostatic effect of the compound (Fig. 3.7.3). At day 2, treatment with NAT47 had inhibited the bacterial growth by >45%. However, at day 4, *M. tuberculosis* growth was fully restored.

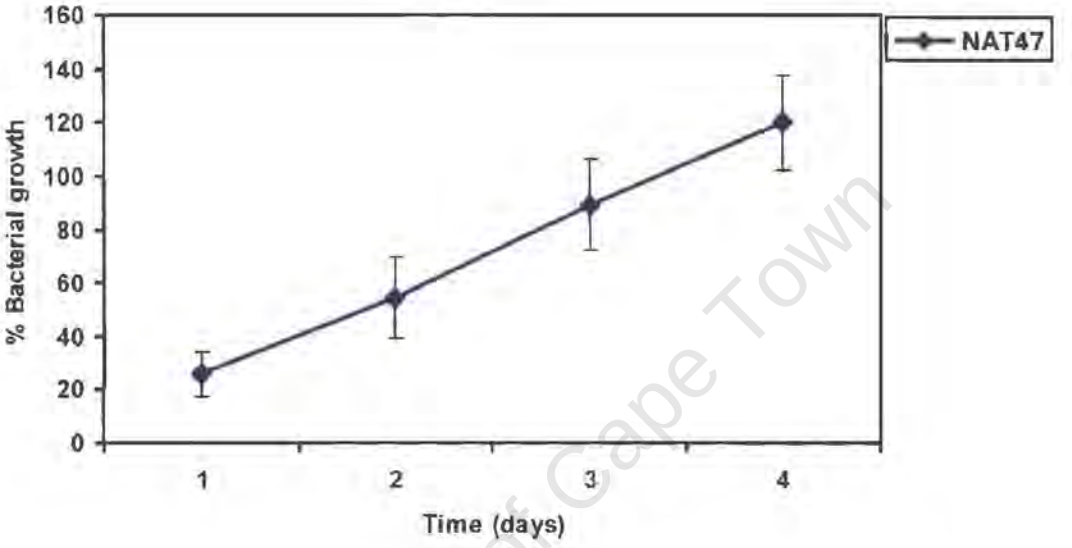


Figure 3.7.3 Kinetics of *M. tuberculosis* growth during treatment with Cysteine Protease inhibitors. Kinetics of *M. tuberculosis* growth was monitored at 24 hours interval for 5 days after adding resazurin. *M. tuberculosis* was incubated with NAT at 10µg/ml for 6 days. Resazurin was added to samples to a final concentration of and incubated further. Plates were read at 570nm and 600nm. Percentage growth was calculated relative to day 2 untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements.

3.8. Efficacy of Thiosemicarbazones

In this study we tested the anti-bacterial efficacy of 25 synthetic derivatives from 2 subclasses of thiosemicarbazones against *M. tuberculosis* using REMA. *M. tuberculosis* was incubated with 10µg/ml of thiosemicarbazones detivatives for 6 days after which resazurin was added and incubated further. All assays included INH and RIF treatment of samples to indicate growth inhibition and untreated samples to serve as controls for bacterial growth.

3.8.1. Ferrocenic Thiosemicarbazones

In this study 18 compounds were assessed for antimycobacterial activity. FM19 and FM48 were the only compounds that showed significant ($p<0.05$) antimycobacterial properties (Fig. 3.8.1). FM19 and FM48 inhibited bacterial growth by >80% compared to INH and RIF which inhibited bacterial growth by >90%.

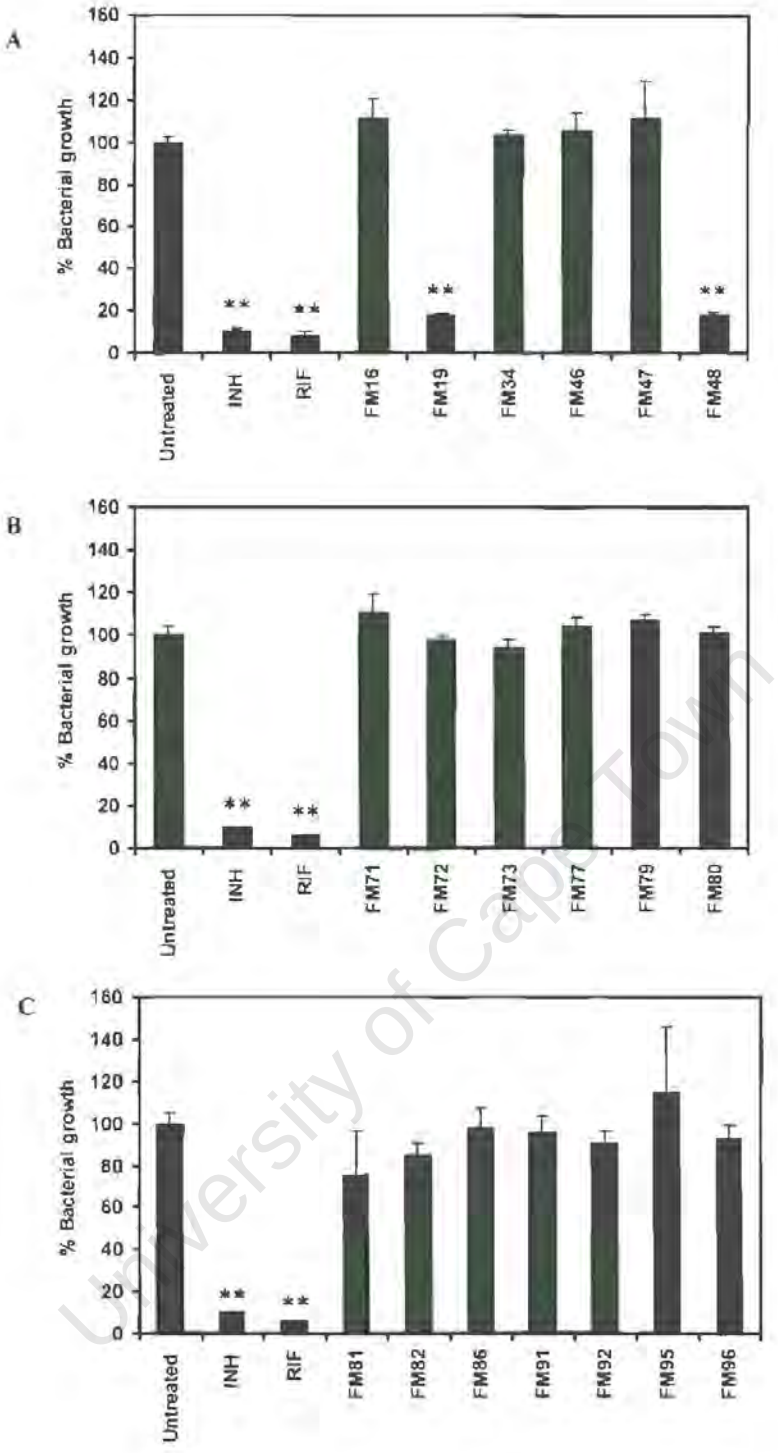
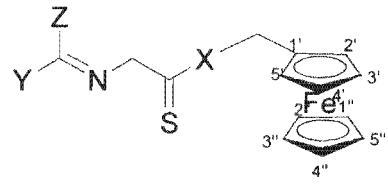


Figure 3.8.1. *In vitro* efficacy of Ferrocenic Thiosemicarbazones. Seventeen compounds from this ALS inhibitors subclass were tested for antimycobacterial activity against *M. tuberculosis* using REMA. *M. tuberculosis* was incubated with Ferrocenic Thiosemicarbazones at 10μg/ml for 6 days. Resazurin was added to samples to a final concentration of 5% (v/v) and incubated further. Plates were read at 570nm and 600nm after 24 hours of adding resazurin. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements. ** represents $p<0.01$.

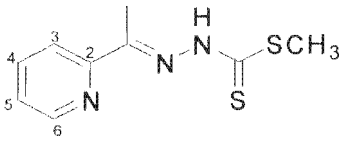


Compound	X	Y	Z
FM16	$C_4H_{10}N_2$	$C_6H_3Cl_2$	CH_3
FM19	$C_4H_{10}N_2$	C_5H_4N	CH_3
FM34	$C_4H_{10}N_2$	C_6H_4Br	CH_3
FM46	N	$C_6H_3Cl_2$	CH_3
FM47	C	C_6H_4Br	CH_3
FM71	N	$C_6H_3(OH)_2$	H
FM72	N	$C_6H_3(OH)_2$	H
FM73	N	C_6H_3OHOMe	H
FM77	N	C_6H_4OH	H
FM79	N	C_6H_4OH	H
FM80	N	$C_{10}H_6OH$	H
FM81	N	C_6H_3OHOMe	H
FM82	N	$C_6H_2(OMe)_3$	H
FM86	N	C_6H_4Br	H
FM91	N	$C_6H_3Cl_2$	H
FM92	N	$C_6H_3NO_2Cl$	H
FM96	N	C_5H_5N	C_5H_5N

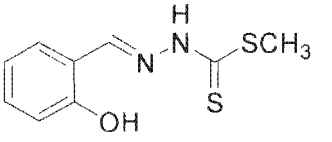
Table 3.4.4 The chemical formulas of ferrocenic thiosemicarbazones

3.8.2. Thiosemicarbazone thioesters

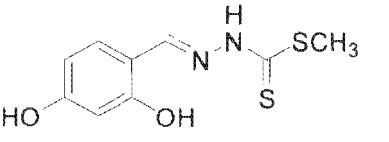
In this study 7 compounds were assessed for antimycobacterial activity. FM04 and FM102 inhibited bacterial growth by approximately 48% and 30% respectively (Fig. 3.8.3). None of the other compounds tested had antimycobacterial activity.



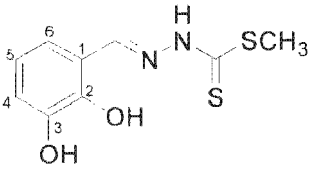
FM04



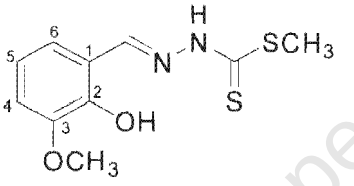
FM52



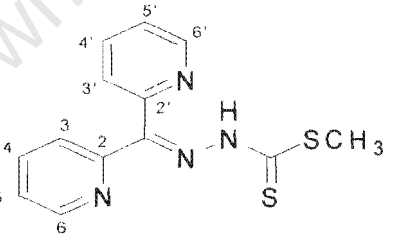
FM61



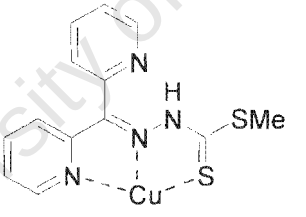
FM60



FM65



FM75



FM102

Figure 3.8.2. The chemical structures of thiosemicarbazones thioesters

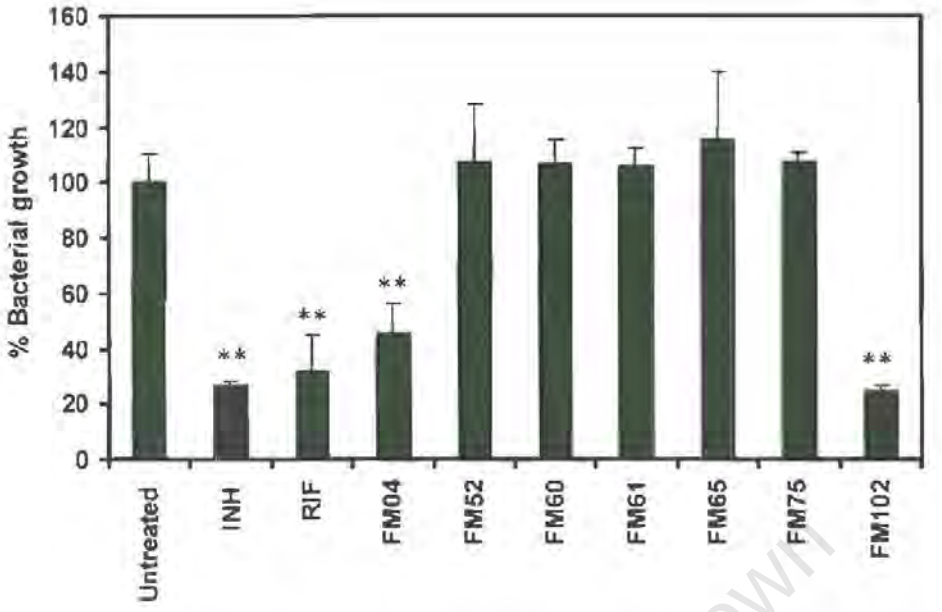


Figure 3.8.3. *In vitro* efficacy of Thiosemicarbazone thioesters. Seventeen compounds from this ALS inhibitors subclass were tested for antimycobacterial activity against *M. tuberculosis* using REMA. *M. tuberculosis* was incubated with Thiosemicarbazone thioesters at 10μg/ml for 6 days. Resazurin was added to samples a final concentration of 5% (v/v) and incubated further. Plates were read at 570nm and 600nm after 24 hours of adding resazurin. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements. ** represents $p<0.01$.

3.9. Kinetics of *M. tuberculosis* growth during treatment with Thiosemicarbazones

Four thiosermicarbazones were found to exert antibacterial activity against *M. tuberculosis*. To further assess the antibacterial properties of these compounds, kinetic inhibition studied against *M tuberculosis* were performed. Mycobacterial growth was monitored for 5 days and expressed relative to growth obtained on day 2 untreated samples.

3.9.1. Ferrocenic Thiosemicarbazones

In this study 2 derivatives were further assesses for kinetic inhibition of *M. tuberculosis* growth. Complete growth inhibition was observed when *M. tuberculosis* was treated with FM19 and FM48 on day 1 (Fig. 3.9.1). Lack of colour change on

samples treated with FM19 and FM48 confirmed this observation. The inhibitory effect was maintained for 5 days and did not recover. These compounds were therefore recorded as bactericidal against *M. tuberculosis*.

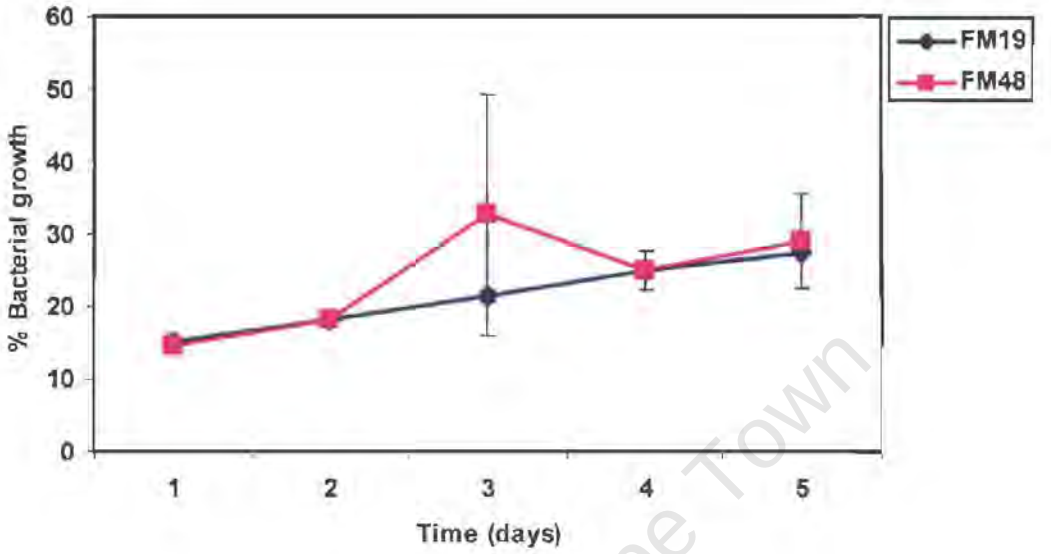


Figure 3.9.1. Kinetics of *M. tuberculosis* growth during treatment with Ferrocenic Thiosemicarbazones. Kinetics of *M. tuberculosis* growth was monitored at 24 hours interval for 5 days after adding resazurin. *M. tuberculosis* was incubated with 10μg/ml of FM19 and FM48 for 6 days. Resazurin was added to samples to a final concentration of 5% (v/v) and incubated further. Plates were read at 570nm and 600nm. Percentage growth was calculated relative to day 2 untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements.

3.9.2. Thiosemicarbazone thioesters

In this study FM04 and FM102 were further assessed for kinetic inhibition of *M. tuberculosis* growth. A retarded bacterial growth was observed with treatment with FM04 and FM102 (Fig. 3.9.2). However, at day 3, >75% of growth occurred and at day 4 *M. tuberculosis* growth was fully restored.

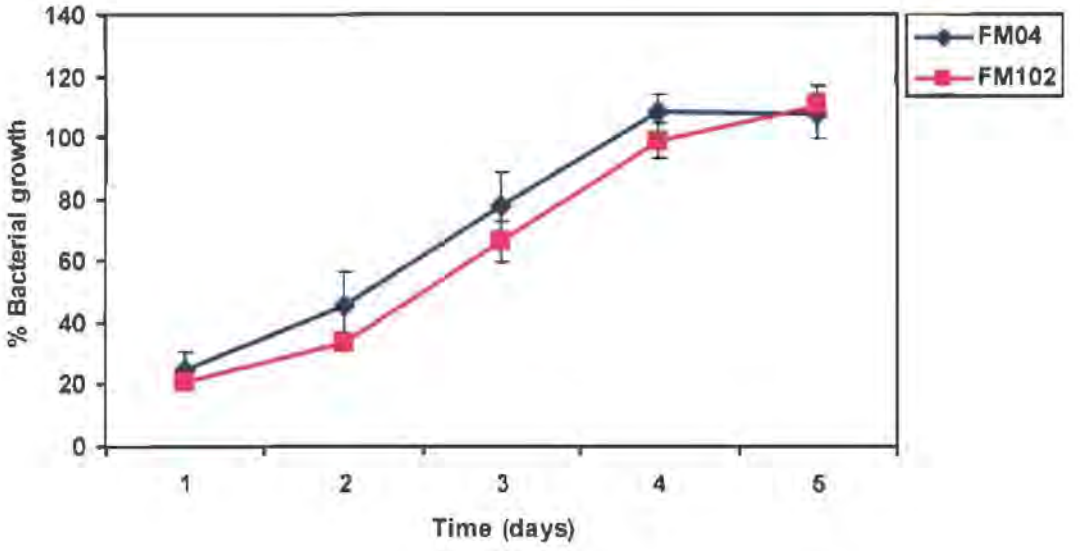


Figure 3.9.2. Kinetics of *M. tuberculosis* growth during treatment with Thiosemicarbazone thioesters. Kinetics of *M. tuberculosis* growth was monitored at 24 hours interval for 5 days after adding resazurin. *M. tuberculosis* was incubated with 10μg/ml of FM04 and FM102 for 6 days. Resazurin was added to samples and incubated further. Plates were read at 570nm and 600nm. Percentage growth was calculated relative to day 2 untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements.

