ANTIMYCOBACTERIAL ACTIVITY OF SYNTHETIC COMPOUNDS ISOLATED FROM SOUTH AFRICAN MEDICINAL PLANTS AGAINST *MYCOBACTERIUM TUBERCULOSIS*

ELIZABETH RAMADIMETSA LEDWABA

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Dissertation submitted in fulfillment of the requirements for the degree of

MASTER OF TECHNOLOGY

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Vaal University Technology

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November 2014

CONFIDENTIALITY CLAUSE

TO WHOM IT MAY CONCERN

This work is of strategic importance.

The contents of this dissertation are to remain confidential and not to be circulated for a period of five years.

Sincerely,

E. R. Ledwaba

Date:

DECLARATION

I **Elizabeth Ramadimetsa Ledwaba** declare that this dissertation has never been submitted to any other University or Academic Institution for purposes of getting an academic award. All the information in this dissertation is based on my observations.

Signed: E.R Ledwaba

Date.....

STATEMENT 1

This dissertation is being submitted in partial fulfillment of the requirements for the degree of Master of Technology (Biotechnology).

Signed: E.R Ledwaba

Date……

STATEMENT 2

The dissertation is the result of my own independent work/investigation, except where otherwise stated. Other sources are acknowledged by giving explicit references. A bibliography is appended.

Signed E.R Ledwaba

Date……..

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DEDICATION

I would like to dedicate this piece of work to my mom and dad Leah and Peter Ledwaba and also to my brothers and sisters for everything they have done for me.

ABSTRACT

Tuberculosis (TB) remains one of the most difficult infectious diseases to control in the world today. The disease spreads easily in overcrowded, badly ventilated places and among people who are undernourished. Trends in the incidence of TB together with the development of multi-drug (MDR-TB) and extensively drug resistant (XDR-TB) strains of TB raises the need to intensify the search for more efficient drugs to combat this disease. Herbal remedies used in traditional medicine provide an interesting and largely unexplored source for the discovery of potentially new drugs for infections such as TB. The aim of the study was to evaluate the *in vitro* antimycobacterial activity of synthesized compounds from medicinal plants against *Mycobacterium tuberculosis* (*M. tuberculosis*). About 40 synthesized compounds isolated from South African medicinal plants were screened against H37RV using microplate alamar blue assay (MABA). Identified active compounds were screened against resistant strains of *M. tuberculosis* (MDR, XDR and pre-XDR) and sensitive clinical isolates of TB. Cytotoxicity and synergistic drug combination studies were done on active compounds to validate their toxicity and synergy levels. Cytotoxicity was done by sulforhodamine assay (SRB) against the C2C12 cell line. Only six compounds showed activity against *M. tuberculosis* with minimum inhibitory concentration (MIC) below 10µg/ml. The results obtained indicated that the cytotoxicity effects of the three compounds on C2C12 cells demonstrated marginal toxicity except for MVB 282/61215 which showed a high toxicity at the lowest concentration of 0.156µg/ml with over 100% viable cells at the highest concentration (5µg/ml). MVB 282/61271 had the highest percentage cell viability (65%) at the lowest concentration. Only two compounds had a higher potency evoking a bigger response at low concentrations with treated cells still viable after 3 days of incubation with the compound which was comparable with the treatment of isoniazid (INH). Synergistic activity of the six compounds was less in INH combination as compared to the rifampicin's (RIF) combination. The results demonstrated that the synergistic interaction between the compounds and RIF could the antituberculosis acitivity. In conclusion the synergistic effects with RIF translate to lower dosing requirements of the compounds and the potential to combat multidrug resistant TB. In deed there is no doubt that natural products, with their range of interesting chemical structures and powerful antimycobacterial effects are certain to remain important participants in the development of new generations of antimycobacterial drugs.

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LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
CFU	Colony Forming Units
CSIR	Council for Scientific and Industrial Research
DCS	Cycloserine
DMEM	Dulbecco's modified eagle media
DMSO	Dimethyl sulfoxide
EBA	Early bactericidal activities
EMB	Ethambutol
ETH	Ethionamide
FIC	Fractional inhibitory concetration
GIC	Growth inhibition concetration
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HTS	High throughput screening
ICL	Isocitrate lyase
INH	Isoniazid
KAN	Kanamycin
MABA	Microplate alamar blue assay
MDR	Multidrug resistant
MIC	Minimum inhibitory concetration
NAD	Nicotinamide adenine dinucleotide
PAS	p-aminosalcyclic acid
PZA	Pyrazinamide

RIF	Rifampicin
SPOTi	Spot culture growth inhibition
SRB	Suforhodamine assay
STR	Streptomycin
TB	Tuberculosis
TCA	Trichloroacetic acid
WHO	World health organization
XDR	Extensively drug resistant

CHAPTER 1

1.1 INTRODUCTION

Tuberculosis (TB) is a contagious infectious disease caused by species belonging to the *Mycobacterium tuberculosis* complex. At present, it is a re-emerging disease, due to co-infection with the human immunodeficiency virus (HIV), but also to global bacterial resistance, and lack of adequate treatment in some places in the world. Approximately one third of the world's population is infected with *M. tuberculosis*, and out of these people, about 1.1 million people die every year of TB (WHO 2011), making this disease the main cause of bacterial infectious death in adolescents and adults all around the world. In 2011 there was an estimation of 8.8 million incident cases and 12.0 million prevalent cases of TB worldwide. *M. tuberculosis* drug-resistant isolates have appeared giving origin to multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains. XDR-TB has been identified in every continent of the planet. By 2012, the World Health Organization was notified of the existence of 53 018 cases of multi-drug resistant TB (MDR-TB) worldwide; a figure that only represents 18% of the TB-MDR estimated cases among reported pulmonary TB cases around the world (WHO 2012). Currently, there is global alarm since the infection with these strains is cured only in 66% of MDR cases and in 60% of the XDR cases (Mitnick, Beyond, Palaceous, Shin, Furin, Alacantara, Sanchez, Sarria, Becerra, Fawzi, Kapiga, Neuberg, Maguire, and Kim & Farmer 2012).

More than sixty years ago, the introduction of the first anti-TB drugs for the treatment of TB (streptomycin (STR), *p*-aminosalcylic acid (PAS), isoniazid (INH) and then later ethambutol (EMB) and rifampicin (RIF) gave optimism to the medical community, and it was believed that the disease would be completely eradicated soon. After a 30-year halt of anti-TB drug Research & Development pipeline, the Global Alliance for TB Drug Development (TB Alliance) started to fill the gap between the existing chemotherapeutics and the clinical need (Sacks & Behrman 2010). The need for new drugs to extend the range of TB treatment options is acute. New chemical entities with novel mechanisms of action will most likely possess activity against MDR-TB (Cohen & Murray 2010). However; these alone will not provide the breakthrough that is needed. The key to improving therapy is to develop new agents with potent sterilizing activity that will lead to shortening of the duration of chemotherapy (O'Brien & Spigelman 2005). In

order to meet the goal of finding the next generation TB medicine within the next decade, a significant increase in effort is required at an early stage of the drug discovery process. The known pipeline is relatively sparse and many potential new drugs will be lost by slow destruction as they proceed through development. It is therefore critical that new leads are identified and then properly resourced leading optimization programmes established. A few new molecules have been disclosed as potential leads for TB drug discovery. These have been identified in complementary screening strategies to secure active entities, which are based on either whole-cell evaluation or profiling against specific biochemical targets. As the treatment of TB infections typically necessitates extended oral dosing regimens, an agent is needed that is both economical to produce and preferably highly specific for mycobacteria to minimize unwanted side effects associated with disturbance of the normal gut flora. Unfortunately, most of the compounds described are interesting only because of their activity against growing *M. tuberculosis*. Further effort must be made to identify compounds acting on key targets that are essential for persistence of *M. tuberculosis* if a real breakthrough in therapy is to be made (Rivers & Mancera 2008).