3.10. Efficacy of Thiolactomycins

In this study we tested the anti-bacterial efficacy of 13 synthetic derivatives from this class of thiolactomycins against *M. tuberculosis* using REMA. All assays included INH and RIF treatment of samples to indicate growth inhibition and untreated samples to serve as controls for bacterial growth. No growth inhibition was observed with any of the thiolactomycins tested (Fig. 3.10). In contrast, INH and RIF inhibited growth by >90%. Interestingly, addition of ISA1 showed a significant ($p<0.05$) increase in mycobacterial growth indicating that it could be acting as a growth supplement for *M. tuberculosis*.

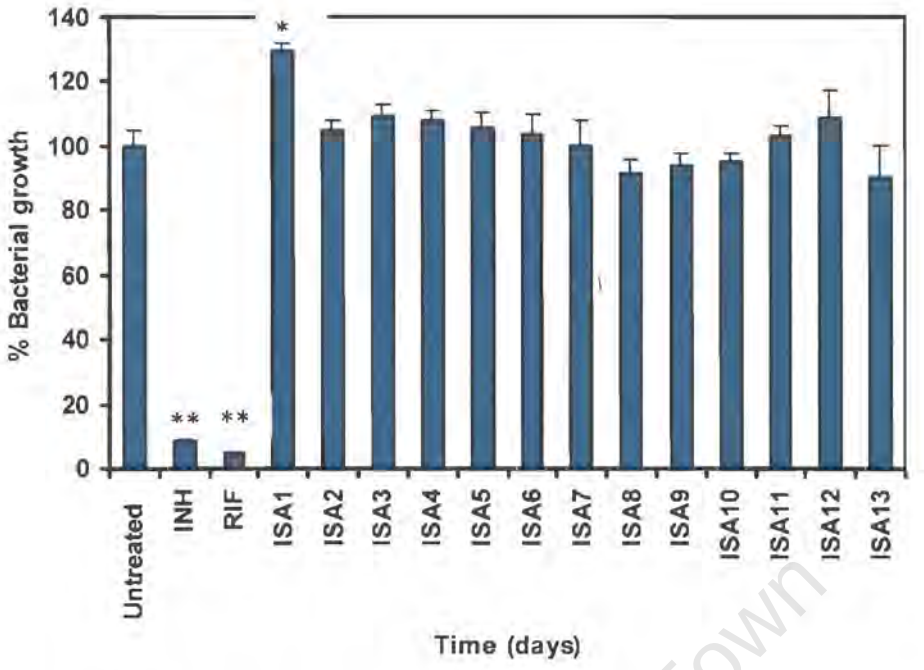
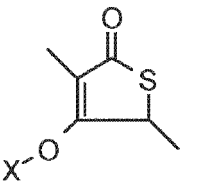


Figure 3.10. *In vitro* efficacy of *Thiolactomycins.* Seventeen thiolactomucins derivatives were tested for antimycobacterial activity against *M. tuberculosis* H37Rv using REMA. *M. tuberculosis* was incubated with thiolactomycins at 10μg/ml for 6 days. Resazurin was added to samples and incubated further. Plates were read at 570nm and 600nm after 24 hours of adding resazurin. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements.* Represents $p<0.05$ and ** $P<0.01$.



Compound	X
ISA1	C_3H_6Br
ISA2	$C_6H_{12}Br$
ISA3	$C_9H_{18}Br$
ISA4	$C_{12}H_{24}Br$
ISA5	C_4H_8Br
ISA6	$C_5H_{10}Br$
ISA7	$C_8H_{16}Br$
ISA8	$C_7H_{17}SN$
ISA9	$C_9H_{21}SN$
ISA10	$C_{10}H_{23}SN$
ISA11	$C_{12}H_{27}SN$
ISA12	$C_{13}H_{29}SN$
ISA13	$C_{16}H_{35}SN$

Table 3.4.5. The chemical structures of Isatin and cyclic amine thiosermicarbazones

3.11. Determination of the Minimum Inhibitory Concentration (MIC) following treatment with synthetic derivatives

Eight derivatives from Acetolactate Synthase inhibitors, Cysteine protease inhibitors, and Thiosemicarbazones inhibited *M. tuberculosis* in the primary screening assay. We therefore investigated the lowest concentration at which these derivatives inhibited *M. tuberculosis* growth using REMA. *M. tuberculosis* was incubated for 6 days with an increasing concentration of compounds. At day 6 resazurin was added and incubated further. The concentrations tested ranged from 0.0195 - 10μg/ml.

3.11.1. The Minimum Inhibitory Concentration of INH

In this study, *M. tuberculosis* was treated with 0-10µg/ml of INH. At day 1, *M. tuberculosis* growth had decreased radically relative to day 0 when treated with >0.039µg/ml (Fig. 3.11.1). The bacterial growth remained almost constant for the duration of treatment. At day 5, an insignificant growth was observed with >0.039µg/ml. Treatment with 0.0195 however, inhibited growth by approximately 50% at day1 and growth was completely restored by day 5. The MIC of isoniazid was therefore recorded as 0.039µg/ml.

3.11.2. Minimum Inhibitory Concentration of RIF

In this study, *M. tuberculosis* was incubated with 0-10µg/ml of rifampicin. Treatment with 0.02µg/ml and 0.039µg/ml of RIF resulted in retarded *M. tuberculosis* growth (Fig. 3.11.2). *M. tuberculosis* growth was completely inhibited when treated with ≥0.078µg/ml. By day 5 approximately 20% of bacilli was actively growing when treated with >0.078µg/ml. Treatment with 0.078µg/ml of RIF showed a slight increase in bacterial growth when compared with treatment with ≥0.156µg/ml of RIF. MIC was therefore recorded as 0.078µg/ml for rifampicin.

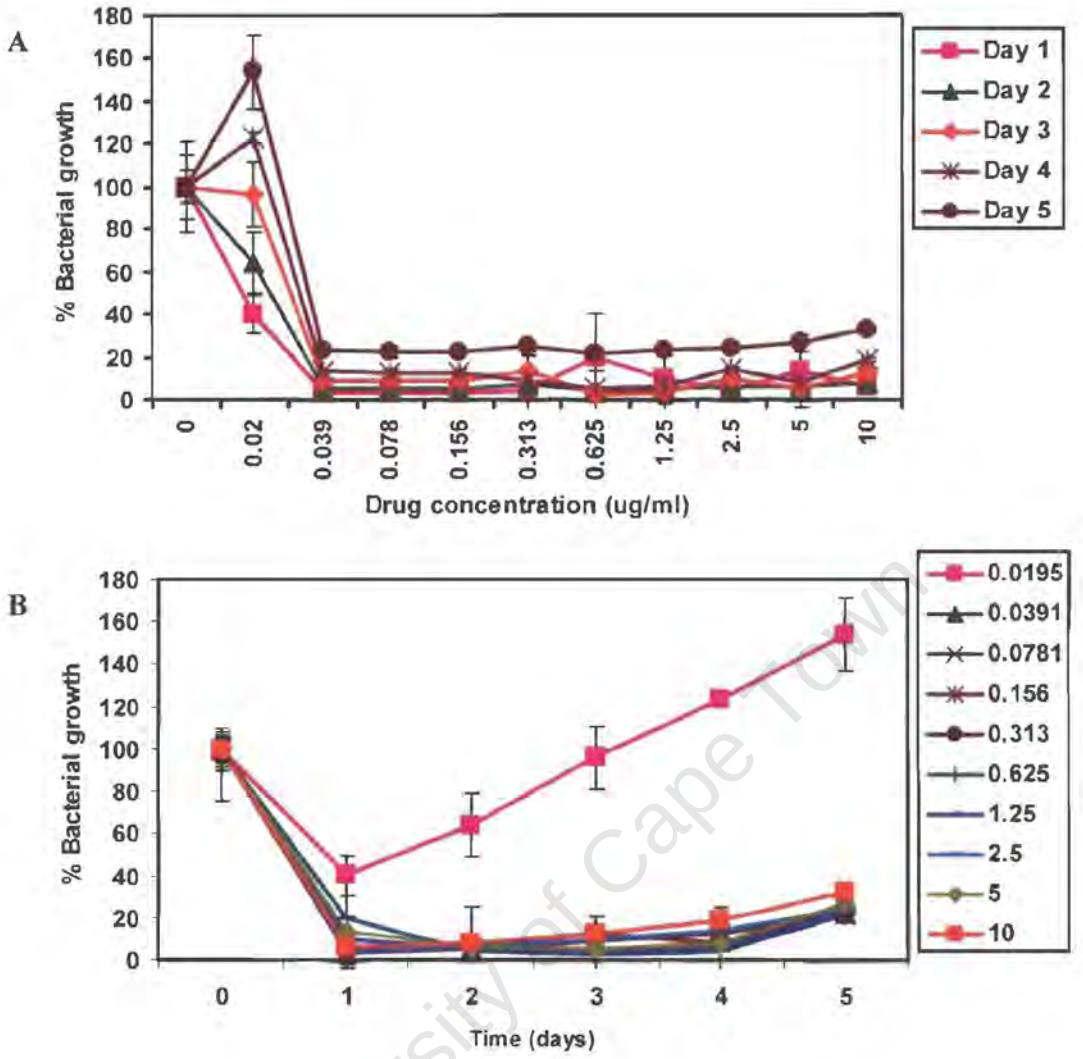


Figure 3.11.1. The Minimum Inhibitory Concentration (A) and kinetics of inhibition (B) of isoniazid. The MIC of INH was determined by incubating *M. tuberculosis* for 6 days with INH at a concentration ranging from 0-10μg/ml. Resazurin was added to samples and incubated further. Plates were read at 570nm and 600nm. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements.

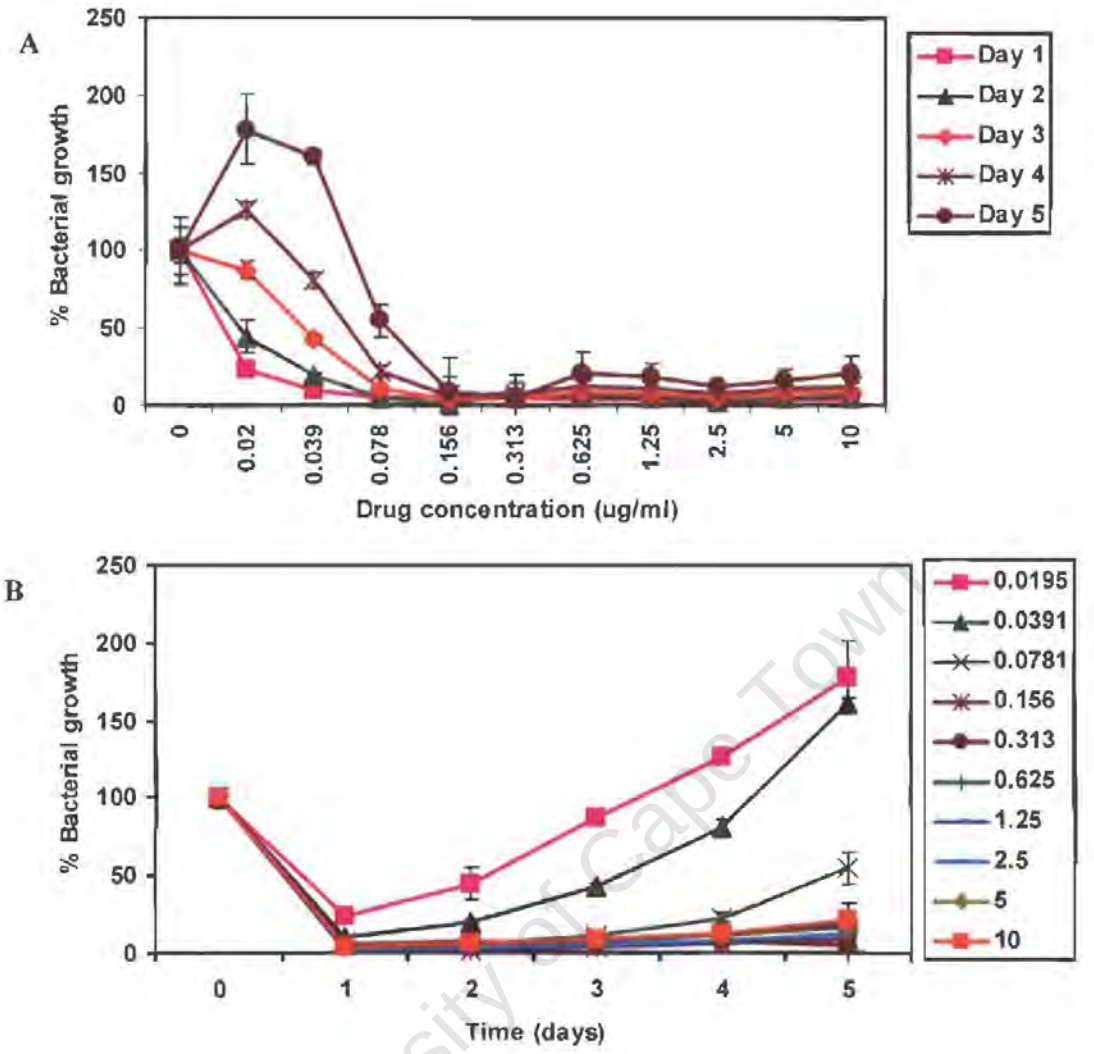


Figure 3.11.2. The Minimum Inhibitory Concentration (A) and kinetics of inhibition (B) of rifampicin. The MIC of RIF was determined by incubating *M. tuberculosis* for 6 days with RIF at a concentration ranging from 0-10µg/ml. Resazurin was added to samples and incubated further. Plates were read at 570nm and 600nm. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium. Values are representative of the mean and standard deviation of quadruplicate measurements.

3.11.3. The Minimum Inhibitory Concentrations of Acetolactate Synthase inhibitors

In this study we assessed RKG162A and RKG1541 for the lowest concentration that inhibited *M. tuberculosis* growth. When *M. tuberculosis* was treated with RKG162A, no growth inhibition was observed with 0-1.25µg/ml of RKG162A (Fig. 3.11.3). The bacilli growth when treated with 2.5 and 5µg/ml had a delayed growth. At day 1, bacterial growth was approximately 30% and 5% respectively. However, at day 5, bacilli growth was completely restored. In contrast, treatment with 10µg/ml resulted in almost complete inhibition of bacilli growth. We therefore recorded 10µg/ml as the MIC of RKG162A.

A greater inhibition capacity was observed with RKG1541 when compared to RKG162A (Fig. 3.11.4). Treatment with 1.25µg/ml of RKG1541 resulted in retarded *M. tuberculosis* growth (Fig. 3.11.4) as opposed to no inhibition observed when *M. tuberculosis* was treated with RKG162A at the same concentration. In samples treated 1.25-10µg/ml of RKG1541, growth was restored by day 5 except for 10µg/ml in which the bacilli growth was at approximately 25% (Fig. 3.11.4). We therefore recorded the MIC of RKG1541 as 10µg/ml.

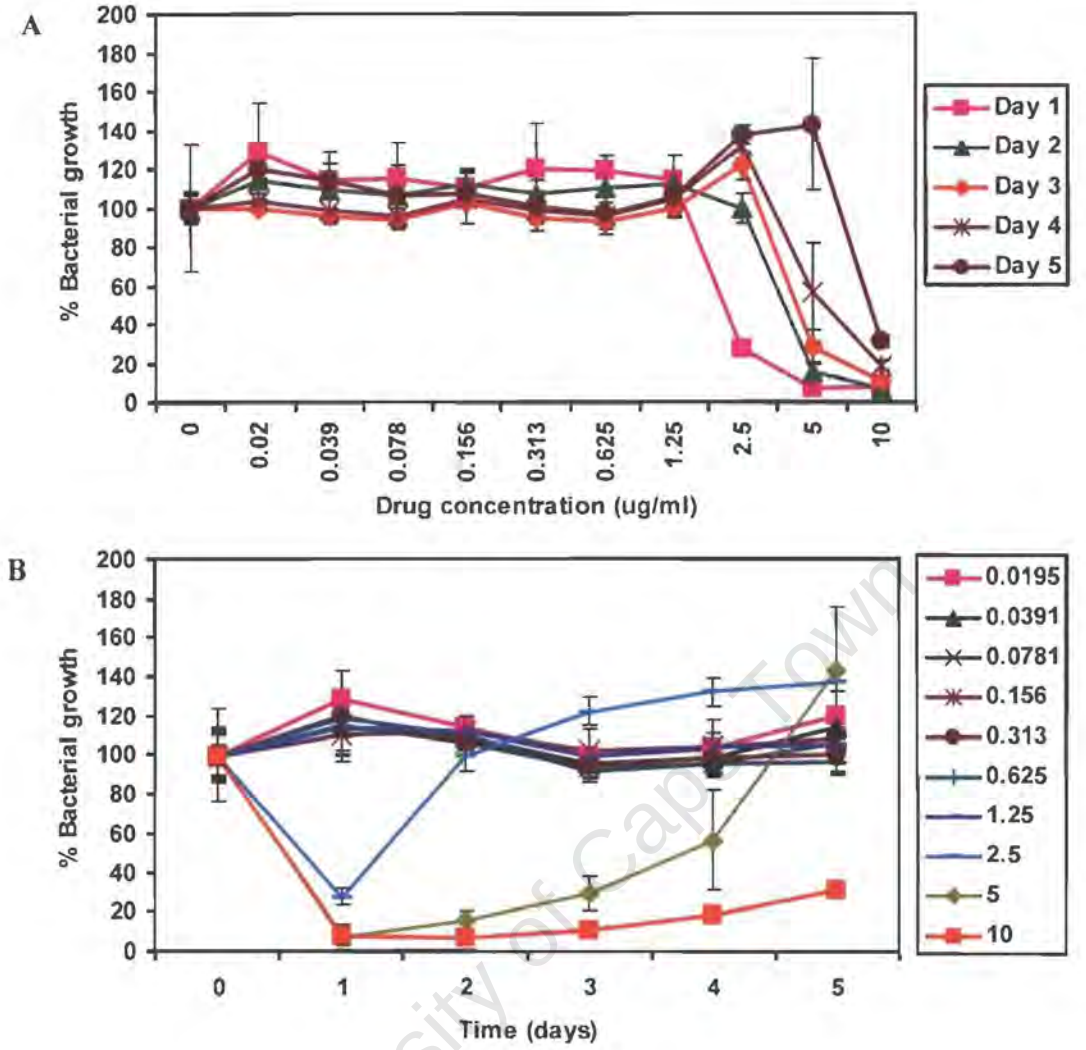


Figure 3.11.3. The Minimum Inhibitory Concentration (A) and kinetics of inhibition (B) of RKG162A. The MIC of RKG162A was determined by incubating *M. tuberculosis* for 6 days with RKG162A at a concentration ranging from 0-10µg/ml. Resazurin was added to samples and incubated further. Plates were read at 570nm and 600nm. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium. Values are representative of the mean and standard deviation of quadruplicate measurements.

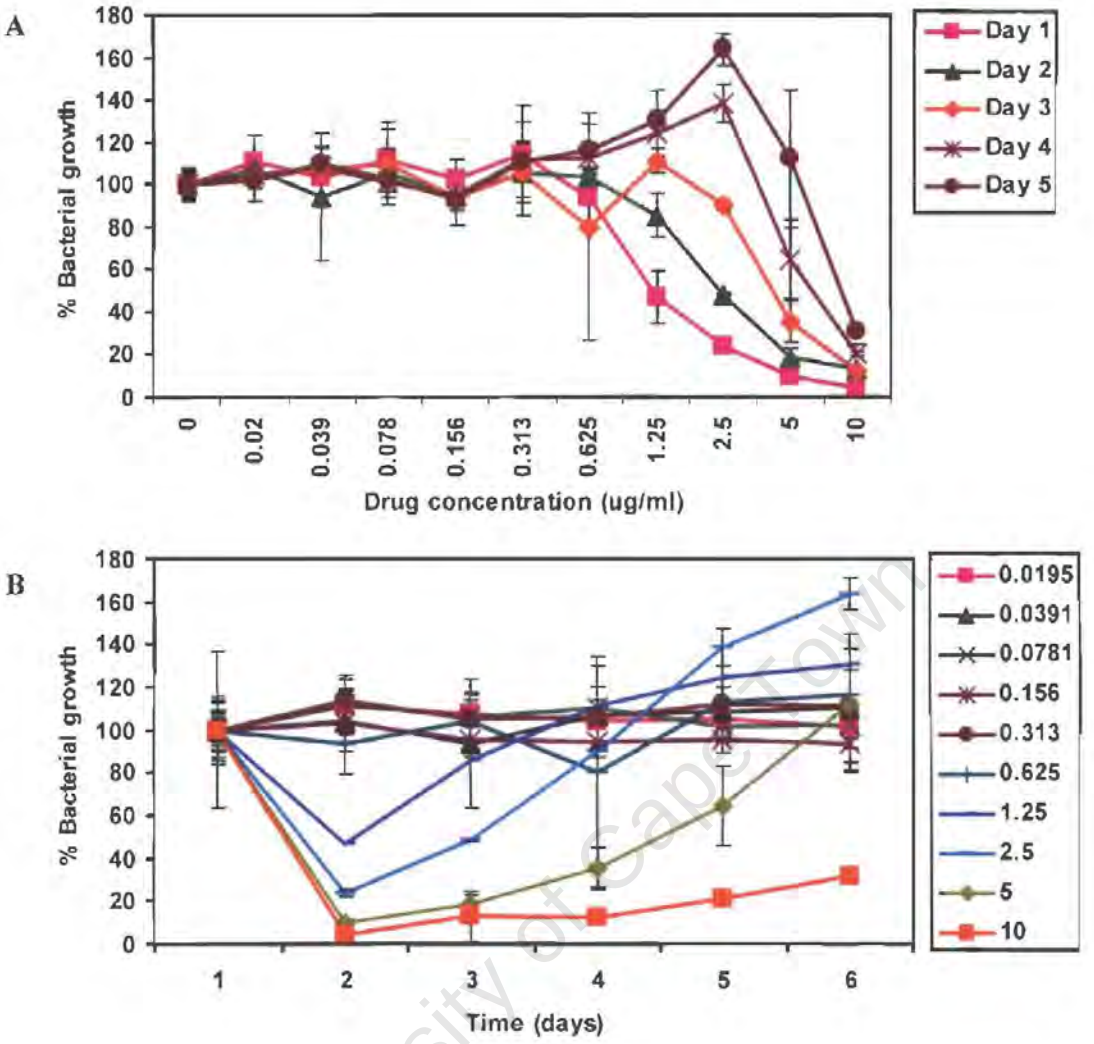


Figure 3.11.4. The Minimum Inhibitory Concentration (A) and kinetics of inhibition (B) of RKG1541. The MIC of RKG1541 was determined by incubating *M. tuberculosis* for 6 days with RKG1541 at a concentration ranging from 0-10μg/ml. Resazurin was added to samples and incubated further. Plates were read at 570nm and 600nm. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium. Values are representative of the mean and standard deviation of quadruplicate measurements.

3.11.4. Minimum Inhibitory Concentrations of Cysteine Protease inhibitors

Four cysteine protease inhibitors namely AXE1, AXE4, AXE5, and AXE29 were found to completely inhibit *M. tuberculosis* growth (section 3.7). We therefore determined the lowest concentration at which AXE1, AXE4, AXE5, and AXE29 inhibited *M. tuberculosis* growth. When *M. tuberculosis* was treated with AXE1, no growth inhibition was observed with treatment concentration ranging from 0-2.5µg/ml (Fig.3.11.5). The same trend of *M. tuberculosis* growth and resazurin reduction was observed with AXE 4 (Fig. 3.11.6) and AXE29 (Fig. 3.11.8). In contrast, treatment with 1.5µg/ml of AXE5 (Fig. 3.11.7) resulted in delayed bacilli growth and its MIC was attained at a lower concentration when M. tuberculosis was treated with AXE1, AXE4, and AXE29. We therefore recorded the MIC of AXE1, AXE4, and AXE29 as 10µg/ml and 5µg/ml for AXE5.

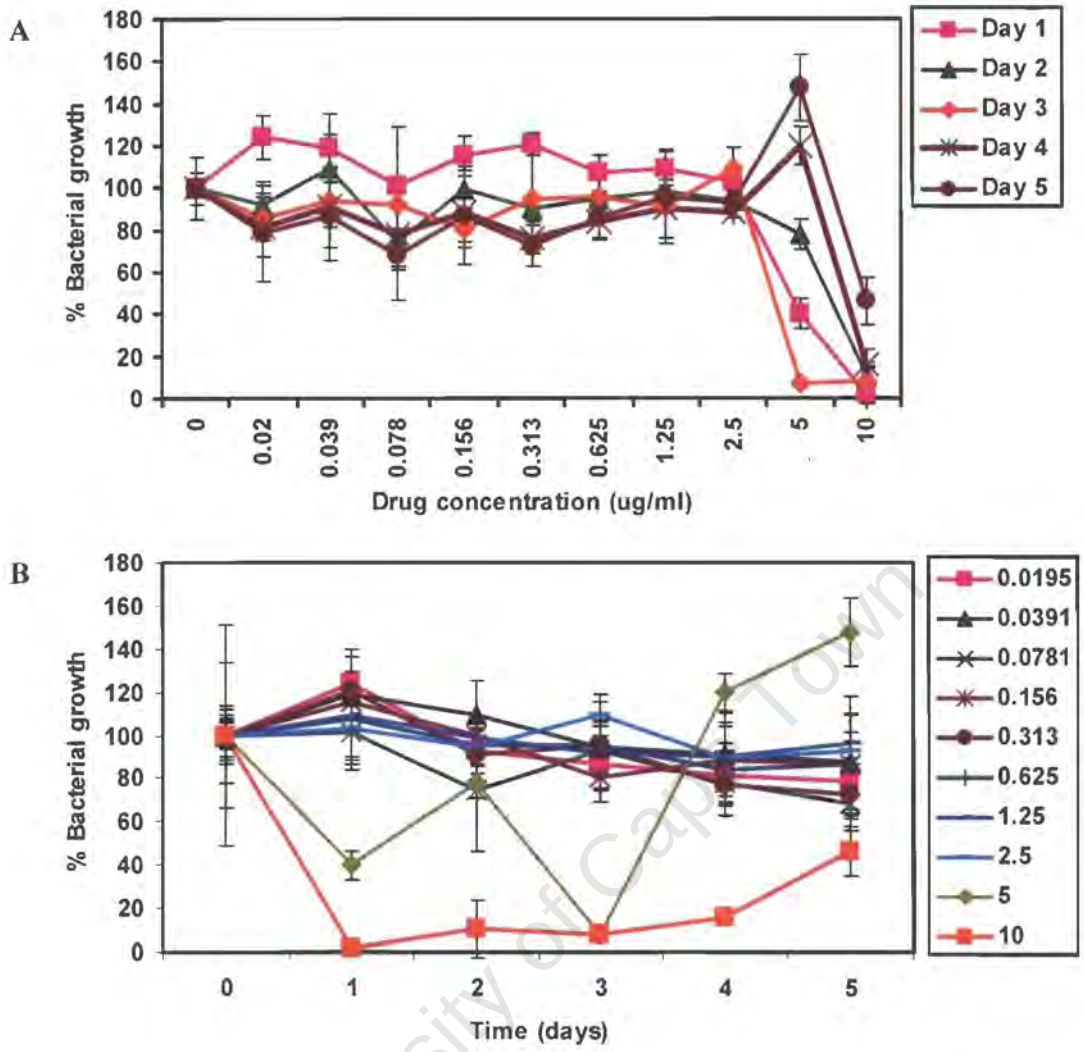


Figure 3.11.5. The Minimum Inhibitory Concentration (A) and kinetics of inhibition (B) of AXE1. The MIC of AXE1 was determined by incubating *M. tuberculosis* for 6 days with AXE1 at a concentration ranging from 0-10µg/ml. Resazurin was added to samples and incubated further. Plates were read at 570nm and 600nm. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium. Values are representative of the mean and standard deviation of quadruplicate measurements.

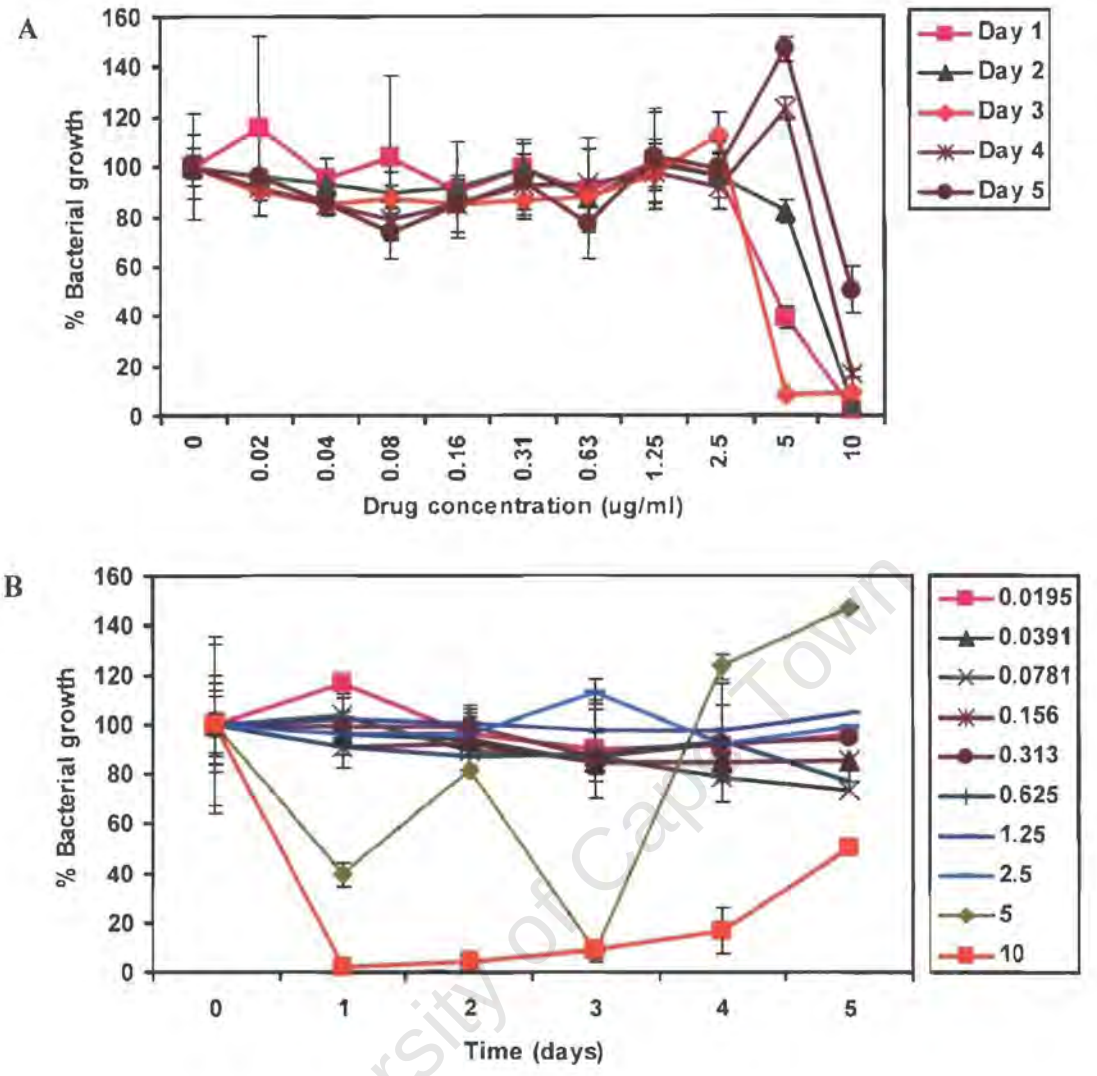


Figure 3.11.6. The Minimum Inhibitory Concentration (A) and kinetics of inhibition (B) of AXE4. The MIC of AXE4 was determined by determining the *M. tuberculosis* growth (A) and resazurin reduced (B) by incubating *M. tuberculosis* for 6 days with AXE4 at a concentration ranging from 0-10µg/ml. Resazurin was added to samples and incubated further. Plates were read at 570nm and 600nm. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium. Values are representative of the mean and standard deviation of quadruplicate measurements.

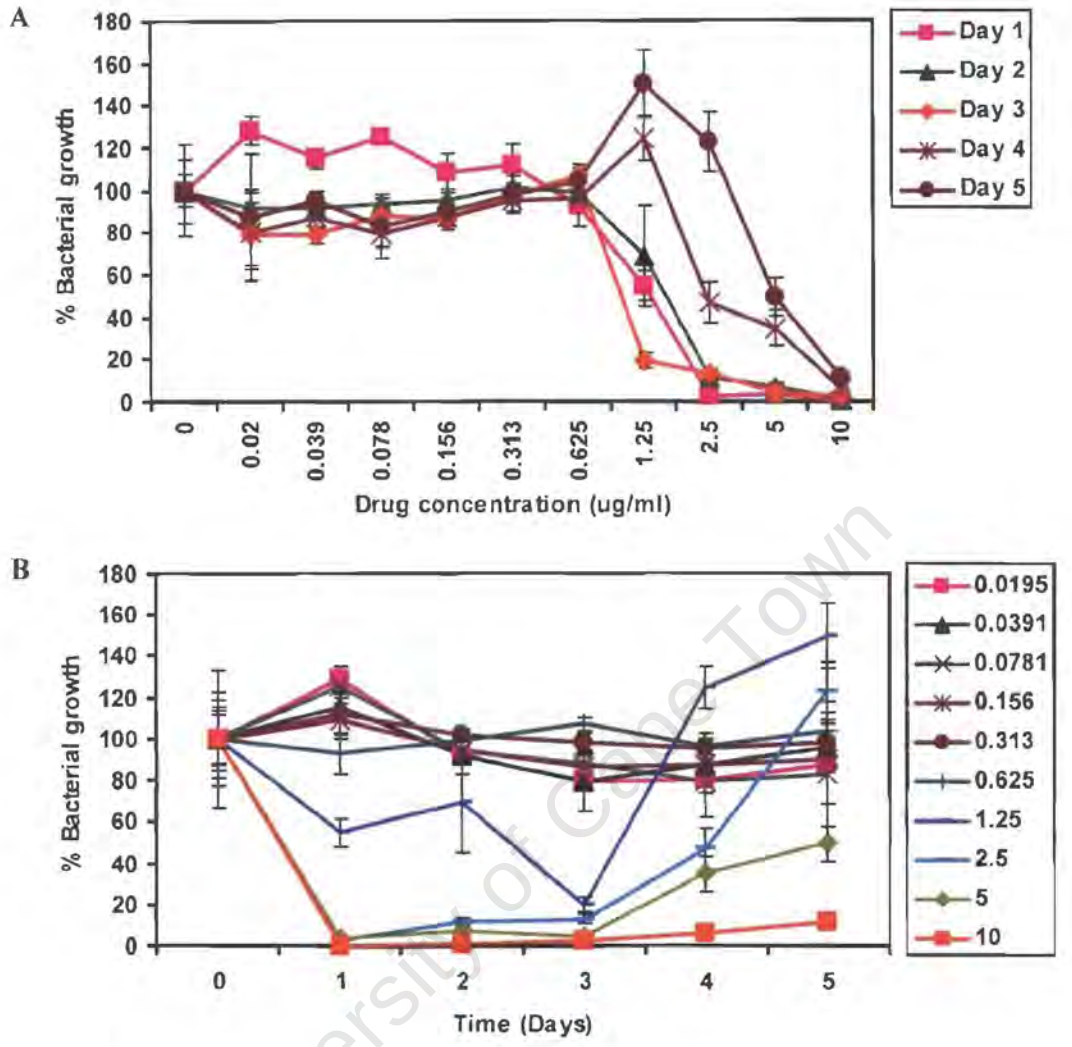


Figure 3.11.7. The Minimum Inhibitory Concentration (A) and kinetics of inhibition (B) of AXE5. The MIC of AXE5 was determined by incubating *M. tuberculosis* for 6 days with AXE5 at a concentration ranging from 0-10µg/ml. Resazurin was added to samples and incubated further. Plates were read at 570nm and 600nm. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements.