Plant products have received considerable attention as potential anti-TB agents with a recent review emphasizing plant products as sources of antimycobacterial extracts and compounds. Most traditionally used plant therapies rely for their effects on a variety of compounds and synergy between these compounds, and there are numerous benefits for isolating and identifying active constituents from these bioactive plants. These benefits include characterizing toxicity profiles, simpler determination of modes of action and new activities of a known compound which adds to wealth of information on phytochemicals. Combining plant extracts and current TB drugs holds advantages such as decreased toxicity profiles, increased bioavailability and activity and reduced onset of microbial resistance (Negi, Kumar, Luqman, Saika & Khanuja 2010).

Natural products continue to play the most significant role in the drug discovery and development process, and plants are recognized as a useful source of highly active antimycobacterial metabolites (Mcgaw, Lall, Meyer & Eloff 2008). Natural products or their semi synthetic derivatives have indeed provided novel drug leads for tuberculosis therapy (Shu

1998). Examples of such compounds include streptomycin and kanamycin from *Streptomyces griseus* and capreomycin isolated from *Streptomycin capreolus* (Copp 2003; Shu 1998). Rifampicin is a semi-synthetic drug that has been derived from Rifamycin; a product of *Amycolatopsis mediterranei* (Tribuddharat & Fennewald 1999). Plant drugs contain chemical compounds that act individually or in combination on the human body to prevent infections and maintain health. The search for used plants is still relevant due to the appearance of microbial resistance to many antibiotics and the occurrence of opportunistic infections. Ethnobotanical data has proved to be useful in the search of compounds isolated from plants. Thus, there has been renewed interest in phytomedicine during the last decade and these days many medicinal plant species are being screened for pharmacological activity.

1.2 PROBLEM STATEMENT

The use of medicinal plants still plays a vital role in covering the basic health needs in developing countries where pharmaceuticals are not available or are unaffordable. Herbal remedies used in traditional medicine provide an interesting and largely unexplored source for the discovery of potentially new drugs for chemotherapy especially to treat infections such as tuberculosis. South Africa has a remarkable diversity of flora that has not been satisfactorily explored in terms of their anti-tubercular activity. It is on this basis that medicinal plants with a reputation of effective use in treatment of tuberculosis will be investigated.

1.3 HYPOTHESIS

Medicinal plants exist that are reputed to be effective against tuberculosis. For this reason it is envisaged that synthetic compounds that have effective anti-mycobacterial activity against *Mycobacterium tuberculosis* can be extracted from such plants.

1.4 AIM

To determine the *in vitro* antimycobacterial activity of synthetic compounds isolated from South African medicinal plants against *Mycobacterium tuberculosis.*

1.5 OBJECTIVES

The objectives of the study were:

- To identify the compounds that has the necessary *in vitro* activity against *M. tuberculosis* by using microplate alamar blue assay technique.
- To determine the toxicity level of identified active compounds by using sulforhodamine (SRB) assay.
- To determine the synergistic antimycobacterial activity of identified active compounds. This was obtained by using the microplate alamar blue assay.

CHAPTER 2

FIRST LITERATURE REVIEW

2.1 MYCOBACTERIUM TUBERCULOSIS COMPLEX

Mycobacterium tuberculosis is the causative agent of TB. Other names given to TB since ancient times are Potts disease or phthisis or scrofula. *M. tuberculosis* belongs to the genus *Mycobacterium* and family *Mycobacteriaceae*. The genus contains over 50 species including *Mycobacterium tuberculosis*, the most common pathogen for humans, *M. africanum* common in West Africa and *M. bovis* that causes infection in animals but can also infect humans (van Ingen, Rahim, Mulder, Boeree, Simeone, Brosch & van Soolingen 2012). Other members of *M. tuberculosis* complex include *M. bovis, M. microti, M. caprae, M. pinnipedii, M. canetti* and *M. mungi.* Furthermore *M. avium* and *M. scrofulaceum* are potentially pathogenic species that have been reported to cause opportunistic infections in HIV/AIDS patients (Parsons, Somaskövi, Gutiererrez, Lee, Paramasivan, Abimiku, Spector, Rascigno & Nkengasong 2011). It is rod (Fig. 1) shaped, 0.2-0.5µm in diameter and 2.4µm in length, aerobic, non-motile and is neither Gram positive nor Gram negative, as it does not retain any Gram stains due to the high lipid content in its cell wall. Therefore, Ziehl-Neelsen staining (involving carbolfuchsin, acid alcohol and methylene blue) is used which allows it to be categorized as an acid fast bacterium (Murray, Kreiswirth, Barry & Barker 2006).

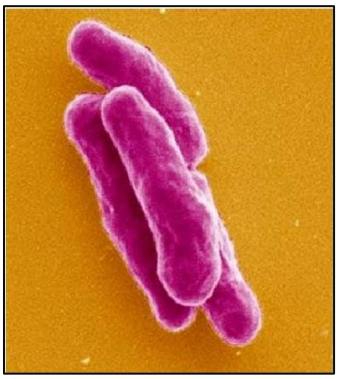

Figure 1: Rods of *M. tuberculosis* (http://en.wikipedia.org/wiki/*Mycobacterium_tuberculosis* _complex)

Mycobacteria are obligate aerobes and derive energy from the oxidation of many simple carbon compounds. Increased carbon dioxide tension enhances growth. The growth rate is much slower than that of most bacteria. The doubling time of tubercle bacilli is about 18 hours (Murray, Kreiswirth, Barry. & Barker 2006). Mycobacteria are rich in lipids; these include mycolic acids, complex waxes, and phospholipids. In the cell, the lipids are largely bound to proteins and polysaccharides. The high lipid content of the cell wall binds fuchsin dye so that it is not destained by acid alcohol, which is why mycobacteria are referred to as "acid - fast bacilli" (AFB's). This important property allows differential staining in contaminated clinical specimens such as sputum. Cox, Ford & Reeder (2009) have suggested that the function of mycolic acids may be involved in the export of proteins secreted by the organism. Pore proteins are thought to mediate the diffusion of particles across the cell wall (Podust, Poulos & Waterman 2001). The cell wall of *mycobacteria* represents numerous potential drug targets. The insoluble cell wall core needs to be maintained for organism viability, for instance, representing a very attractive target (Pfyffer 2007). Enzymes responsible for the incorporation of mycolic acids in the cell wall are also expected to be potent targets.

Although mycobacteria are normally cultured from clinical material by inoculation onto enriched agar media containing bovine serum albumin, they can grow on a chemically defined medium containing asparagine, glycerol, and micronutrients. Even under ideal culture conditions *M. tuberculosis* grows very slowly, with doubling times in the order of 18 to 24 hours (Velayati & Farnia 2012).

2.2 EPIDEMIOLOGY OF TB

TB is contagious and airborne. It is a disease of poverty affecting mostly young adults in their most productive years (Dye & Williams 2010). Out of 100%, 95% of TB deaths are in the developing countries (WHO 2011). In 2011 there were an estimated 8.7 million incident cases of TB (range, 8.3 million–9.0 million) globally, equivalent to 125 cases per 100 000 population. Most of the estimated number of cases in 2011 occurred in Asia (59%) and Africa (26%); one smaller proportion of cases occurred in the Eastern Mediterranean Region (7.7%), the European Region (4.3%) and the Region of the Americas (3%) (WHO 2012). The World Health Organization (WHO) suggests that the number of new TB cases world-wide will rise from the

current 7 million a year to 10 million by 2015. It is estimated that between the years 2000 and 2020, nearly one billion people will be newly infected, 200 million people will get sick and 70 million will die from the disease if the control of the disease is not strengthened (WHO 2011). Within the black population of South Africa TB is endemic. In South Africa over three in every thousand people die of TB, the highest rate in the world. TB is the most commonly notified disease in South Africa and the fifth largest cause of death among the black population. In the United States, the number of TB cases steadily decreased until 1986 after which an increase was noted; TB cases have continued to rise since (Brewer & Heymann 2011).