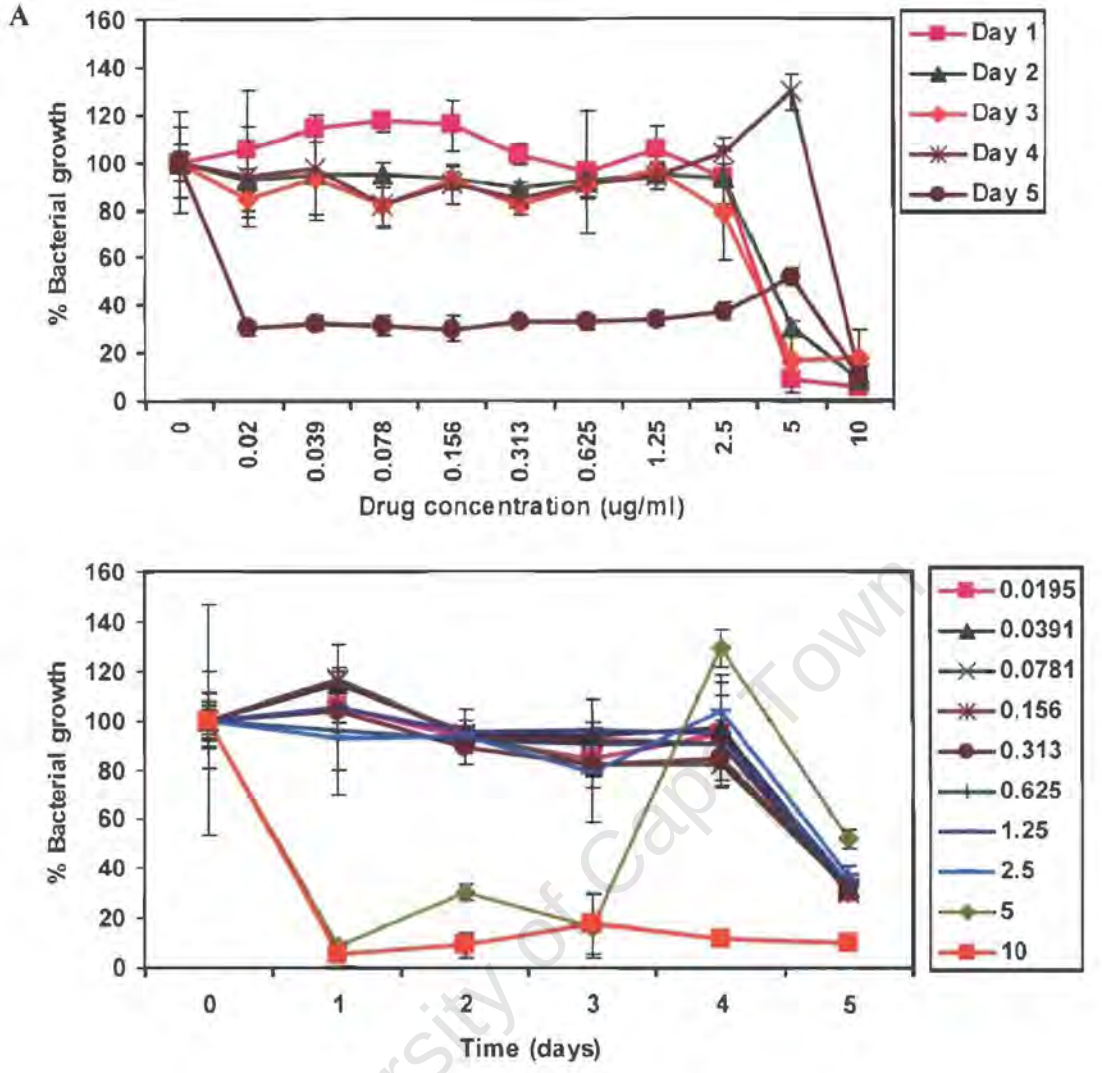


Figure 3.11.8. The Minimum Inhibitory Concentration (A) and kinetics of inhibition (B) of AXE29. The MIC of AXE29 was determined by determining the *M. tuberculosis* growth (A) and resazurin reduced (B) by incubating *M. tuberculosis* for 6 days with AXE29 at a concentration ranging from 0-10μg/ml. Resazurin was added to samples and incubated further. Plates were read at 570nm and 600nm. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements.

3.11.4. Minimum Inhibitory Concentrations of Thiosemicarbazones

In this study, we determined the lowest concentration at which FM19 and FM48 inhibited *M. tuberculosis* growth. Similar growth to untreated samples was observed when *M. tuberculosis* was treated with 0.02-0.625μg/ml of FM19 (Fig. 3.11.9). At 1.25μg/ml the inhibition potential of FM19 had decreased and therefore resulted in a delayed bacilli growth (Fig. 3.11.9). In contrast, treatment with ≥2.5μg/ml was bactericidal.

FM48 was less potent than FM19. Treatment with 0-2.5μg/ml gave similar bacilli growth (Fig. 3.11.10) to that of day 0 untreated samples and concentrations ≥5μg/ml were bactericidal. We therefore recorded the MIC of FM19 and FM48 as 2.5 and 5μg/ml respectively.

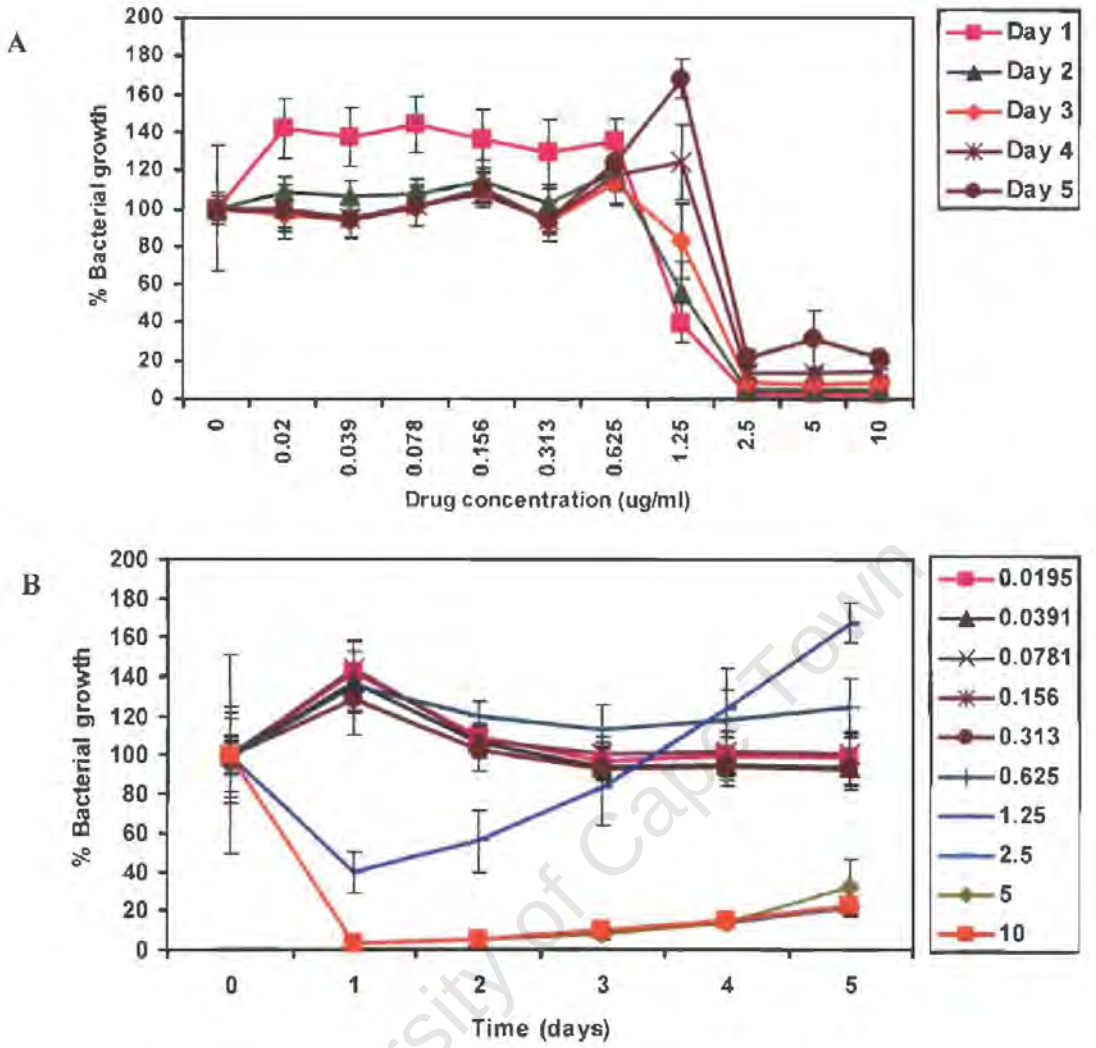


Figure 3.11.9. The Minimum Inhibitory Concentration (A) and kinetics of inhibition (B) of FM19. The MIC of FM19 was determined by incubating *M. tuberculosis* for 6 days with FM19 at a concentration ranging from 0-10µg/ml. Resazurin was added to samples and incubated further. Plates were read at 570nm and 600nm. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium. Values are representative of the mean and standard deviation of quadruplicate measurements.

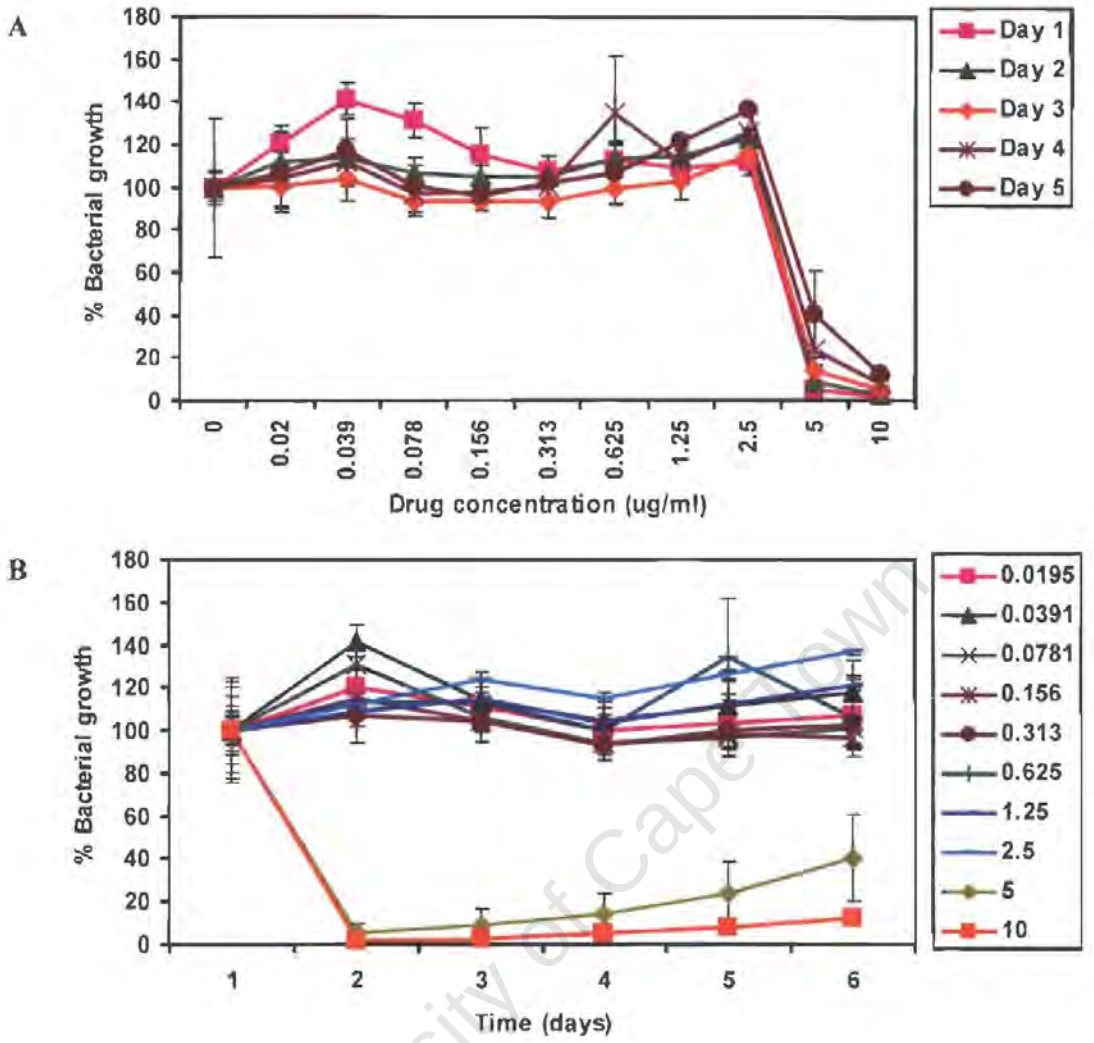


Figure 3.11.10. The Minimum Inhibitory Concentration (A) and kinetics of inhibition (B) of FM48. The MIC was determined by incubating *M. tuberculosis* for 6 days with FM48 at a concentration ranging from 0-10μg/ml. Resazurin was added to samples and incubated further. Plates were read at 570nm and 600nm. Percentage growth was calculated relative to untreated samples containing *M. tuberculosis* and medium only. Values are representative of the mean and standard deviation of quadruplicate measurements.

3.12. *In vitro* cytotoxic effects of DMSO on peritoneal macrophage cell culture

M. tuberculosis is an intracellular pathogen. Upon entry into the host system, *M. tuberculosis* is engulfed by macrophages. We therefore wanted to test the ability of the identified active compounds to traverse the phagosome and kill *M. tuberculosis*. In order to test this, we needed to assess the cytotoxicity of these compounds in a cell based assay to ensure that the host cells engulfing *M. tuberculosis* will be able to withstand the effects of treatment. Cytotoxicity of compounds was therefore tested against the peritoneal macrophages obtained from C57BL/6 female mice. For cytotoxicity studies, derivatives were solubilized in 100% DMSO. Prior to the cytotoxicity study of synthetic derivatives, we determined the maximum concentration of DMSO that could be tolerated in our assay without affecting viability of macrophage cells and distinguish between the effect of the synthetic derivatives and the solvent. Macrophage cells were incubated with 0-25% (v/v) DMSO for 8 days. Medium was removed, cells washed and viability of cells tested using MTT assay. DMSO had an *in vitro* cytotoxicity (IC_{50}) of 2.4% (Fig. 3.12.1). Compounds were therefore solubilized in ≤1.5% of DMSO, a concentration at which >86% of macrophages were metabolically active in subsequent experiments in order to minimize the cytotoxic effects of DMSO.

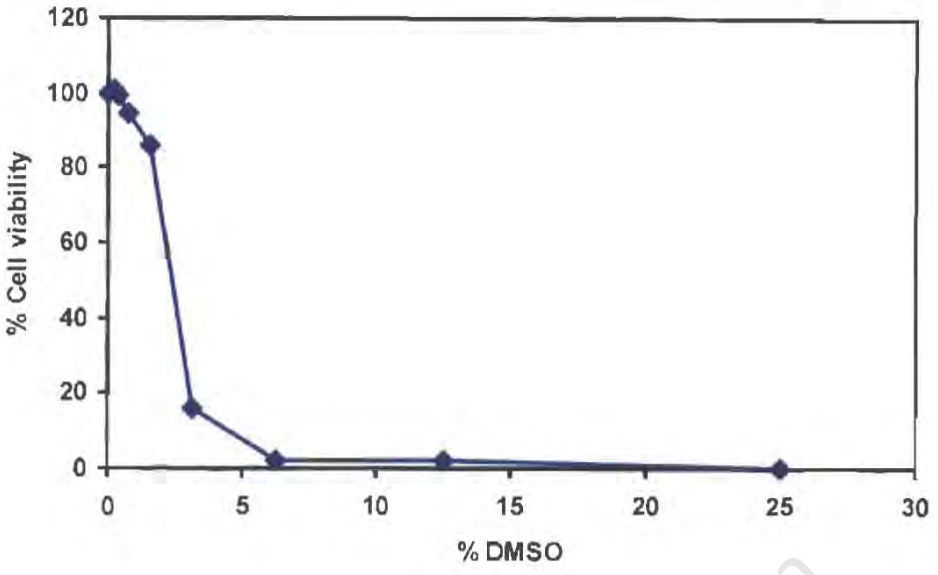


Figure 3.12 *In vitro* cytotoxicity of DMSO.DMSO was tested for cytotocixity on a cell based culture using C57BL/6 peritoneal macrophages. Macrophage cells were incubated with DMSO at an increasing concentration of 0-25% (v/v) for 8 days. Cells were tested for viability using MTT assay. Formazan formed was read at 550nm and 690nm. Cell viability was calculated relative to the untreated samples. Values are representative of the mean and standard deviation of quadruplicate measurements.

3.13. *In vitro* cytotoxic effects of INH and RIF on peritoneal macrophage cell culture

INH and RIF, the two drugs known to inhibit *M. tuberculosis* growth were tested for cytotoxicity on peritoneal macrophage cells. These drugs were tested for cytotoxicity in a cell based system. Macrophages cells were incubated with INH and RIF for 8 days. Medium was removed, cells washed and viability of cells tested using the MTT assay. INH had the lowest toxicity on macrophage cells. At 100µg/ml, 79% of macrophages were metabolically active (Fig. 3.13). RIF however, had IC_{50} of 27.35µg/ml.

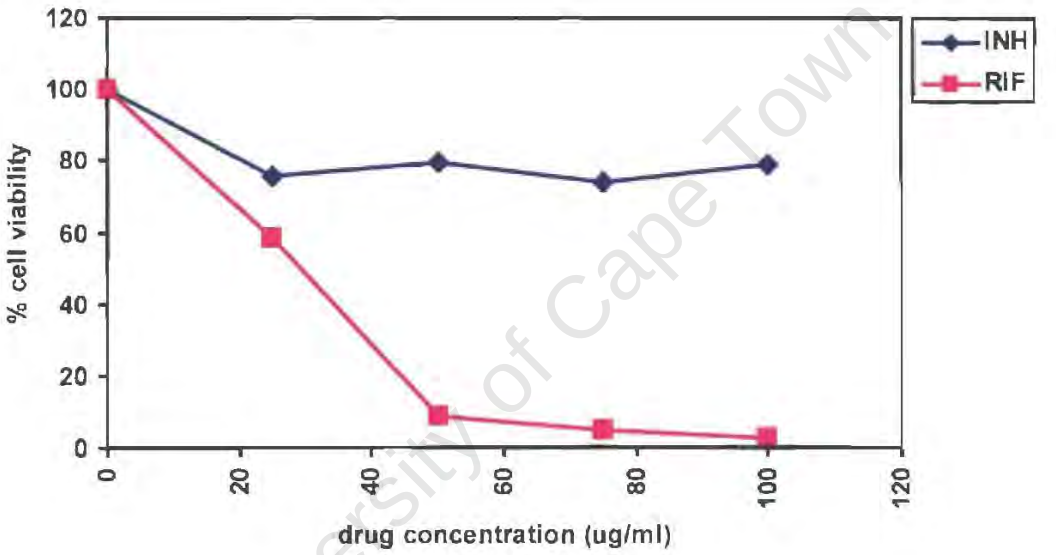


Figure 3.13. *In vitro* cytotoxicity of INH and RIF. INH and RIF were tested for cytotocixity on a cell based culture using C57BL/6 peritoneal macrophages. Macrophage cells were incubated with INH and RIF at an increasing concentration of 0-100% (v/v) for 8 days. Cells were tested for viability using MTT assay. Formazan formed was read at 550nm and 690nm. Cell viability was calculated relative to the untreated samples. Values are representative of the mean and standard deviation of quadruplicate measurements.

3.14. *In vitro* cytotoxic effects of identified synthetic derivatives on peritoneal macrophage cell culture

Eleven identified synthetic derivatives from different classes were tested for *in vitro* cytotoxicity on peritoneal macrophage cells. These compounds were shown to have antibacterial properties in previous experiments. They either completely inhibited bacterial growth or resulted in retarded growth of *M. tuberculosis*. Macrophages cells were incubated with derivatives for 8 days. Medium was removed, cells washed and viability of cells tested using MTT assay.

3.14.1. Acetolactate Synthase inhibitors

In this study RKG162A and RKG1541 were tested for *in vitro* cytotoxicity on peritoneal macrophages. RKG162A and RKG1541 had an IC_{50} of 17.6 µg/ml and 6µg/ml respectively indicating that the both compounds were partially cytotoxic to macrophage cells with the cytotoxic effects of RKG1541 3x higher than RKG162A (Fig. 3.14.1).

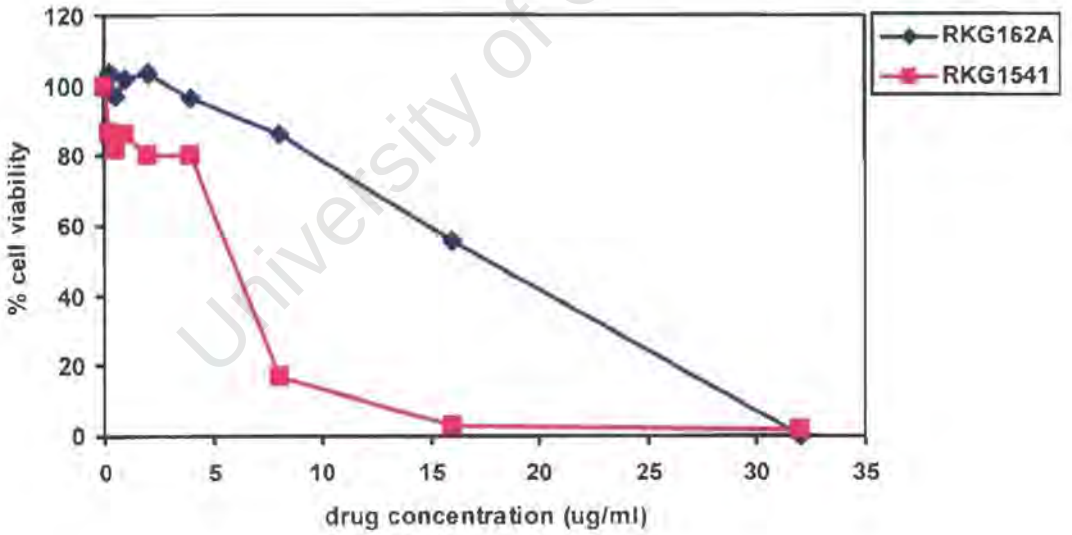


Figure 3.14.1. *In vitro* cytotoxicity of ALS inhibitors. RKG162A and RKG1541were tested for cytotocixity on a cell based culture using C57BL/6 peritoneal macrophages. Macrophage cells were incubated with RKG162A and RKG1541 at an increasing concentration of 0-32µg/ml for 8 days. Cells were tested for viability using MTT assay. Formazan formed was read at 550nm and 690nm. Cell viability was calculated relative to the untreated samples. Values are representative of the mean and standard deviation of quadruplicate measurements.

3.14.2. Cysteine Protease inhibitors

3.14.2.1. Isatin-derived cysteine protease inhibitors

Four compounds from this subclass were tested for *in vitro* cytotoxicity on peritoneal macrophages. AXE1, AXE4, and AXE5 were highly toxic to macrophages with IC_{50} of <0.25µg/ml (Fig. 3.14.2.1). In contrast, AXE29 displayed partial toxic effects with IC_{50} of 16.08µg/ml.

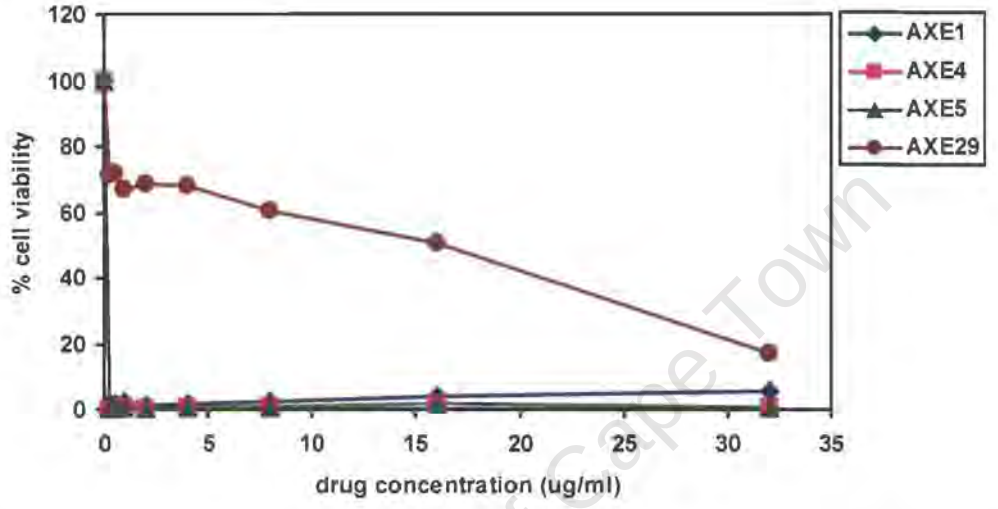


Figure 3.14.2.1. *In vitro* cytotoxicity of Isatin-derived Cysteine Protease inhibitors. AXE1, AXE4, AXE5, and AXE29 were tested for cytotocixity on a cell based culture using C57BL/6 peritoneal macrophages. Macrophage cells were incubated with AXE1, AXE4, AXE5, and AXE29 at an increasing concentration of 0-32µg/ml for 8 days. Cells were tested for viability using MTT assay. Formazan formed was read at 550nm and 690nm. Cell viability was calculated relative to the untreated samples. Values are representative of the mean and standard deviation of quadruplicate measurements.

3.14.2.2. Peptidyl Cysteine Protease inhibitors

NAT 47 was the only compound that was tested for cytotoxicity on peritoneal macrophages from this subclass. IC_{50} of NAT47 was 9.75μg/ml (Fig. 3.14.2.2).

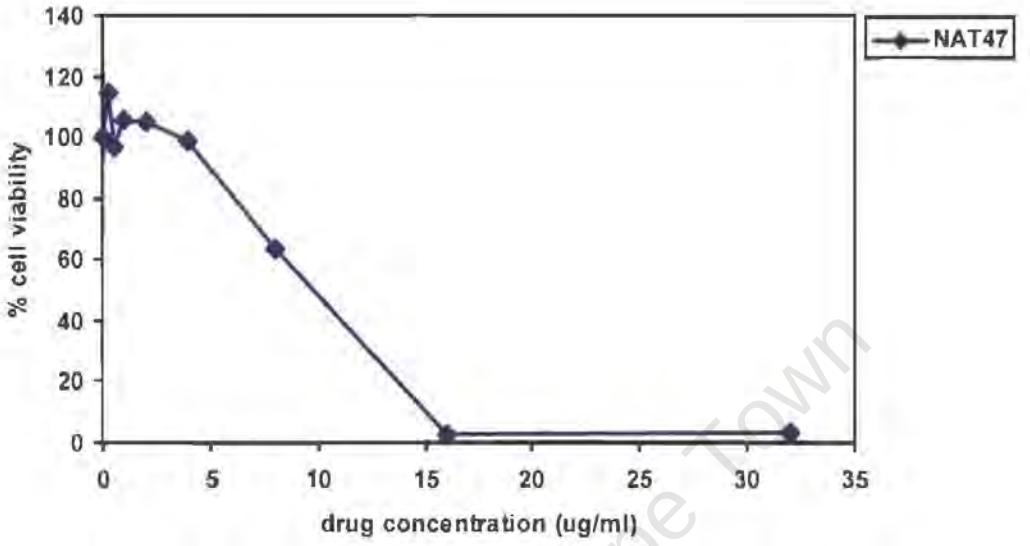


Figure 3.14.2.2. *In vitro* cytotoxicity of a Peptidyl Cysteine Protease inhibitor. NAT 47 was tested for cytotocixity on a cell based culture using C57BL/6 peritoneal macrophages. Macrophage cells were incubated with NAT 47 at an increasing concentration of 0-32μg/ml for 8 days. Cells were tested for viability using MTT assay. Formazan formed was read at 550nm and 690nm. Cell viability was calculated relative to the untreated samples. Values are representative of the mean and standard deviation of quadruplicate measurements.

3.14.3. Thiosemicarbazones

In this study, 4 derivatives namely FM04, FM19, FM48, and FM102 were tested for *in vitro* cytotoxicity on peritoneal macrophages. All 4 compounds were highly toxic to macrophage cells (Fig. 3.14.3). All cells were killed at 0.25μg/ml, the lowest concentration that was tested.

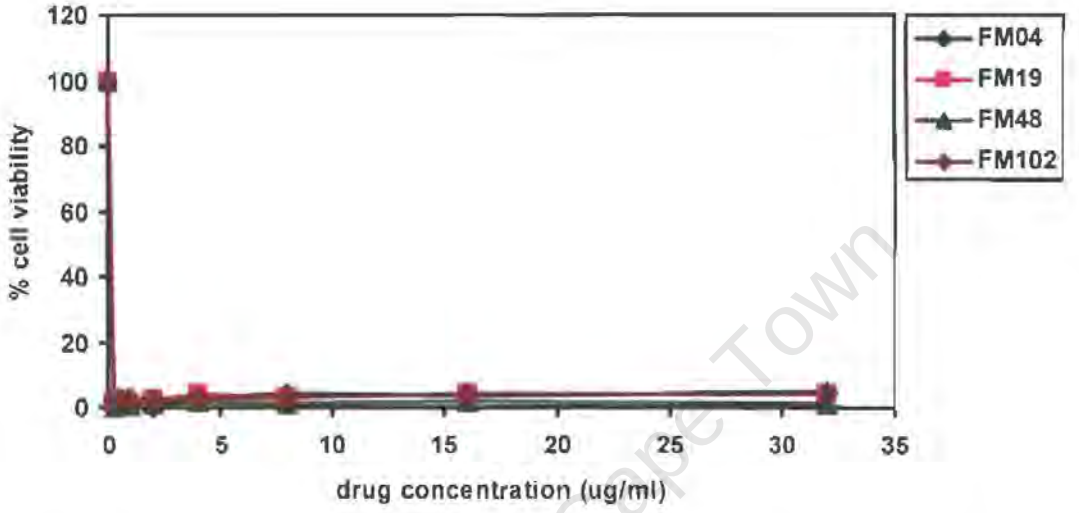


Figure 3.14.3. *In vitro* cytotoxicity of Thiosermicarbazones. FM04, FM19, FM48, and FM102 were tested for cytotocixity on a cell based culture using C57BL/6 peritoneal macrophages. Macrophage cells were incubated with thiosemicarbazones derivatives at an increasing concentration of 0-32μg/ml for 8 days. Cells were tested for viability using MTT assay. Formazan formed was read at 550nm and 690nm. Cell viability was calculated relative to the untreated samples. Values are representative of the mean and standard deviation of quadruplicate measurements.

3.15. Intracellular killing of *M. tuberculosis* by synthetic derivatives

M. tuberculosis is an intracellular pathogen and its target cells are macrophages within the mammalian hosts. Antimycobacterial drugs should be able to cross the membrane of the target cells and kill mycobacteria. We therefore investigated the ability of the AXE29 and RKG162A to traffic into the macrophage phagosome containing bacteria and destroy the bacilli. INH and RIF were incorporated as positive control drugs with known capability to kill intracellular bacilli. Phagocytosed *M. tuberculosis* was treated with 1 and 10μg/ml of drug for 5 days. Macrophages were lysed and CFU enumerated. As expected, INH and RIF inhibited bacterial growth significantly ($p<0.01$) at both 1 and 10μg/ml. Unlike RIF, increase in INH concentration did not result in increase in bacterial growth inhibition. No significant inhibition was observed when internalized *M. tuberculosis* was treated with 1μg/ml of AXE29 and RKG162A. Treatment with 10μg/ml however resulted in significant ($p<0.05$) bacterial growth inhibition for both AXE29 and RKG162A.

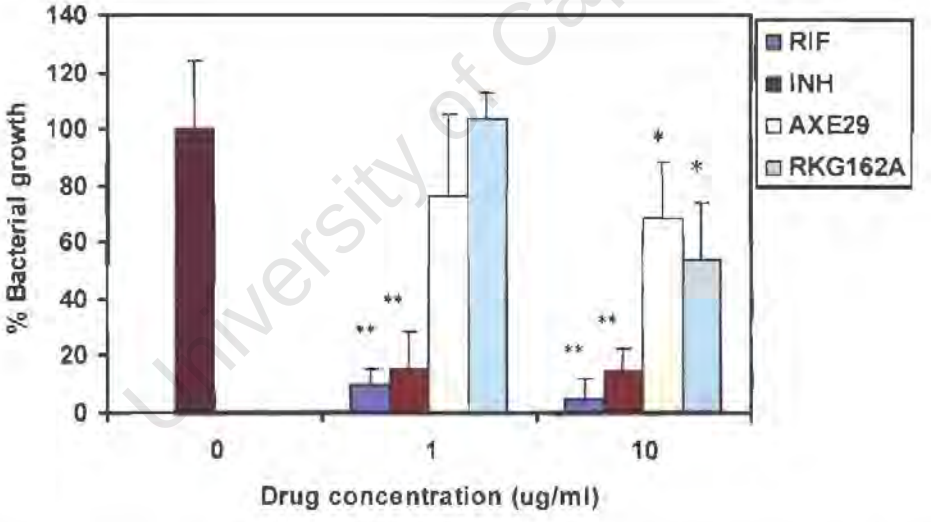


Figure 3.15. Intracellular killing of *M .tuberculosis* by synthetic derivatives. INH, RIF, AXE29, and RKG162A were tested for the ability to cross C57BL/6 peritoneal macrophages and destroy the bacilli. Macrophage phagocytosed *M. tuberculosis* was incubated with INH, RIF, AXE29, or RKG162A for 5 days. Macrophage cells were lysed and CFU enumerated. Percentage bacterial growth was calculated relative to the untreated samples. Values are representative of the mean and standard deviation of quadruplicate measurements. * represents $p<0.05$ and ** represents $p<0.01$.