TB has already been recognized as one of the most frequent opportunistic infections in persons with HIV infection in developing countries. HIV results in an impairment of the immune system and entails a substantial risk of TB in those individuals who are or become infected with the tubercle bacillus (Grenier, Pinto, Nair, Steingart, Dowdy, Ramsay & Pai 2012). The diagnosis of infection caused by *M. tuberculosis* is of public health concern following an increase in the number of TB cases in developing countries and a major increase in developing countries is associated with the spread of HIV infection. TB accounts for almost one third of AIDS deaths worldwide, which kills 2 million people each year and infects 8 million more; almost all of them in developing countries. It causes about 40% of AIDS-related deaths in Africa and Asia (Lönnroth, Castro, Chakaya, Chauhan, Floyd, Glaziou & Raviglione 2010). Control of TB epidemic linked with HIV infection will depend largely on the adequate treatment of TB, and possibly of effective chemoprophylaxis not just for HIV infected persons but for the community as well (Frieden 2002).

2.3 TRANSMISSION AND PATHOGENESIS TB

M. tuberculosis, the infectious agent of TB, is a thin, slightly curved bacillus that is an obligate aerobe. In comparison to other bacteria, *M. tuberculosis* has a cell wall with a very high lipid content that resists staining by the usual Gram method (Flynn & Chan 2001). However, it accepts basic fuchsin dyes and is not easily decolourized even with acid alcohol; this resistance to decolourization by acid-alcohol is termed acid-fast borne droplet nuclei that are produced when persons with pulmonary or laryngeal TB cough, sneeze, speak, or sing. The particles, which measure 1–5μm in size, can be kept airborne by normal air currents for prolonged periods

of time, resulting in dispersion throughout a room or building. The presence of acid-fast bacilli in the sputum smear is the main indicator of potential for transmission (Flynn & Chan 2001).

Infection occurs when a susceptible person inhales droplet nuclei that contain tubercle bacilli. As the distribution of inhaled droplet nuclei is determined by the ventilatory pattern and volumes of the various lung lobes, the site of implantation preferentially occurs in the middle and lower lung zones, although any lobe may be affected. Once lodged in the alveolus, *M. tuberculosis* is ingested by alveolar macrophages (Phillips & Ernst 2012).

Resistance to the establishment of tuberculous infection is known to be under genetic control, and the course of infection depends on the interaction between the inherent microbiocidal power of the alveolar macrophage and the virulence of the ingested bacillus. If the alveolar macrophage cannot destroy or inhibit *M. tuberculosis*, the bacilli multiply within its intracellular environment, causing the host macrophage or its progeny to burst (Druszczynska, Kowalewicz-kubat, Fol, Wodarczyk & Rudnicka 2012).

The cycle continues as released bacilli are ingested by other alveolar macrophages and monocytes are recruited from the blood. During this period of rapid growth, tubercle bacilli are spread through lymphatic channels to regional hilar and mediastinal lymph nodes and through the bloodstream to more distant sites in the body. The logarithmic phase of bacillary growth is arrested with the development of cell-mediated immunity and delayed-type hypersensitivity at 2–10 weeks after the initial infection. Development of specific immunity is usually adequate to limit further multiplication of the bacilli; the host remains asymptomatic, and the lesions heal (Sakamoto 2012). Some of the bacilli remain dormant and viable for many years, and this condition referred to as latent TB infection may be detectable only by means of a positive purified protein derivative tuberculin skin test or radiologically identifiable calcification at the site of the primary lung infection or in regional lymph nodes (Guptaa, Kaula, Tsolakib, Kishoreb & Bhakta 2012).

2.4 CLINICAL SIGNS AND SYMPTOMS

The clinical signs and symptoms of pulmonary TB in an infected adult are often nonspecific; complete absence of symptoms occurs in approximately 5% of active adult cases. Systemic manifestations include low-grade fever, anorexia, fatigue, night sweats, and weight loss that may persist for weeks to months. Cough is the most frequent symptom referable to the site of lung infection. Early in the disease, it may be nonproductive, but subsequently there usually is production of mucoid or mucopurulent sputum. Hemoptysis (coughing up blood) may also occur. Inflammation adjacent to a pleural surface can cause pleuritic chest pain (Bark, Dietzec, Okwerad, Quelapio, Thiel & Johnson 2012).

2.5 TREATMENT OF TB

Effective treatment of TB involves targeting the multiple populations of bacteria that reside in the host. Since the control measures for TB such as Bacillus Calmette- Guérin (BCG) vaccination and chemoprophylaxis appear to be unsatisfactory, treatment with anti-tubercular (anti-TB) drugs becomes the only option available. The goals of treatment are to ensure cure without relapse and to prevent the emergence of drug resistance. Long-term treatment with a combination of drugs is required. Treatment of active TB with a single drug should never be attempted, and a single drug should never be added to a failing regimen, the result being development of MDR TB (Streicher, Müller, Chihotaa, Tait, Pillay, Trollip, Hooke, Sirgel, van Pittius, van Helden, Victor & Warren 2012). As suggested by WHO, treatment of TB and drug resistant cases requires multi-drug therapy, comprising of an initial intensive phase of RIF, INH, pyrazinamide (PZA), and ethambutol (EMB) daily for 2 months. Secondly a continuation phase of RIF and INH for a further 4 months, either daily or 3 times per week is to be administered. Isoniazid eradicates most of the rapidly replicating bacilli in the first 2 weeks of treatment, together with streptomycin and ethambutol (Parsons *et al.* 2012). Thereafter, rifampicin and pyrazinamide have an important role in the sterilization of lesions by eradicating organisms; these two drugs are crucial for successful 6-month treatment regimens. Rifampicin kills low or non-replicating organisms and the high sterilizing effect of pyrazinamide serves to act on semi-dormant bacilli not affected by any other anti-TB agents in sites hostile to the penetration and action of the other drugs. Isoniazid and rifampicin, the two most potent anti-TB drugs, kill more

than 99% of tubercular bacilli within 2 months of initiation of therapy (Velayati & Farnia 2012). Using these drugs in conjunction with each other reduces anti-TB therapy from 18 to 6 months.

Streptomycin (SM) is nearly as effective as EMB; however, several factors have discouraged its use in more recent times. Oral formulations in multidrug regimens are not feasible requiring frequent patient's visits to health care facilities. Globally, the highest level of resistance to an anti-TB drug is also observed for streptomycin (Dale, Bothamley, Proniewski, Gillepsie, Mchugh & Pitman 2005). Streptomycin may be used as a first-line or second-line drug for treating patients with failing therapy or MDR-TB provided the *M. tuberculosis* strain is susceptible to streptomycin (Ahmad & Mokaddas 2010).

2.6 TARGETS AND MODE OF ACTION OF ACTIVE PRINCIPLES CURRENTLY USED IN THE TREATMENT OF TB

Current TB chemotherapy is based on the combination of four anti-TB drugs which inhibit the bacterial metabolism, particularly the cell wall synthesis. During the therapy, the goal of this drug combination strategy is to effectively prevent the mutational events. According to their action mode, first and second line anti-TB drugs are grouped into cell wall inhibitors (INH, EMB, ethionamide (ETH), and cycloserine (DCS)), protein synthesis inhibitors (RIF, fluoroquinolones, STR, kanamycin (KAN)), and membrane energy metabolism inhibitors (PZA) (Brewer & Heymann 2011).

Current chemotherapy principally inhibits cell processes such as cell wall biosynthesis and DNA replication, and they only turn to be active regarding bacteria in active growth (Kolyva & Karakousis 2012). This implies that the chemotherapeutic agents in use are efficient bacteriocides but are poor sterilizers, not able to kill "dormant" *M. tuberculosis* which persists in macrophages after the death of the active bacteria. RIF and PZA have a partial sterilizing activity and they play an important role in the decrease of therapy from 18 to 6 months, even though there is a persistent population surviving these two agents. Consequently the current therapy ensures a clinical cure but fails to obtain a bacteriological cure (Udwadia, Amale, Ajbani & Rodriques 2012)

2.7 WHY NEW TB DRUGS ARE NEEDED?

Whereas it is true that TB can be cured with the current active principles, treatment is complex and long, involving four drugs for two months and two drugs for four months more as a minimum. During the initial chemotherapy phase (2 months), actively dividing bacilli rapidly die mostly because of INH bactericidal activity. Thereafter bacilli of low metabolic activity suffer from a slow death under the effects of RIF and PZA. There is evidence that persistent bacillary population existing in the lesions usually determines the duration of therapy (WHO 2012). Therefore efforts need to be made to target every physiological state of *M. tuberculosis* thus shortening the time of therapy and inhibiting the appearance of drug resistance.

Since the start of the chemotherapeutic era, physicians have realized the slowness and difficulty of achieving an effective cure. McDermott, Griffiths-Johnson & Nicholls proved in 1956 that the *in vitro* efficacy of first-line TB drugs does not correlate to their *in vivo* efficacy (Casenghi 2006).

Table 1: Reported MIC and molecular targets drugs of first and second-line drugs used in the treatment of TB (Ribon 2012)

Active principle (Year of discovery)	**Source**	**MIC (µM)**	**Action Mechanism**	**Target Site**	**Genes involved in the resistance**
Isoniazid (1952)	Synthetic	0.182	Mycolic acids synthesis, inhibition, multiple effects on DNA, Lipids & carbohydrates	Enoylreductase (InhA)	*KatG, InhA,ndh*
Rifampicin (1966)	Semi-synthetic	0.486	RNA synthesis inhibition	RNA polymerase β sub-unit	*rpoB*
Pyrazinamide (1952)	Sythetic	490 pH 5.5	Breakage of transport membrane & energetic depletion	Membrane energy metabolism	*pncA*
Ethambutol (1961)	Synthetic	2.45	Arabinogalatanbiosynthesis inhibition	Arabinosyltransferase	*embC AB*
Streptomycin (1944)	Natural	1.72	Protein synthesis inhibition	rRNA ribosomal proteins S12 and 16S	*rpsL, rrs*
Kanamycin (1957)	Natural	3.43	Protein synthesis inhibition	rRNA ribosomal proteins S12 and 16S	*rpsL, rrs*
Amikacin (1972)	Semi-synthetic	0.85-1.7	Protein synthesis inhibition	rRNA ribosomal proteins S12 and 16S	*rpsL, rrs*
Floroquinolones (1980's)	Synthetic	0.6-1.4	DNA replication & transcription inhibition	DNA gyrase	gyrA, grB
Ethionamide (1956)	Synthetic	1.5	Mycolic acid biosynthesis inhibition	Enoylreductase (InhA)	*inhA, eta/ethA*
Prothionamide	Synthetic	2.77	Mycolic acid biosynthesis inhibition	Thymidilate synthase	*thyA*
p-aminosalicilic acid (1946)	Sythetic	1.9-6.5	Inhibition of thymidilate synthase and iron acquisition	D-alanine racemase	*alrA, ddl*
Cycloserine (1952)	Natural	245	Peptidoglycan synthesis inhibition	D-alanine racemase	*alrA,ddl*

Cultures of *M. tuberculosis* in exponential growth are sterilized *in vitro* in a few days by first line agents such as INH and RIF, while the same combination requires months to achieve the same result in host tissue. It has been stated that mycobacterial persistency is due to the physiologic heterogeneity of the bacillus in the tissues, the existence of subpopulations with completely different rate-determining factors. Despite an urgent need for new therapies targeting persistent bacteria, our knowledge of bacterial metabolism throughout the course of infection remains rudimentary (WHO 2012). Mitchison proposed in 1979 that, in lesions, *M. tuberculosis* exists under at least four different population stages as listed below:

- Bacteria in active growth, susceptible to INH.
- Bacteria with intermittent metabolism period, susceptible to RIF.
- Low metabolic activity bacteria residing in acidic pH, susceptible to PZA.
- "Dormant" or "persistent" bacteria, non-susceptible to any current active principle.

During the initial chemotherapy phase (2 months), actively dividing bacilli rapidly die mostly because of INH bacteriocidal activity. Thereafter bacilli of low metabolic activity suffer from a slow death under the effects of RIF and PZA. There is evidence that persistent bacillary populations existing in the lesions usually determine the duration of therapy (Lienhardt, Raviglione, Spigelman, Horner, Jaramillo, Hoelscher, Zumla & Gheuens 2012). Therefore efforts need to be made to target every physiological state of *M. tuberculosis* thus shortening the time of therapy and inhibiting the appearance of drug resistance.

That leads to the second reason why new anti-TB drugs are needed. Drug resistance has emerged as a phantom from the dark, threatening every corner of the world. RIF-resistance often correlates to MDR category (resistant to INH and RIF). XDR *M. tuberculosis* is an MDR strain resistant to any fluoroquinolone and at least one injectable agent. Prognosis is less favourable for patients harbouring XDR-bacilli compared to patients with MDR, with a five times higher risk of death, and requiring longer hospitalization or treatment times. However it has been shown that within an aggressive treatment, XDR-TB patients have been successfully cured in 60% (Jiminéz-

Levi 2011). Treatment of M/XDR-TB usually takes more than two years, and requires the use of more toxic, less effective and more expensive drugs. In resource-limiting countries, supplies of second-line drugs cannot be guaranteed.

TB infection in an immune-compromised population leads to severe cases, possibly affecting other parts of the body, such as the pleura, meninges, the lymphatic system, the genitourinary system, and the bones (Phillips, Gillepsie, Boeree, Heinrich, Aarnoutse, Mchugh, Pletschette, Lienhardt, Horner, Mgone, Zumla, Nunn & Hoelscher 2012). It has been estimated that HIV infected patients are 100 times more likely to develop TB. Although the studies support a decrease of mortality for TB patients after the introduction of antiretroviral therapy, evidence exists of the existence of interactions between Highly Active Antiretroviral Therapy (HAART) and TB chemotherapy. HAART is based on a combination therapy normally involving two reverse-transcriptase inhibitors and a non-inhibitor (Ribon 2012). P450 Cytochrome typically metabolizes reverse-transcriptase inhibitors; however this cytochrome is also induced by RIF. TB chemotherapy may significantly reduce the concentrations of anti-retroviral drugs which may lead to treatment failure or resistance. An increase of the nevirapine dose to compensate for this interaction increases the risk of toxic effects and hepatotoxicity in patients who already present a low body mass index and high level of CD4 lymphocytes (Cantón & Ruiz-garbajosa 2011). Physicians prefer to avoid the concomitant use of nevirapine and RIF; consequently there is a clinical need for mycobacteriocidal agents devoid of P450 catabolism.

2.8 THE NEW TB DRUG PIPELINE

Antibiotic discovery began in the early 1930s when different classes were discovered (Theuretzbatcher 2011). At the end of the 1950 decade, the combined regime was established and was thought to eradicate the disease completely. In the following thirty years after the introduction of the last first-line anti-TB drug, RIF, the regimen remained unchanged. The landscape changed in 1993 when the WHO declared TB a global health emergency (Nguyen 2011). Until recently, research in development of new anti-TB drugs was poor. These days, the TB Alliance has emerged as a non-profit organization promoting and funding anti-TB drug development by creating consortia over a defined project, often involving big pharmaceutical companies, institutes of research, and universities. Interest in drug discovery has been placed on

both phenotypic and target-based approaches to set in motion a strong pipeline with the joint effort of the Working Group on New TB Drugs, Stop TB Partnership and other societies.