CHAPTER FOUR

Therapeutic and toxic effects of identified synthetic derivatives in an experimental tuberculosis murine model

Summary

One compound from the ALS inhibitors (RKG162A) and one from the cysteine protease inhibitors (AXE29) were highly active against *M. tuberculosis in vitro*. We therefore evaluated AXE29 and RKG162A for the *in vivo* efficacy against *M. tuberculosis* in a murine model. C57BL/6 mice were infected by aerosol inhalation with 100CFU/lung of virulent *M. tuberculosis* H37Rv. Chemotherapy with AXE29 and RKG162A was initiated at day 1 of infection and sustained for 2 weeks. Drugs were administered daily intraperitoneally. INH was included as a known inhibitor of *M. tuberculosis in vivo* and DMSO as a solvent control. RKG162A exhibited mortality at 50mg/kg in *M. tuberculosis* infected mice. In contrast, in a survival study, when uninfected mice were treated for 10 days with 50mg/kg of RKG162A mice survived and no weight loss was observed. At the end of the treatment phase of *M. tuberculosis* infected mice with AXE29, there was no significant difference in bacterial burden in the lungs of these mice when compared with the untreated group. In contrast, at day 14 of INH treatment, no bacilli could be detected in the organs of these mice. Treatment with AXE29, RKG162A, INH and DMSO was not accompanied by weight loss. The untreated, DMSO, and AXE29 treated groups had similar levels of lung infiltration whereas INH had a significantly less infiltration relative to the untreated group. These findings suggest that although AXE29 and RKG162A were highly active *against M. tuberculosis in vitro*, they do not exhibit any therapeutic capacity *in vivo* at the tested concentrations. Therefore further manipulation of these compounds is necessary to increase the *in vivo* activity and decrease the toxic effects of these compounds.

4.1. Preparation of synthetic derivatives for *in vivo* efficacy study

DMSO is widely used in the field of biosciences and the toxicity of DMSO for cultured cells or animals has been reported (Worthley and Schott 1969; Cavaletti, Oggioni et al. 2000). For this study all synthetic derivatives were solubilized in DMSO. In order to minimize the toxicity of DMSO to mice, DMSO-dissolved compounds were diluted in different solvents to lower the final DMSO concentration for *in vivo* application. However, the dilution of AXE29 and RKG162A dissolved in DMSO resulted in the formation of precipitate and were therefore unsuitable for further application. Solvents that were used to dilute compounds are shown in table 4.1.

Solvent	Final concentration
Tween-80	0.02%
Distilled water	
Acidified water	pH 2
Ethanol	20%
Methyl cellulose	0.5%
Na_2CO_3	5%
DMSO	5%
Olive oil	

Table 4.1. Solvents for DMSO-dissolved derivatives dilution

4.2. Toxicity of DMSO in a murine model

Because none of the solvents stated in table 4.1 could be used to dilute DMSO-dissolved AXE29 and RKG162A, the effect of DMSO alone was assessed for its toxicity *in vivo*. Fifty microlitres of DMSO was administered to uninfected mice daily for 10 days and the condition and mortality of mice monitored. Mice from both the treated and the untreated group survived and there was no significant change of mouse body weight during the treatment phase (Fig. 4.1). DMSO was therefore used as a solvent in subsequent experiments.

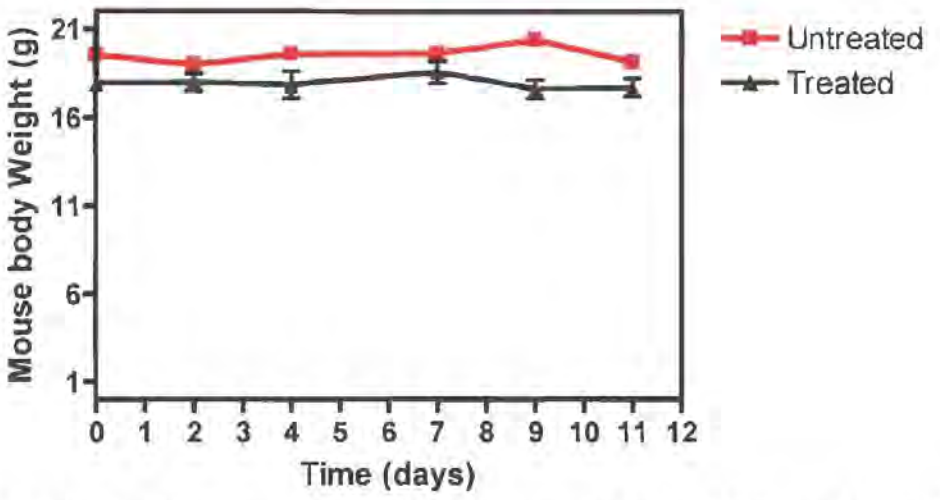


Figure 4.1. Mouse weight measurement during treatment with DMSO. Uninfected C57BL/6 mice were administered with 50µl DMSO intraperitoneally daily for 10 days. Mice were weighed at intervals for the duration of treatment.

4.3. Toxicity of identified synthetic derivatives in a murine model

When mice were treated with DMSO, no toxic effects were observed. To investigate the potential toxicity of AXE29 and RKG162A, uninfected mice were treated with each of the compounds at 25, 50, and 100mg/kg for 7 days to determine the maximum tolerated dose (MTD). Treatment with 100mg/kg of either AXE29 or RKG162A resulted in mortality (see table 4.2 & 4.3). Treatment with AXE29 was accompanied by an insignificant body weight loss. Seventy five percent of mice had succumbed at day 7 of treatment with RKG162A whereas 25% of mice died after treatment with AXE29. However, mice treated with 25 and 50mg/kg of both compound survived and no weight loss was observed.

Day	25mg/kg	50mg/kg	100mg/kg
0	4/4	4/4	4/4
1	4/4	4/4	2/4
2	4/4	4/4	2/4
3	4/4	4/4	1/4
4	4/4	4/4	1/4
5	4/4	4/4	1/4
6	4/4	4/4	1/4
7	4/4	4/4	1/4

Table 4.2. Effect of RKG162A on survival of uninfected C57BL/6 mice. RKG162A was administered at 50µl to uninfected mice intraperitoneally for 1 week at 25, 50 and 100mg/kg of body weight.

Day	25mg/kg	50mg/kg	100mg/kg
0	4/4	4/4	4/4
1	4/4	4/4	3/4
2	4/4	4/4	3/4
3	4/4	4/4	3/4
4	4/4	4/4	3/4
5	4/4	4/4	3/4
6	4/4	4/4	3/4
7	4/4	4/4	3/4

Table 4.3. Effect of AXE29 on survival of uninfected C57BL/6 mice. AXE29 was administered at 50µl to uninfected mice intraperitoneally for 1 week at 25, 50 and 100mg/kg of body weight.

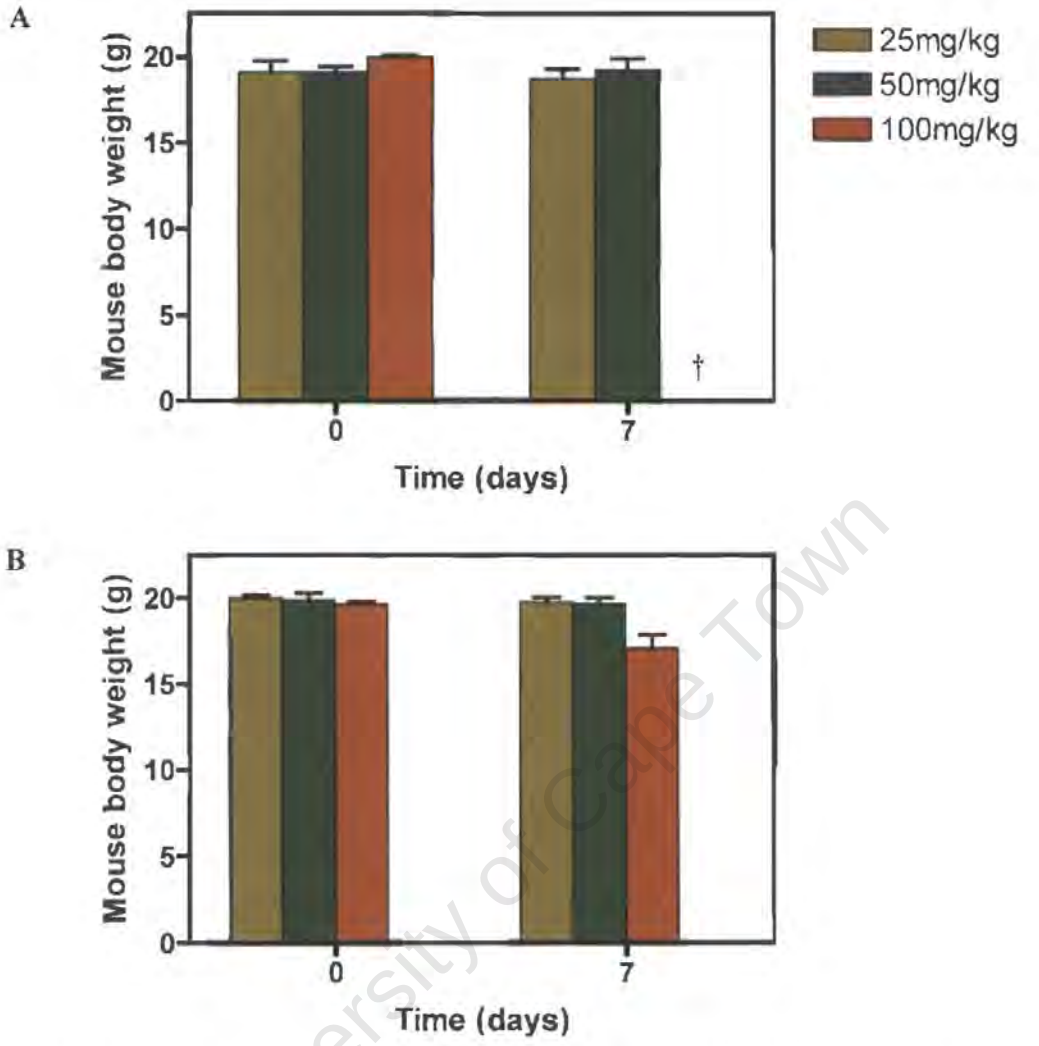


Figure 4.2. Mouse body weight measurement during treatment with AXE29 (A) and RKG162A (B). Uninfected C57BL/6 mice were administered with 50µl of either AXE29 or RKG162A intraperitoneally daily for 1 week. Mice were weighed for the duration treatment. Values are representative of the mean and standard deviation of 5 mice. † represents mortality.

4.4. Therapeutic effect of identified synthetic derivatives in a murine model

Therapeutic potential of AXE29 and RKG162A was investigated in an experimental tuberculosis murine model. Mice were challenged with 100CFU/lung of *M. tuberculosis* by aerosol inhalation. Treatment was initiated at the onset of infection and sustained for 2 weeks. Efficacy of AXE29 and RKG162A were assessed by *M. tuberculosis* burden in mice organs, mouse body weight, and gross lung lesions.

4.4.1. Body Weight

Mice were weighed before onset of treatment and subsequently once a week. DMSO and INH were incorporated as solvent and positive controls during treatment. Mice body weights are shown in Fig. 4.3. The weight of untreated, INH, and DMSO treated groups remained invariable throughout the treatment phase. In contrast, weight loss was observed in RKG162A and AXE29 treated mice. Mortality (100%) was observed in RKG162A treated mice by day 7.

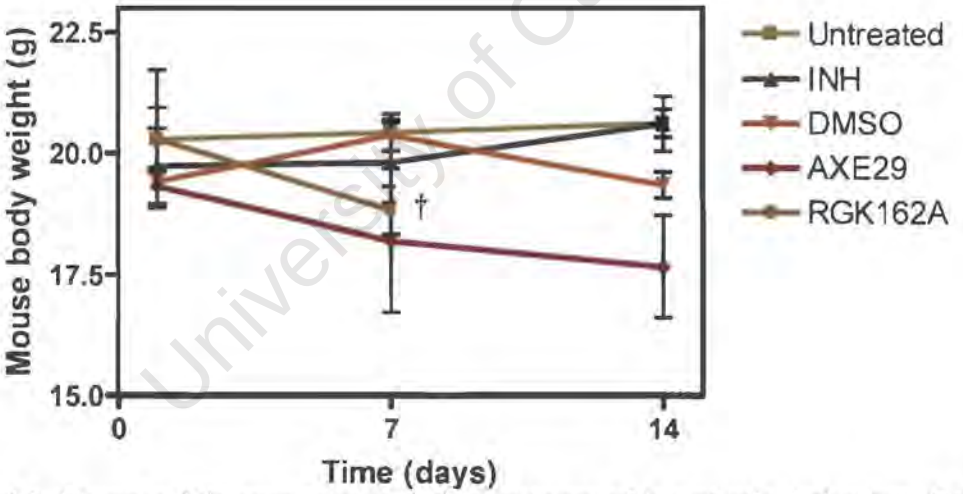


Figure 4.3. Mouse weight measurement during treatment with synthetic derivatives. C57BL/6 mice were challenged with 100CFU/lung of *M. tuberculosis* by aerosol inhalation. Treatment with INH, DMSO, AXE29, and RKG162A was initiated at day 1 of infection and sustained for 2 weeks. An untreated group was included as a control for body weight profile during infection with *M. tuberculosis*. Mice were weighed once a week for the duration of treatment. Values are representative of mean and standard deviation of 4 mice per group. † represents mortality.

4.4.2. Enumeration of CFU in organs during treatment

In this study the pulmonary bacilli burden was determined as an indication of the killing potential of AXE29 and RKG162A. Treatment phase was initiated at day 1 was and sustained for 14 days. At the end of treatment phase, the lung, liver, and spleen of mice that had received INH were culture negative (fig 4.4). For lungs (Fig. 4.4A), the untreated, AXE29, and DMSO treated groups had increased lung log CFU at day 14 with small but insignificant difference between the 3 groups. For the liver (Fig. 4.4B), the untreated and AXE29 treated groups had increased bacilli burden, while the INH and DMSO treated groups were culture negative. For the spleen (Fig. 4.4C) however, all the groups except the untreated group were culture negative at the end of treatment.

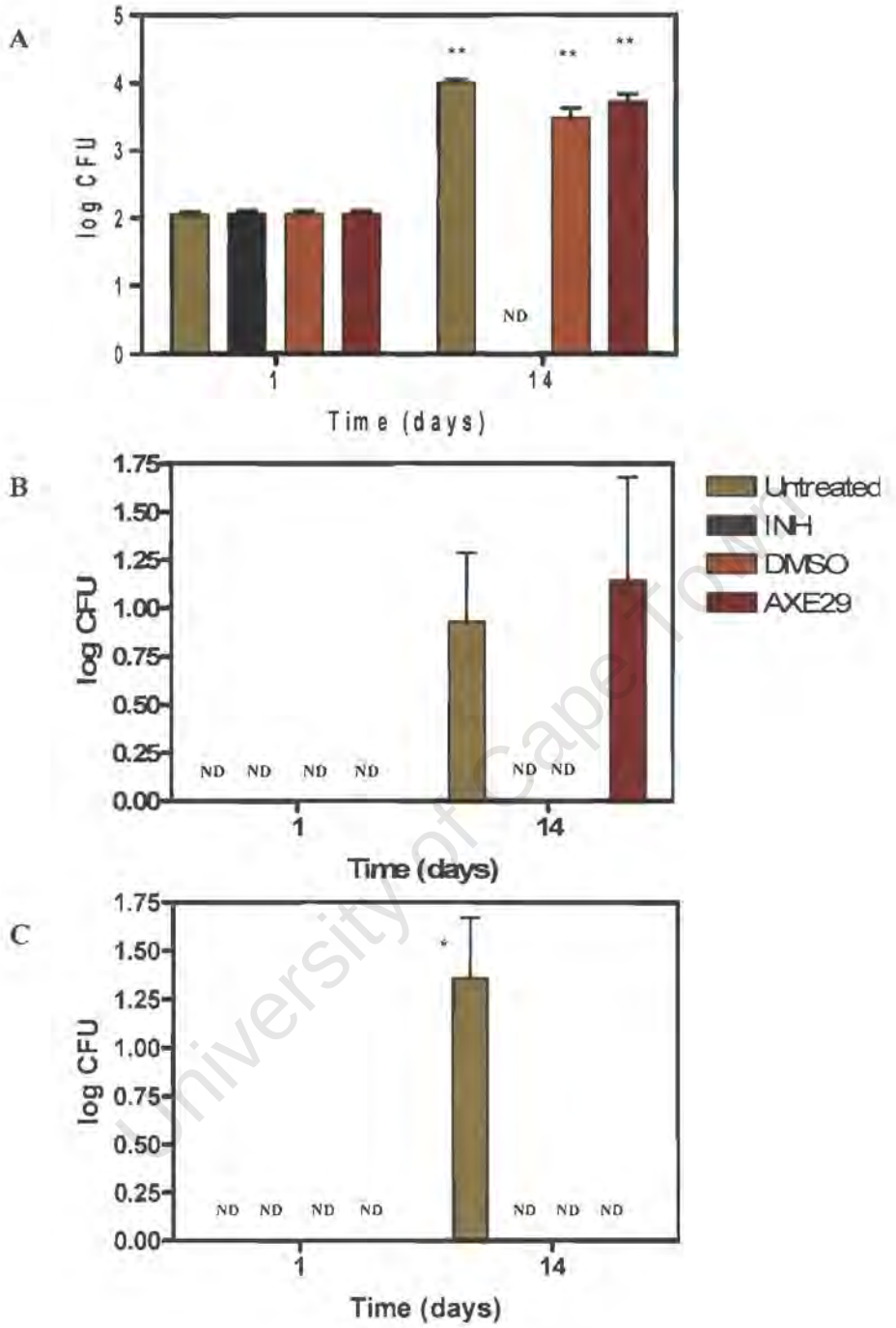


Figure 4.4. Bacilli burden in mouse organs. C57BL/6 mice were challenged with 100CFU/lung of *M. tuberculosis* by aerosol inhalation. Treatment with INH, DMSO and AXE29 was initiated at day 1 of infection and sustained for 2 weeks. Untreated group was included as a control for bacilli burden profile during infection with *M. tuberculosis*. Colony forming units (CFU) for lung (A), liver (B), and spleen (C) were enumerated at the beginning and the end of treatment. ND=not detected

4.4.3. Gross lung lesions

In this study, the morphology of lungs of *M. tuberculosis* infected mice following treatment with INH, AXE29, and DMSO was assessed. Treatment of infected mice was initiated at day 1 and sustained for 2 weeks. At the end of the treatment phase, lungs were stained for morphology. At day 14, inflammation was observed in untreated mice (Fig. 4.4A). No difference in the extent of inflammation was observed in lungs of AXE29 (Fig. 4.4D) and DMSO (Fig. 4.4B) treated mice when compared to the untreated mice. In contrast, treatment with INH resulted in reduce inflammation (Fig. 4.4C). No defined granulomas could be observed at day 14 of treatment. When lungs were stained for acid-fast bacilli, no *M. tuberculosis* was observed (data not shown).