SQ-109, bedaquiline and linezolid are candidates in clinical trial 9 (Brien 2012). There are other promising compounds (CPZEN-45, BTZ043, AZD5847, DC-159a and others), but a handful of scientists believe that the gap is large and there is no certainty whether there will be a full new regimen in the next decade (Villemagne, Crauste, Flipo, Baulard, Déprez & Willand 2012).

Of the eleven new or repurposed TB drugs under clinical investigation, four are in Phase III (efficacy) trials and seven are in Phase II (early bacteriocidal activity and sputum culture conversion) trials. Two of the Phase III trials are evaluating 4-month combination regimens in which a fluoroquinolone (gatifloxacin or moxifloxacin) is substituted for either ethambutol or isoniazid; results were expected in 2013 (Diacon, Dawson, Hanekom, Narusky, Venter, Hittel, Geiter, Wells, Paccaly & Donald 2011). A third Phase III trial is evaluating the use of rifapentine (a rifamycin that has a longer half-life than rifampicin) as part of a 4-month regimen for the treatment of drug-susceptible TB. Since mid-2011, the delamanid (OPC-67683) compound, which is being tested as an addition to optimized background therapy for the treatment of MDR-TB, has moved from a Phase II to a Phase III trial (Lienhardt *et al.* 2012).

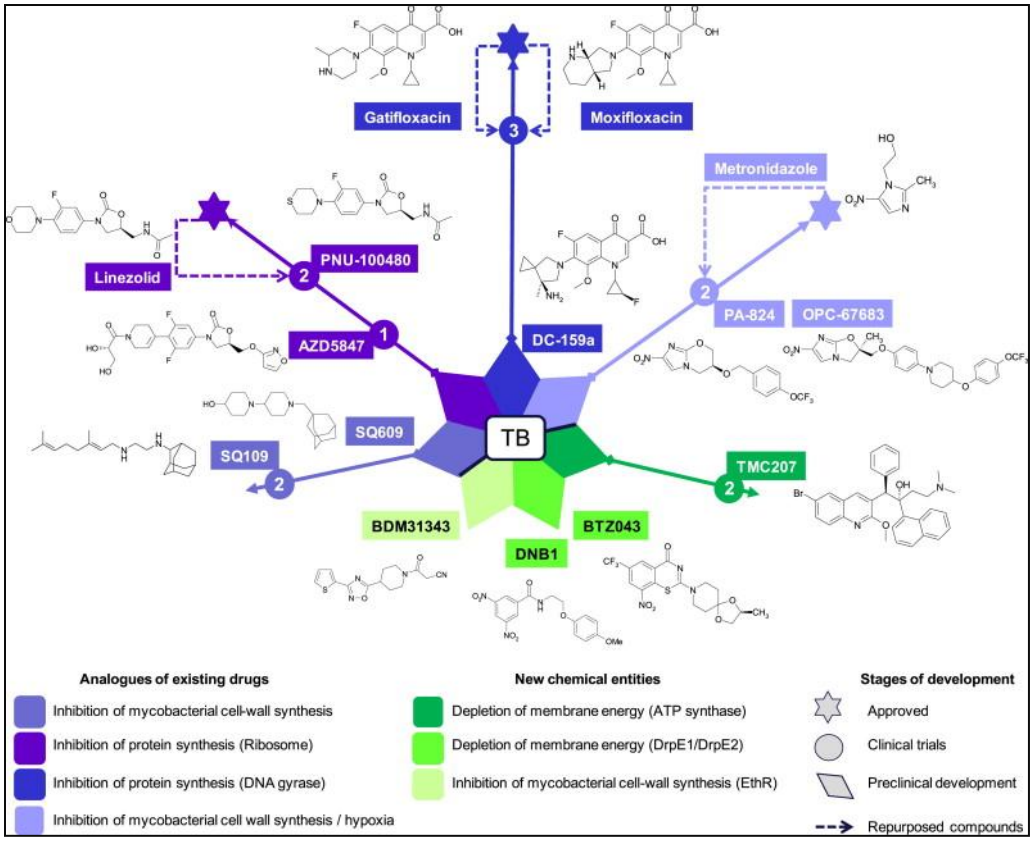

Figure 2: Anti-tubercular compounds in development and their targets (Villemagne *et al*. 2012).

Besides individual compounds, very promising results on the early bacteriocidal activity of a novel TB regimen (NC-001) that includes three drugs (PA-824, moxifloxacin and pyrazinamide) became available in July 2012. NC-001, also known as New Combination 1 is a trial of a novel TB regimen that includes the drug candidate PA-824 combined with moxifloxacin and the standard first-line anti-TB drug, pyrazinamide (PaMZ). The trial is being conducted in partnership with and sponsored by the TB Alliance (Schoubben, Blasi, Marenzoni, Barberini, Giovagnoli, Cirotto & Ricci 2013). The regimen has been tested for early bacteriocidal activity against pulmonary TB over a 2-week period, and the results were encouraging. The regimen had bacteriocidal activity at least comparable to a standard regimen of isoniazid, rifampicin, pyrazinamide and ethambutol. This study also validated a new approach to the development of new anti-TB drug regimens, which has the potential to reduce the time required to complete clinical trials from decades to years. Research on NC-001 has also included testing of some novel combinations of two drugs that may form the "core" of future regimens, thus informing

other clinical trials being planned during the next 18 months and beyond (Lawn, Suthar, Del amo, Getahun, Dye, Sculier, Sterling, Chaisson, Williams, Harries & Granich 2012).

The testing of the PaMZ regimen advanced to a 2-month trial (called NC-002) in March 2012. In this trial, carried out in Brazil, South Africa and Tanzania, PaMZ is being tested for patients with drug-sensitive TB and patients with drug-resistant TB who are sensitive to the drugs included in the new regimen. The NC-002 trial is a landmark trial; it is the first to simultaneously investigate treatment of both drug sensitive and drug-resistant diseases using the same regimen. Results are expected in the third quarter of 2013 (WHO 2012).

These major advances in drug development mean that multiple trials will be needed in various high-burden countries. This presents several challenges. Trials are lengthy and costly, since patients need to be followed up for an extended period of time after completing treatment. New drugs have to be tested in various drug combinations with current and/or newly re-purposed drugs, and to facilitate this, novel biomarkers for treatment response and sterilizing activity, new approaches to the design of clinical trials and increased capacity to implement trials in accordance with international standards are required (Coghlan 2012).

Several research groups and institutions worldwide are working to address and overcome these challenges. A good example is the NIH-funded AIDS Clinical Trials Group, whose goal is to transform TB treatment (including HIV-associated TB) by developing and optimizing regimens to treat and prevent TB more quickly and effectively (Sharma, Sing, Kumar & Singh 2012). The group is working on the identification of biomarkers to better understand TB pathogenesis and treatment response and to shorten future clinical trials by using surrogate markers for clinical end-points (Yadav, Deolekar & Mishra 2012). This is closely linked to strengthening the capacity of clinical trial sites and building laboratory and pharmacology research capacity; efforts are coordinated with other clinical trial networks to optimize efforts to develop new combination regimens. The Critical Path to New TB Drug Regimens' initiative, whose goal is to accelerate the development of novel regimens that will shorten TB treatment, is also an important example of a global effort to ensure that the necessary trials can be implemented (www. newtbdrugs.org/pipeline-discovery).

2.9 PLATFORM FOR THE DEVELOPMENT OF ACTIVE PRINCIPLES IN THE TREATMENT OF TB

Both basic and clinical pharmacology have contributed to the progress in the discovery of drugs applying their experience to the development and validation of hypotheses of new action targets in order to produce novel drugs. In this sense, researchers need to be innovative and they must have a wide vision over the interpretation of the results (Trist & Davies 2011). The choice of a therapeutic candidate is probably the most important decision to make in the discovery and development of a medication. The chemical structure of a drug confers its biologic, pharmacokinetic, physicochemical, and toxicological properties (Rossi & Braggio 2011). On the other hand, the discovery and development of new drugs is a complex and costly process requiring large amounts of resources and time. The cost of launching a new drug to the market ranges from US$ 800 million to 1000 million, and it may take between 8 and 17 years depending on the disease and the treatment (Fig. 3) (Showalter & Denny 2008).

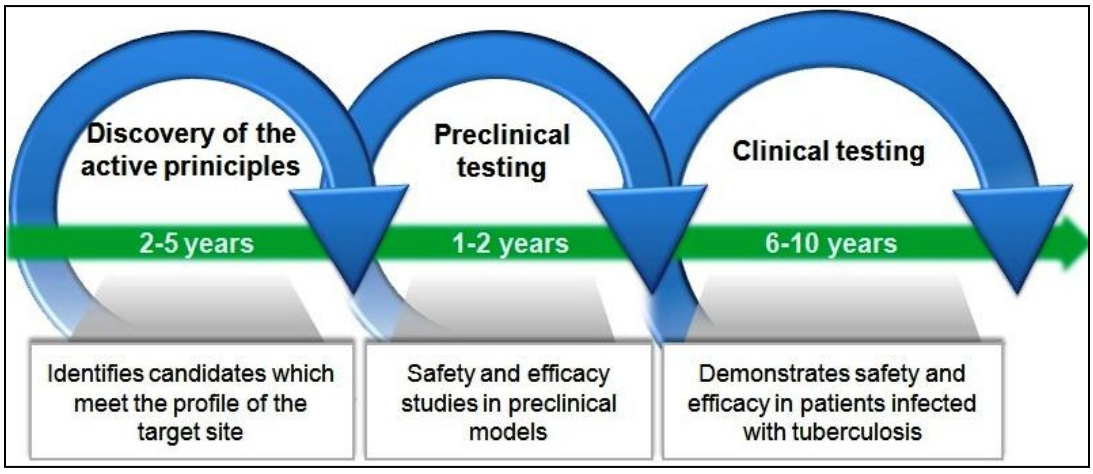

Figure 3: TB drug pipeline**.** From the discovery bench through preclinical and clinical studies for novel anti-TB agents, a process that could last more than 15 years (Ribon *et al*. 2012).

Ideally; an antibacterial agent must show bacteriocidal activity often impeding an essential function for the survival of the microorganism (Orme, 2011)

2.10 DISCOVERY OF ACTIVE COMPOUNDS

The parameter most commonly determined to examine the *in vitro* antibacterial activity of a specific molecule is the MIC which represents the concentration required to inhibit 99.9% of the growth of bacilli. The main limitations of these trials are that they do not describe the percentage of dead bacteria (which critically depends on cell density) or the metabolic state of the bacteria, if we aim to examine the persistent antimicrobial effects of a certain drug (Singh & Tam 2011). Most publications include at least a compound with a MIC lower than 6.25µg/ml (Ballell *et al*. 2011; Orme 2011). It is recommended that active compounds under a colorimetric assay (Resazurin, Alamar Blue, and MTT) are reconfirmed using agar-based techniques or MGIT. A simple and easy to use agar-based method using Middlebrook 7H10 was introduced in 2004 by Bhakta *et al*. for measuring MIC values (Bhakta, Besra, Upton, Parish, Sholto-Douglas-Vernon, Gibson, Knutton, Gordon, da Siva, Anderton & Sim 2004; Balganesh, Balaubramanian & Kumar 2010).

The spot culture growth inhibition assay (SPOTi) has now been used to screen at least more than 1000 compounds. Simultaneously, the cytotoxicity in different types of mammalian and/or macrophages is carried out. The selectivity index (SI) is determined by dividing the growth inhibitory concentration 50 (GIC50) corresponding to the concentration of a compound capable of killing half of the mammalian cells by the MIC using the same concentration units. If the SI is larger than 10, infection of a macrophage with a selected strain of mycobacteria and treating with the drug candidate can help to determine its intracellular potential (Fig. 4) (Orme 2011).

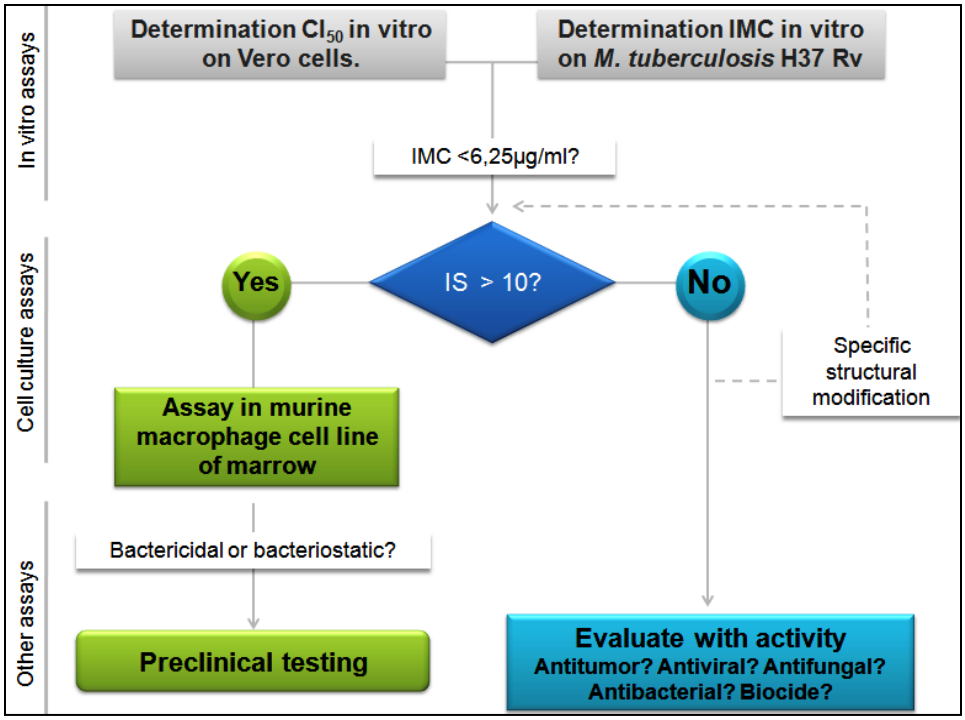

Figure 4: Research and development of new TB active compounds**.** In an attempt to promote pre-clinical studies of early leads, Orme proposed this rapid diagram based on the selectivity ration between a bacteria and a mammalian cell line (Orme 2011)

The success of a discovery program of antibacterial principles is founded on three factors: identification of key elements contributing to pathogenicity of the microorganism, the understanding of the existing relationships between the microbe and the host, and importantly, properties of the chemical compound (Balganesh *et al*. 2004). Two pathways have been traced with the aim of discovering active principles. One is the empirical pathway, mainly based on chemistry and phenotypic screening; and the more modern is the mechanistic, based on genomics, biochemistry and molecular biology. The former begins with the identification of an active principle with potent antimicrobial activity in *in vitro* conditions. The active principle is discovered by chance or by random screening after which it is subjected to trial on rigorous toxicological assays before using animal models. Some candidates may eventually be selected for human trials (Fig. 4). The limitation of the empirical pathway is the lack of information on the specific target or the action mode, sometimes this lack of understanding can lead to high failure rates mostly for toxicity problems (Balganesh *et al*. 2004). On the other hand, the mechanistic pathway started with the age of molecular biology and genomics which allowed the

identification of specific targets of the microbe, absent or structurally different in human hosts. This strategy can be upgraded to a high-throughput screening (HTS) platform and to evaluate a large amount of substances in a short time. Crystallization of the target proteins and X-ray diffraction spectroscopy, together with an analysis of the active site in the presence of the natural substrate and inhibitors allow the detailed study of the crucial structural structure.