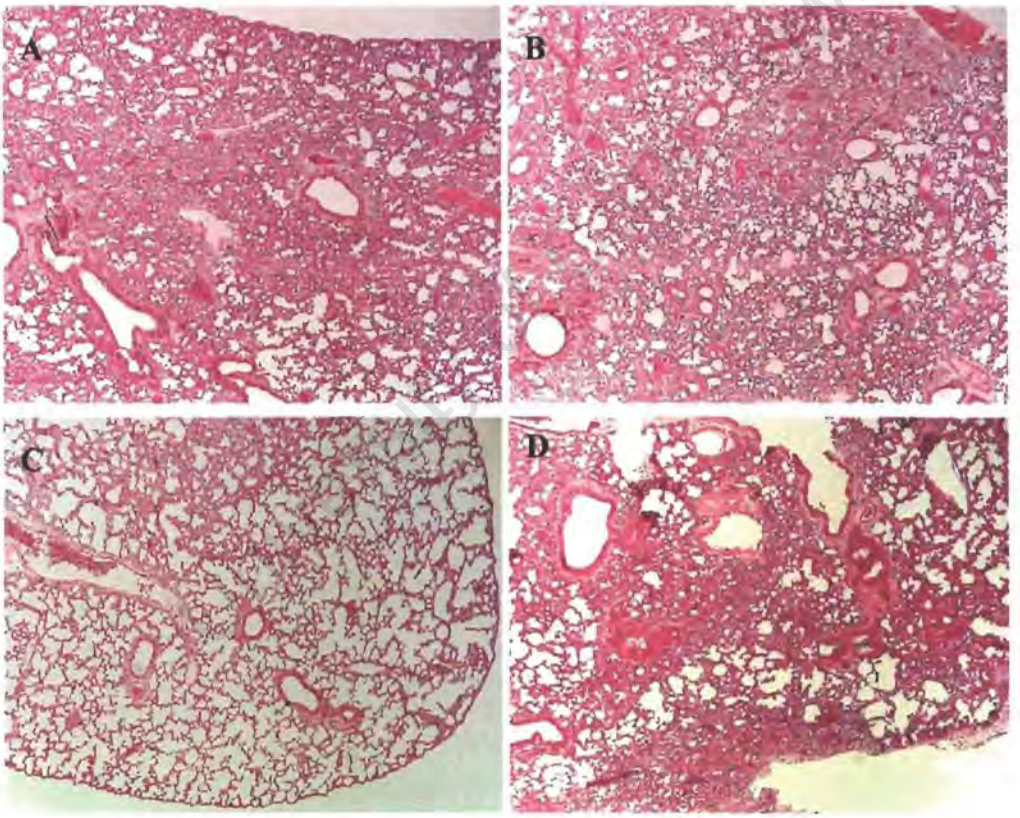


Figure 4.5. Lung morphology of C57BL/6 mice. Mice were challenged with 100CFU/lung by aerosol inhalation. Treatment with DMSO (B), INH (C), and AXE29 (D) was initiated at day 1 and sustained for 2 weeks. Untreated group (A) was included as a control for lung morphology during infection with *M. tuberculosis*. At day 14, mice were sacrificed and lungs fixed in PBS buffered formalin. Tissue sections were prepared and stained with haematoxylin and eosin for morphology (magnification=40X).

CHAPTER FIVE

Discussion and Conclusion

There is an increase in the development of drug resistance towards INH and RIF, two of the frontline antimycobacterial drugs currently used in therapeutic regimes (WHO 2005). The resistance arises from mutations in target genes for current TB drugs and the lack of compliance by patients to the current lengthy treatment regimen. MDR-TB is defined as resistance to at least INH and RIF. As an attempt to address drug resistance, the World Health Organization started implementing the DOTS strategy in 1991 and by 2003 this strategy had been implemented in 182 of 211 WHO countries (WHO 2005). Moreover, new chemical libraries of potential antituberculosis drugs have been designed and synthesised. The focus of drug development is to shorten the duration of the current regimen, improve treatment of multi-drug resistant tuberculosis or exhibit sterilizing activity (O'Brien 2001).

In this study we therefore investigated the potential of specifically identified synthetic derivatives from different classes of compounds to inhibit *M. tuberculosis* growth *in vitro*. Four classes of compounds namely the acetolacteate synthase (ALS) inhibitors, cysteine protease inhibitors, thiosemicarbazones, and thiolactomycins were tested for antimycobacterial properties. The compounds were selected on the basis of rationale drug design. We assessed the bioactivity of specific derivatives in a resazurin assay as described by (Collins and Franzblau 1997). In this assay, resazurin is reduced to form resorufin by metabolically active *M. tuberculosis*. This was compared to samples in which no *M. tuberculosis* was not present. As expected, no reduction of resazurin was observed in samples in which no *M. tuberculosis* was included (Fig. 3.1.2). Prior to testing of derivatives, we investigated the kinetics of reduction of resazurin by metabolically active *M. tuberculosis*. INH and RIF were included in this study as known inhibitors of *M. tuberculosis*. The amount of resazurin that was reduced peaked at day 2 following addition of resazurin (Fig. 3.2.2). Subsequent to day 2, a decrease in the amount of resazurin was observed. However, the observed decrease was not due to death of *M. tuberculosis* but further reduction of resazurin to its colourless form dihydroresorufin (Fig. 3.2.1) (Erb and Ehlers 1950). In an *in vitro* assay, INH exhibited an MIC of 0.039µg/ml (Fig. 3.11.1). Interestingly the MIC of RIF was higher than that of INH. RIF exhibited an MIC of 0.078µg/ml. These results were similar to results obtained by Alvirez-Freites *et al.* (2002) in which the MIC of INH against *M. tuberculosis* AT35801 (strain Erdman) was 0.03µg/ml and 0.06µg/ml for RIF. The slight difference in our results and those of Alvirez-Freites (2002) may

be attributable to the mycobacterial strain difference as in our study the MIC was determined against *M. tuberculosis* H37Rv. INH was found to be mildly cytotoxic as approximately 80% of the cultured macrophages were still viable at the highest concentration (100µg/ml) tested (Fig. 3.13). In contrast, RIF was more cytotoxic relative to INH and had an IC_{50} of 27.35µg/ml.

All derivatives were solubilized in DMSO prior to determining their antituberculosis potential. We therefore wanted to assess the lowest concentration at which *M. tuberculosis* growth was inhibited. Furthermore we investigated the cytotoxic potential of these compounds in elicited peritoneal macrophages. This was compared to DMSO alone to distinguish between the effect of the synthetic derivatives and the solvent. The MIC of DMSO against *M. tuberculosis* was attained at 2.5% and the *in vitro* (IC_{50}) cytotoxicity at 2.4% DMSO. We selected the DMSO concentration used for the primary screening directly against *M. tuberculosis* to be below 1.25% and below 1.5% for the intracellular killing assay. Therefore we could attribute with confidence any antimycobacterial activity or cytotoxicity obtained directly to the effect of the synthetic derivatives tested.

Acetolactate synthase is an enzyme that catalyses the first step of the branched chain amino acids pathways such as Leucine, Isoleucine, and Valine pathways(Grandoni, Marta et al. 1998). *M. tuberculosis* has to synthesize the branched-chain amino acids in order to survive within the host (Grandoni, Marta et al. 1998). ALS is found in plants and bacteria but not humans (Grandoni, Marta et al. 1998) therefore having ALS inhibitors as TB drugs would be beneficial and not interfere with the human system. By targeting ALS we could potentially inhibit *M. tuberculosis* growth and therefore tested ALS inhibitors for antimycobacterial activity. Of the compounds that were tested from this class, two compounds namely RKG162A and RKG1541 demonstrated antibacterial activity against *M. tuberculosis*. Both RKG162A and RKG1541 inhibited bacilli growth at 10µg/ml for 5 days after adding resazurin to samples and were therefore considered bactericidal, an important property of an antibacterial drug. Interestingly, these two compounds belonged to the same ALS inhibitors subclass namely Acyl thioureas. Substituting bromo benzene on RKG1541 by bromo pyridine to form RKG1542 resulted in loss of antibacterial activity of RKG1541. Similarly when the RKG1541 bromobenzene was substituted with a different aromatic ring to give RKG1543, antibacterial activity was lost. In creating

the Acyl thiourea library, the functional groups of a sulphonyl urea the functional groups of RKG1533PC were substituted with different functional groups except for chlorobenzene an aromatic ring present in proguanil. Proguanil, forms part of Malarone, a drug that is used in the treatment and prophylaxis of uncomplicated *Plasmodium falciparum* malaria (Wiesner, Ortmann et al. 2003). Upon entry into the human host, proguanil is metabolised to form cycloguanil (Birkett DR, vol 37, pg 413, 1994). On the structure of cycloguanil, chlorobenzene, the proguanil aromatic ring is maintained suggesting that this functional group could be of significant importance in the inhibition of the dihydrofolate reductase of the malaria parasites. None of the 13 commercially available sulfonyl ureas, known to inhibit plant ALS were active against *M. tuberculosis*. The lowest concentration of RKG162A and RKG1541 that inhibited *M. tuberculosis* growth was 10µg/ml (Fig.3.11.2). RKG162A and RKG1541 were partially toxic to peritoneal macrophages with an IC_{50} of 17.6 µg/ml and 6µg/ml respectively (Fig. 3.14.1). In summary, the study yielded 2 novel compounds that are highly active against *M. tuberculosis*. Despite their partial toxicity they may be investigated and modified further to enhance its antitubercular potential and decrease its cytotoxicity. Nonetheless, the potential of these compounds are increased as it is targeted towards the pathogen but not the host.

Thiosemicarbazones inhibit the growth of microorganisms by withholding iron from the organism including *M. tuberculosis*, one of the factors that determine the occurrence and outcome of tuberculosis (Kochan 1973). From this class we tested 25 derivatives some of which belonged to the thiosemicarbazones thioesters subclass and the other the ferrosenic thosemicarbazones subclass. Thiosemicarbazone thioesters are termed as such because of the presence of two sulphur molecules on their chemical structures. Ferroceenic thiosemicarbazones on the other hand, contain the ferrocene, a molecule that grant these compounds a greater lipophilicity relative to the thiosemicarbazone thioesters. Due to the high lipophilicity of these compounds, they are more likely to traverse the phagolysosome and destroy the bacilli more readily. This characteristic is important because *M. tuberculosis* is an intracellular pathogen. Two methods of iron chelation by thiosermicarbazones are possible. They can either bind and remove iron from the active site of *M. tuberculosis* and therefore depriving mycobacteria of iron or form a complex with iron while on the active site of *M. tuberculosis*. This results in a toxic complex which causes *M. tuberculosis* death.

Most of the thiosemicarbazones tested act by removing iron from the active site and therefore depriving *M. tuberculosis* of iron. Of 18 derivatives from the ferrocenic thiosemicarbazones, FM19 and FM48 displayed bactericidal activity against *M. tuberculosis*. Two other compounds namely FM04 and FM102 displayed bacteriostatic properties. Both these compounds belonged to the Thiosemicarbazone thioesters. FM19 and FM48 contain the pyridine ring on the left hand side of its chemical structure. It is the pyridine ring that could be exerting the activity against *M. tuberculosis*. FM48 has a secondary amine and FM19 has a tertiary amine on the right hand of their chemical structures. Because of to this difference, FM19 and FM48 were expected to exhibit different mycobacterial inhibition. To our surprise there was no significant difference between the bacterial inhibitions by these compounds. FM19 and FM48 inhibited *M. tuberculosis* by approximately 80%. The structure of FM04 is similar to that of FM75 except that FM75 has an extra pyridine ring. FM04 resulted in delayed growth of *M. tuberculosis* and no activity was observed with FM75. The lack of activity could have been due to the introduction of the extra pyridine ring in FM75. Moreover, we postulate that the lack of activity may be attributed to the presence of two available sides for chelation on FM75. The presence of two possible binding sites could have reduced the selectivity of FM75. However, when FM75 was allowed to form a complex with copper to form FM102, tardy mycobacterial growth was observed. FM71 on the other hand has a similar structure to FM48 except that nitrogen and hydrogen on its aromatic ring were substituted with hydroxyl groups. Moreover, the methyl group on the FM48 is substituted with hydrogen. These substitutions resulted in a loss of antimycobacterial activity that was demonstrated by FM48. The remaining thiosemicarbazones showed no activity against the pathogen. This could be attributed to the absence of pyridine ring as an aromatic ring. FM48 displayed an MIC of 5µg/ml (Fig. 3.11.3). The most potent was FM19 with an MIC of 2.5µg/ml. The difference in the MICs of FM19 and FM48 can be attributed to the presence of a primary amine on FM19 and secondary amine on FM48. Thiosemicarbazones were the most toxic class of derivatives to macrophage cells. The bactericidal thiosemicarbazones (FM19 and FM48) and those that were bacteriostatic (FM04 and FM102) had an IC_{50} of 0.25µg/ml. This suggest that these derivatives need to be modified further in order to reduce the cytotoxic effects of the derivatives

Thiolactomycin (TLM) is the first thiolactone to demonstrate antibiotic activity and was initially isolated from a soil bacterium, *Norcadia* spp (ATCC 31319) (Oishi, Noto et al. 1982). TLM is effective against a broad spectrum of pathogenic bacteria, including Gram-negative, Gram-positive, and *M. tuberculosis* with low toxicity (Miyakawa, Suzuki et al. 1982; Noto, Miyakawa et al. 1982). We therefore tested the thiolactomycins derivatives for antituberculosis activity. No growth inhibition was observed with any of the thiolactomycins tested (Fig. 3.10).The lack of antibacterial activity by these derivatives could also be attributed to drug (compound) metabolism by *M. tuberculosis* that resulted in inactive compounds. Relative to the other isatin derivatives with an R-group as a carbon chain with bromine at the end, ISA1, a derivative that had the shortest carbon chain enhanced *M. tuberculosis* suggesting the usage as a supplement for *M. tuberculosis* growth.

Cysteine protease inhibitors are known to inhibit the hemozoin formation, thus causing a build up of toxic heme/hematin which kills the malaria parasite. Hemozoin is a pigment formed by conversion of the heme group when haemoglobin is hydrolyzed by cysteinse proteases of the *Plasmodium* parasites and therefore releasing the globin. Hemoglobin hydrolysis is important in provide the malarial parasites with amino acids (McKerrow, Sun et al. 1993). Cross species activity of compounds have been reported for isatin-derivatives (Imam and Varma 1975; Webber, Tikhe et al. 1996) and thiosemicarbazones (Condit, Easterly et al. 1991; Finch, Liu et al. 1999). We therefore tested the cysteine protease inhibitors that were designed to inhibit hemozoin formation for malaria parasites for its potential antituberculous activity. Some of these compounds were isatin-derived cysteine protease inhibitors while the others were peptidyl-cysteine protease inhibitors. The peptidyl-cysteine proteases were designed using the dual drug concept (bi-therapeutic strategy). This design was to prevent/slow down the emergence of drug resistance. The double drug molecules consist of two independent modes of action merged via a covalent linker. Out of seven derivatives from this subclass that were tested for antituberculous activity, NAT47 showed bacteriostatic activity (Fig. 3.10). NAT46, NAT47, and NAT48 had similar chemical structures. NAT47 however, had a longer carbon chain on the left hand side of the linker molecule. The delayed growth could be attributed to the higher number of carbon atoms present. NAT46, NAT47, and NAT48 had a quinoline attached to the carbon chains present on either side of the linker. In NAT32, NAT50,

and NAT60, the right hand quinoline-attached carbon chain was substituted by other functional groups. This resulted in loss of activity that was displayed by NAT47. No activity was observed with NAT18, a derivative in which both the carbon chain attached quinoline structures present on NAT47 were substituted with a hydroxyl group and an aromatic ring. NAT47 exhibited an IC_{50} of 9.75µl/ml (Fig. 3.14.2). In isatin-derived cysteine protease inhibitors, 24 derivatives were tested for antibacterial activity against *M. tuberculosis*. Four compounds derivatives namely AXE1, AXE4, AXE5, and AXE29 were bactericidal. On the other hand, AXE2, AXE3, and AXE35 were bacteriostatic.

Structural similarities were observed between AXE4 and AXE5 while AXE1 and AXE29 had different chemical structures suggesting that these compounds may have different mechanism of *M. tuberculosis* growth inhibition. The difference between AXE1, AXE4 and AXE5 was the absence of the aromatic ring on AXE1. However, similar inhibition capacity was observed with these compounds. This could be brought about the presence of electron rich sulphur on AXE1, nitrogen on N-methylpirerizene of AXE4, and oxygen on morpholine of AXE5. These electron rich atoms increase the binding capacity of these derivatives by easily forming bonds using lone pairs of electron on the active sites. Partial antitubercular activity of AXE2 and AXE3 may be attributed to the absence of electron rich atoms on the aromatic rings of these compounds. When one hydrogen on isatin of AXE1 was substituted with bromine, chlorine, fluorine of nitrogen dioxide forming AXE9, AXE10, AXE11, AXE12, AXE17, the activity observed with AXE1 was lost. Most of the 4-aminoquinolines isatin thiosemicarbazones were not active against *M. tuberculosis*. However, substituting one hydrogen on the isatin molecule of AXE34 with a methyl group form AXE35 increased the activity of the AXE34 to partial inhibition of *M. tuberculosis*. These could be due to the bulkier size of methyl group resulting in a better fit into the active site. However, when the carbon chain of AXE35 was elongated to form AXE36 and AXE37, activity of AXE35 was lost. MICs for AXE1, AXE4, AXE5, and AXE29 were 10µg/ml, 10µg/ml, 5µg/ml, and 10µg/ml respectively (Fig. 3.11.3). AXE1, AXE4, and AXE5 were highly toxic to macrophage cells with IC_{50} of <0.25µg/ml (Fig. 3.14.2.1). In contrast, AXE29 displayed partial toxic effects with IC_{50} of 16.08µg/ml.

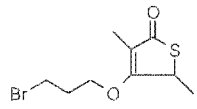
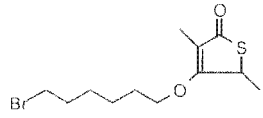
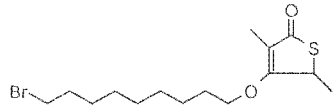
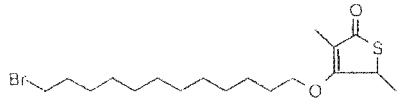
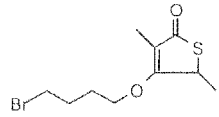
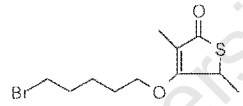
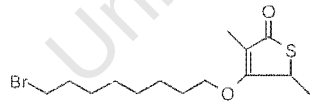
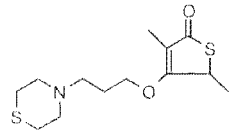
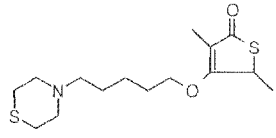
M. tuberculosis is an intracellular pathogen thus the anti-TB drugs are expected to traverse the *M. tuberculosis* target cells' membrane and destroy the bacilli. AXE29 and RKG162A were the two active compounds that displayed less toxic effects on macrophages. We therefore investigated the ability of AXE29 and RKG162A to traverse the phagolysosome and destroy the bacilli. Two known TB agents INH and RIF were included as known compounds with the ability of to cross the cell membrane. *M. tuberculosis* was extensively decreased when treated with INH and RIF at both 1μg/ml and 10μg/ml (Fig. 3.15). In contrast, there was no significant difference between the untreated samples and those treated with AXE29 and RKG162A at 1μg/ml. However, increase in concentration of AXE29 and RKG162A increased activity of the two derivatives. From this it could be concluded that AXE29 and RKG162A have the ability to cross cell membranes implying that *in vivo* chemotherapy to target *M. tuberculosis* with the two compounds was possible.

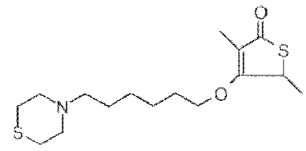
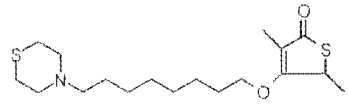
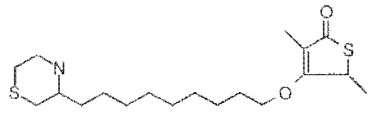
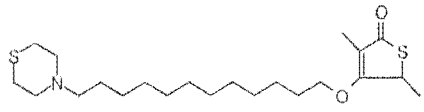
The potential of AXE29 and RKG162A as possible TB agents was shown in chapter 4. In this study the therapeutic potential of these compounds was investigated in a murine TB model. In preparing DMSO-dissolved AXE29 and RKG162A for *in vivo* application, a variety of solvents was used to lower the DMSO concentration of the above mentioned derivatives (Table 4.1). Both AXE29 and RKG162A precipitated out suggesting that they were unsuitable for *in vivo* use. We therefore tested DMSO for toxic effects in a mouse model. No mortality or weight loss was observed when mice were treated daily with DMSO for 10 days suggesting that any mortality that would result during treatment of mice with DMSO-dissolved derivatives would be attributable to the derivatives. Prior to therapeutic testing of AXE29 and RKG162A, we determined the maximum tolerated dose (MTD) of these derivatives in uninfected mice. Mice were treated daily with 25, 50, and 100mg/kg of compound for 1 week. Both AXE29 and RKG162A had an MTD of 50mg/kg (Table 4.3 & 4.4). AXE29 resulted in 25% mortality and 75% mortality for RKG162A. Surprisingly, when *M. tuberculosis* infected mice were treated with 50mg/kg RKG162A, mice survived for 1 week (Fig. 4.3). At the end of treatment phase (day 14), the bacilli burden had increased significantly ($p<0.05$) in the organs of the untreated group (fig 4.4). As expected, the organs of the INH treated mice were culture negative (Fig. 4.4). The lungs and spleen of DMSO and AXE29 treated mice had similar bacilli load (Fig. 4.4A&C). The liver of the DMSO treated mice was

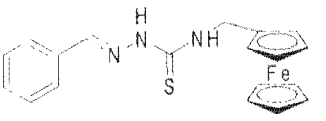
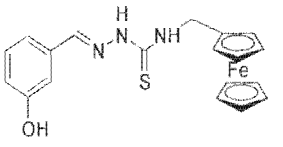
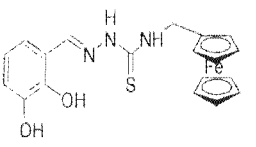
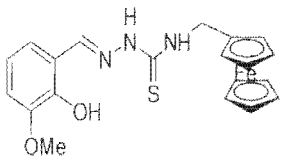
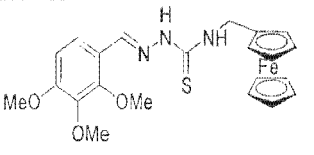
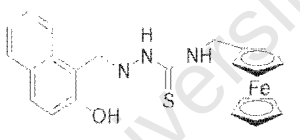
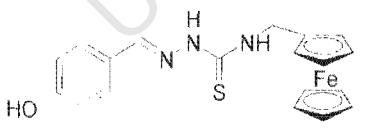
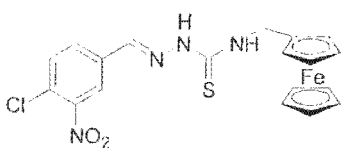
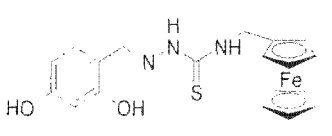
culture negative and the bacilli burden of AXE29 treated mice was at approximately log 1.2 (Fig. 4.4B). The extent of infiltration was similar for the untreated, DMSO, and AXE29 treated group (Fig. 4.5). In contrast, the extent of infiltration was less when compared with the untreated group. These findings suggest lack of therapeutic properties of AXE29. The lack of AXE29 *in vivo* antitubercular activity could have been brought about by a few factors including bioavailability of compounds, low concentration, duration of treatment, and metabolism. For derivatives to exhibit active, they have to be absorbed into the blood stream and transported to the different organs for derivatives to be available in the various organs. The lack of AXE29 *in vivo* antitubercular efficacy could have been due to lack of absorption of this compound resulting in no bioavailable AXE29 to kill *M. tuberculosis*. The lack of activity could also be attributed to drug metabolism of AXE29 by the mice liver resulting in inactive metabolite/s. Also it could have been that the duration of treatment was too short to show the potential of AXE29 to kill mycobacteria or that the AXE29 concentration was too low to kill the bacilli.

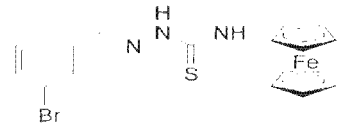
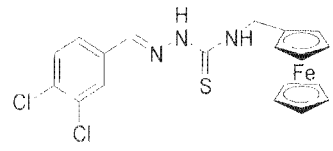
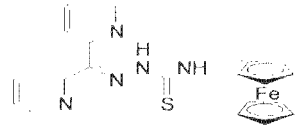
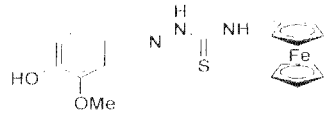
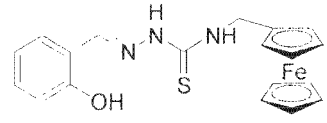
The investigation of the *in vitro* antimycobacterial activity of cysteine protease inhibitors, ALS inhibitors, thiolactomycins, and thiosemicarbazones has indicated that 8 derivatives namely FM19, FM48, AXE1, AXE4, AXE5, AXE29, RKG162A and RKG1541 have emerged as new compounds with antimycobacterial activity. However FM19, FM48, AXE1, AXE4, AXE5, and RKG1541 displayed high toxicity AXE29 and RKG162A mild toxicity against elicited peritoneal macrophage cells. The mildly cytotoxic RKG162A and RKG162A were tested in a murine model for therapeutic efficacy. In this study, AXE 29 did not show any therapeutic properties and RKG162A resulted in 100% mortality by day 7 of treatment phase. In conclusion, although the above mentioned 8 derivatives where either toxic against the elicited peritoneal macrophages in vitro, toxic in an *in vivo* model, or did not demonstrate any therapeutic efficacy *in vivo*, they can be used as scaffolds in construction of new chemical library with potential antimycobacterial properties.

Appendix

Code	Structure	MF
ISA 1		C9H13BrO2S
ISA2		C12H19BrO2S
ISA3		C15H25BrO2S
ISA4		C18H31BrO2S
ISA 5		C10H15BrO2S
ISA 6		C11H17BrO2S
ISA 7		C14H23BrO2S
ISA 8		C13H21NO2S2
ISA 9		C15H25NO2S2

ISA 10		C16H27NO2S2
ISA 11		C18H31NO2S2
ISA 12		C19H33NO2S2
ISA 13		C22H39NO2S2

FM 95		C19H18FeN3S
FM78		C19H18FeN3OS
FM71		C19H18FeN3O2S
FM73		C20H19FeN3O2S
FM82		C22H21FeN3O3S
FM80		C23H20FeN3OS
FM79		C19H18FeN3OS
FM92		C19H16ClFeN4O2S
FM72		C19H18FeN3O2S

FM86		C19H17BrFeN3S
FM91		C19H16Cl2FeN3S
FM96		C23H20FeN5S
FM81		C19H18FeN3O2S
FM77		C19H18FeN3OS

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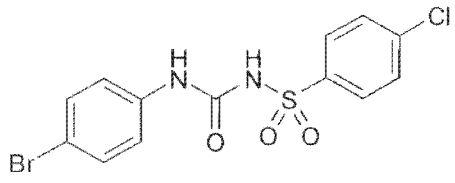
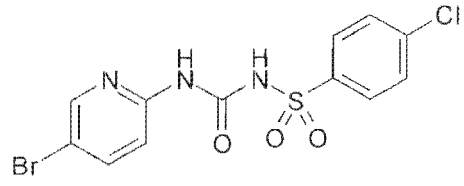
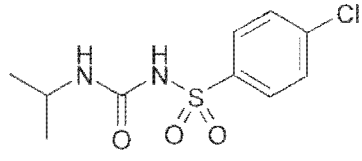
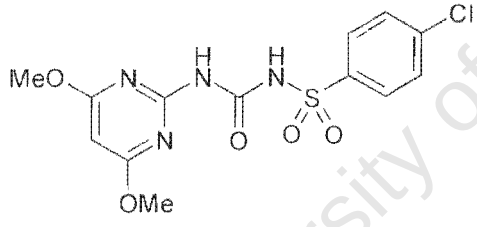
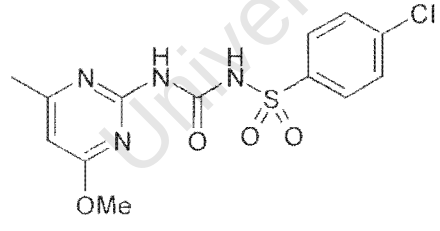
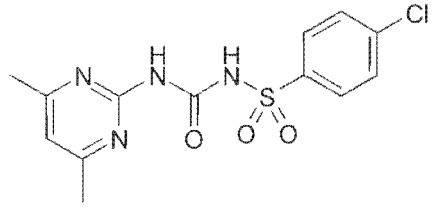
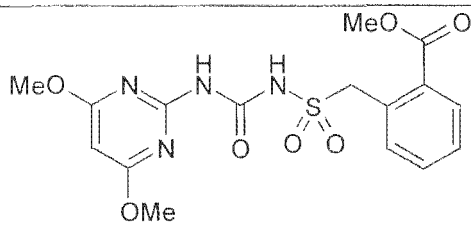
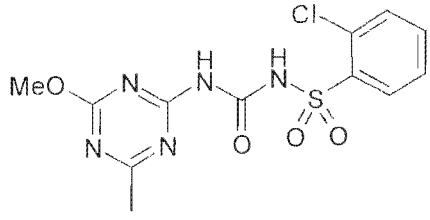
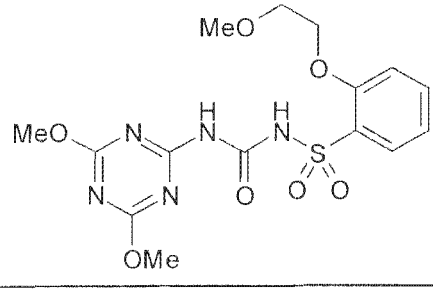
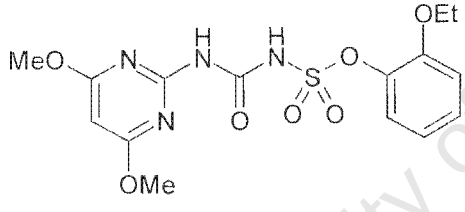
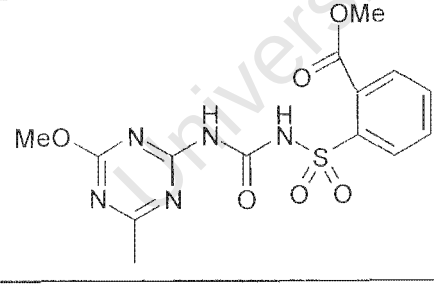
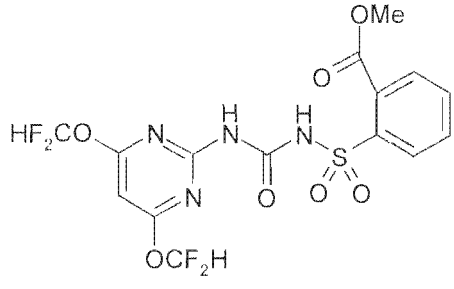
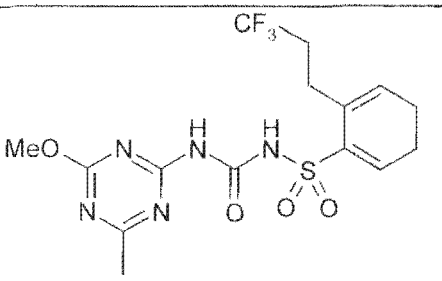
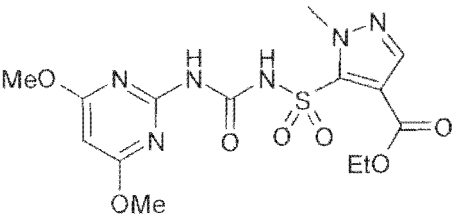
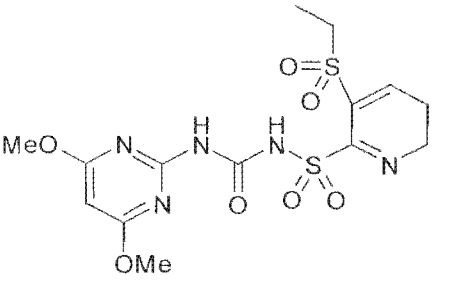
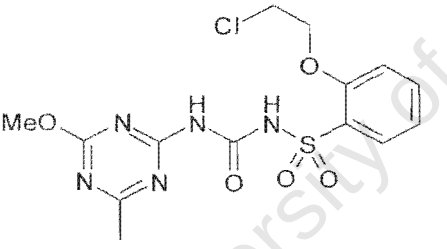
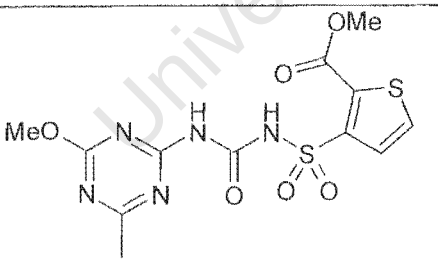
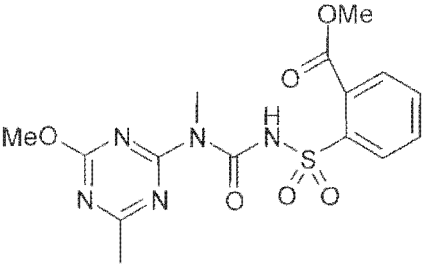
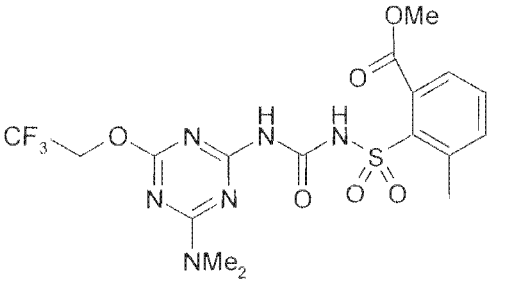
RKG1491		C13H10N2O3SClBr
RKG1492		C12H9N3O3SClBr
RKG1493A		C10H13N2O3SCl
RKG1531		C13H13N4O5SCl
RKG1532		C13H13N4O4SCl
RKG1533PC		C13H13N4O3SCl

RKGC2Be		C16H18N4O7S
RKGC3Ch		C12H12N5O4SCl
RKGC4Ci		C15H19N5O7S
RKGC5Et		C15H18N4O7S
RKGC6Me		C14H15N5O6S
RKGC7Pri		C15H12F4N4O7S

RKGC8Pro		C15H16F3N5O4S
RKGC9Py		C14H18N6O7S
RKGC10Ri		C14H17N5O7S2
RKGC11Tria		C14H16N5O5SCl
RKGC12Th		C12H13N5O6S2
RKGC13Trib		C15H17N5O6S

RKGC14Trif		C17H19F3N6O6S

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