In the mechanistic approach discovery usually involves firstly the identification and validation of the antimycobacterial target macromolecule to be inhibited. Large collections of compounds can be screened directly against the protein if a high-throughput method of assay is available. Alternatively if there is structural information it is possible to computationally interrogate the target against a defined set of computer-based compounds. The preferred targets are generally the ones occurring in *M. tuberculosis* and not represented in the human genome. By means of comparative genomics, the targets are present in the human genome. For example, nicotinamide adenine dinucleotide (NAD) is generated in humans either by *de novo* biosynthesis, or by DNA and RNA degradation.

Another approach is related to the genomics of virulence. Some mycobacterial genes are only expressed in granuloma but not inside the macrophages. Isocitratelyase enzyme is fundamental in the persistence of bacilli in chronic infection in mice and its function is related to obtaining carbon during its persistence in the host (McKinney, Honer, Bentrup, Munoz-Elias, Miczak, Chen, Chan, Swenson, Sacchettini, Jacobs & Russell 2000; Murphy & Brown 2008). The extracellular repetition protein (*Erp*) is another essential protein involved in *M. tuberculosis* virulence that was the first discovered virulence factor. The mutant Δ-*erp* that does not correctly express the extracellular repetition protein does not show any alteration in standard *in vitro* culture, but maintains an essential function for *in vivo* survival (Chopra, Meena & Singh 2003; Berthet, Lagranderie, Gounon, Laurent-Winter, Ensergueix & Chavarot 2004). This protein is also a potential target for the development of anti-TB active principles. Two independent proteins (FadD28 and MmpL7) have been identified contributing to the early growth of *M. tuberculosis* in mice lungs and are related to the synthesis and transport of a complex lipid associated to the cell wall (Chopra *et al*. 2003). Although the function of this lipid is unknown, it is suspected that it plays a role in the decrease of the host's immune response. There is no doubt

about the remarkable progress that the sequencing of the *M. tuberculosis* genome has brought to the anti-TB drug discovery area of research. The functional annotation of all these genes remains a considerable amount of experimental work. Sequencing of other related organisms such as *M. marinum*, *M. leprae*, *M. aurum* and others often offers clues about the essentiality of specific set of genes and operon distribution.

2.11 TARGET OR COMPOUND TYPE IN DISCOVERY STAGE

2.11.1 Analogues of existing drugs

2.11.1.1 Nitroimizadoles (PA-824), Delaminid (OPC-67683)

Initially, bicyclic nitroimidazofurans were investigated as potential radio sensitizing agents for use in cancer radiotherapy but were also found to display some antitubercular activities in both *in vitro* and *in vivo* models. Very promising results were obtained for the lead compound of this chemical series, CGI 17341, but associated mutagenecity discouraged further research with this compound. Nevertheless, the strong activities obtained with these compounds suggested that the bicyclic nitroimidazole might be an interesting pharmacore. Based on this observation, a chemical library of 328 nitroimidazopyrans was designed and evaluated on *M. tuberculosis* (Diacon *et al.* 2011). One member, PA-824 was identified as a promising antitubercular agent.

PA-824 is a prodrug that needs the mycobacterial glucose-6-phosphate dehydrogenase (FDG1) or its cofactor, coenzyme F420, to be transformed into an active form. Activated PA-824 inhibits the synthesis of proteins and cell wall lipids. Its activity is active in susceptible and resistant *M. tuberculosis* strains. No cross–resistance with standard anti-TB drugs has been observed. It has demonstrated good activity in *in vitro* and *in vivo* mouse models. It has also been discovered by Otsuka Pharmaceuticals that PA-824 could be useful in treatment of MDR and XDR TB strains. Its optimal formulation and its role in TB treatment in humans still need to be established. PA-824 is currently in phase II clinical trials for the treatment of TB and the outcome may determine the future directions of drug development for anti-tubercular nitroimizadoles (Murray *et al.* 2012).

OPC- 67683 is a nitro-dihydroimodazooxazole, and was designed from CGI 17341 with the objective to enhance its TB-activity and to lower its toxicity. It acts by inhibiting mycolic wall

synthesis. Mycolic acids are important for the survival of *M. tuberculosis;* they are able to fight against hydrophobic drugs and dehydration, and inside macrophages. Due to the importance of the mycolic acids for maintenance of the targets for the development of novel drugs against TB. (de Souza, de Lima Ferreira, Pinheiro, Saraiva, de Almeida & Valle 2008). It is bacteriocidal and has good activity *in vitro* and in experimental animals. Currently it is in phase II trials, to evaluate the proportion of MDR TB patients receiving either 100 or 200mg twice daily of orally administered OPC-67683 in addition to a standardized second-line TB regimen which achieves sputum-culture conversion to negative within two months, as well as pharmacokinetics and safety (http// www. clinical trials.gov).

2.11.1.2 Diamine derivative: SQ 109

SQ 109 is a synthetic analogue of ethambutol. It was discovered from a focused library of ethylene diamine analogues containing 63, 238 compounds. The exact mechanism of action of SQ 109 is not yet known. SQ 109 ethambutol was shown to over-produce the ATP-dependent DNA/RNA helicase and to reduce the production of β-ketoacyl –acyl carrier protein synthase which may explain its action on mycobacterial cell wall synthesis (Chen, Protopova, Einck & Nacy 2012). Interestingly, SQ 109 is believed to act in a different manner from ethambutol. It was also shown that SQ109 is active against rifampicin resistant strains.

In vitro activity against *M. tuberculosis* has shown that SQ 109 minimum inhibitory concentration (MIC) reported in the literature ranges from 0.3μM to 1.56μM on drug sensitive strains, is equal to 0.9μM on EMB-resistant strain, 1.4μM on INH-resistant strain and 0.7μM and 0.7μM on RIF-resistant strain. SQ109 and TMC207 have been studied in combination on *M. tuberculosis* H37Rv culture. Activity of TMC207 was improved by 4-8- fold in the presence of SQ109. Moreover, the rate of killing bacteria was higher in combination than for drugs alone. SQ109 also showed synergistic effect with antitubercular drugs isoniazid and rifampicin (Sacksteder, Protopova, Barry, Andrie & Nacy 2012).

SQ109 has completed three phase 1 studies in the United States of America (USA). A phase 1 double-blind, placebo-controlled study performed on 62 healthy adults showed that oral doses of SQ109 up to 300mg were safe and well tolerated. In 2009 a study to determine safety and

pharmacokinetics of multiple-doses of SQ109 was performed in healthy volunteers. Results are not yet published. SQ109 is currently being evaluated in a phase 2a trial in adults with smear-positive pulmonary TB (http//www.SQ109 clinical trials.gov).

2.11.1.3 Gatifloxacin and Moxifloxacin

The fluoroquinolones gatifloxacin and moxifloxacin were marketed in 1999 for the treatment of respiratory tract infections. These two molecules are currently in phase 3 clinical trials for the treatment of TB. *M. tuberculosis* does not possess any type IV topoisomerase. Thus, fluoroquinolones specifically target the mycobacterial topoisomerase II DNA gyrase. This implies that large spectra fluoroquinolones suffer from suboptimal inhibition of topoisomerase II and thus can be improved (Villemagne *et al.* 2012).

Gatifloxacin and moxifloxacin have better *in vitro* activity against *M. tuberculosis* than the older fluoroquinolones ciprofloxacin and ofloxacin. MIC against *M. tuberculosis* H37Rv is 0.12-0.25µg/ml for gatifloxacin and 0.18-0.5µg/ml for moxifloxacin. Gatifloxacin has a slightly better activity against *M. tuberculosis* clinical isolates than moxifloxacin. Indeed, the range of MIC_{90} is 0.007-0.12µg/ml for gatifloxacin and 0.031-0.12µg/ml for moxifloxacin (Okumura, Hirata, Onodera, Hoshino, Otani & Yamamoto 2012).

In phase I, in healthy volunteers, moxifloxacin and gatifloxacin showed a high bioavailability (90% and 96% respectively). After a single oral dose of 400mg, a C_{max} of 4.3mg/mL for moxifloxacin and 3.4mg/mL for gatifloxacin was reached in 1.0 hour and 1.5 hour, respectively. In phase II, in TB patients, moxifloxacin and gatifloxacin at 400mg once daily have good early bactericidal activities (EBA) from day 0 to day 2 but less than isoniazid at 300mg (0.67 log colony forming unit (CFU)/ml/day). Another study showed that the EBA of a 5 day-monotherapy with moxifloxacin (400mg daily) is comparable to that of isoniazid (6mg/kg once daily) (Onodera, Hirata, Hoshino & Otani 2012).

In a phase 2b study, addition of moxifloxacin to the standard regimen (RIF/INH/PZA/EMB) in the first two months shortened the time to culture conversion and led to a higher culture conversion rate after 6 weeks of treatment (82% in the moxifloxacin group compared to 61% in

the standard regimen group) . Substitution of moxifloxacin for isoniazid in the standard regimen did not significantly increase the culture negativity of patients after 8 weeks of therapy. However replacement of ethambutol with moxifloxacin or gatifloxacin in the standard regimen significantly improved culture conversion after 8 weeks of treatment and might shorten tuberculosis therapy (Sekiguchi, Disratthakit, Maeda & Doi 2011).

Gatifloxacin and moxifloxacin are currently being evaluated in a phase 3 clinical trial. The aim of these studies is to evaluate the efficacy and safety of gatifloxacin and moxifloxacin and the possibility of reducing the duration of tuberculosis therapy from six to four months (Disratthakit & Doi 2012).

2.11.1.4 Linezolid

Linezolid was introduced in the USA for the treatment of patients with infections caused by Gram-positive pathogens (*Staphylococci, Streptococci* and *Entercocci*) responsible for skin and soft tissue infections, and pneumonias. Linezolid is used for courses of treatment up to 28 days (Schecter, Scott, True, Raftery, Flood & Mase 2010). This compound belongs to the (S)-oxazolidin-2-one family class of compounds and is a direct analogue of DuP105 and DuP721 described to have MIC of 0.3-1.25mg/ml against *M. tuberculosis* and of which development was discontinued in Phase 1 due to toxicity issues.

Linezolid presents a unique mechanism of action which was supported by the lack of cross-resistance between oxazolidinones and other antibiotics. It binds to the 23S RNA in the 50S ribosomal subunit and limits the growth of bacteria by disrupting its production of proteins in the first step of the synthesis by inhibiting formation of the initiation complex. Linezolid has a high *in vitro* antibacterial activity (MIC of 0.125-0.5µg/ml) against *M. tuberculosis* (Zhang, Post-Martens & Denkins 2012).

Only a little Phase 1 and 2 clinical data have been published so far. EBA and extended EBA of linezolid have been studied in patients with pulmonary TB. Linezolid at 600mg/day presented weak early and extended bacteriocidal activities when given once or twice daily to patients with pulmonary TB. Efficacy of linezolid to treat MDR-TB in combination regimens was assessed in

two studies with a total of 11 patients (Yang, Lei, Meng, He, Tong, Zhu, Jian & Dong 2012). Doses of 600 and 1200mg/day allowed sputum and cultures to become negative and some patients were cured after treatment; however toxic side effects such as peripheral and optic neuropathy were common. Today linezolid is used off-label as a third-line agent in combination regimens to treat MDR-TB or XDR-TB (Alffenaar, van der Laan, Simons, van der War, van der Kasteele, de Leeling & Soolingen 2012).

2.11.1.5 PNU-100480

The use of linezolid is limited by adverse effects that occur with long-term administrations. Therefore, new analogues showing identical or better *in vivo* activities and a better therapeutic index would be useful. The development of PNU-100480, a close structural analogue of linezolid was initiated by Upjohn (PNU-100480 clinicaltrials.gov). *In vitro* studies against *M. tuberculosis* showed that PNU-100480 exhibits a MIC range of 0.03-0.50µg/ml against a panel of 5 sensitive and 5 drug resistant strains of *M. tuberculosis*, which makes it in average, 3.2 times more efficient than linezolid. Their sulfoxide and sulfone metabolite (PNU-101603 and PNU-101244) was shown to contribute to its activity (Wallis, Jakubiec, Kumar, Bedarida, Silvia, Paige, Zhu, Mitton-Fry, Ladukto, Campbell & Miller 2011).

Trials with PNU-100480 have been completed and published. These studies were designed to assess safety, tolerability, and pharmacokinetics and for the first time mycobacteriocidal activities, measured in *ex vivo* whole-blood culture, of single or multiple ascending doses of PNU-100480 (Villemagne *et al.* 2012). In both studies, doses up to 1200mg/day were well tolerated; exposure increased linearly with dose and antimycobacterial activity was superior to linezolid. No haematologic safety signals in healthy volunteers were observed with optimal doses of PNU-100480, 600mg twice daily for 28 days. In the multiple-dose study, a synergistic effect with PZA was observed. A phase 2a to assess EBA of PNU-100480 is ongoing (WHO 2012).

2.11.1.6 AZD5847

New oxazolidinone derivative, AZD5847 (also known as AZD2563) currently in phase 1 clinical trials, is developed by AstraZeneca. Two studies, a single ascending dose and multiple ascending dose over 14 days, have now been completed for AZD5847. Bioavailability in fasted volunteers was reported to decrease with increasing doses, declining from 100% at 50mg to less than 30% at 1200mg (AZD5847 clinical trials.gov); however, this tendency was corrected by food intake. AZD5847 was tolerated over 14 days in healthy volunteers. The doses selected for investigation in phase 2 studies are 500mg once and twice daily, 800mg twice daily and 1200mg once daily and will be compared to rifafour (RIF/INH/PZA/EMB) 1 pill per gram once daily. A phase 2a was scheduled to start in 2012 (Balasubramaniam & Butler 2011).

2.11.2 New Chemical entities

2.11.2.1 Diarylquinoline TMC 207

TMC207 (R207910, Bedaquiline) is a novel diarylquinoline belonging to a new class of anti-tuberculosis drugs. This compound was discovered by Johnson & Johnson through a whole-cell screening on *Mycobacterium smegmatis* and it is currently being clinically developed by Tibetan in collaboration with the TB Alliance. It is being tested as an addition to optimized background therapy for the treatment of MDR-TB. TMC207 inhibits the proton transfer chain of the mycobacterial ATP synthase (Haagsma, Podasca Koula, Andries, Guillemont, Lill & Bald 2011). The mechanism of action of the diarylquinolone was originally proposed after isolation of mutant strains of *M. tuberculosis* and *M. smegmatis* that were resistant to TMC207 (Villemagne *et al.* 2012). Their genomes were sequenced and compared to susceptible strains. The only common mutation was localized in the *atpE* gene, encoding for subunit C of ATP synthase which was further validated as the compound's precise target. Whereas derived from quinolones, TMC207 has no inhibitory effect on the DNA gyrase. TMC207 is now in phase III clinical trials (WHO 2012).

2.11.2.2 Nitrophenyl derivative: BTZ043

BTZ043, a new class of sulphur containing heterocyclics called benzothiazinones (BTZ) has recently been described as potent antimycobacterial agents. The structural activity relationships study showed that a sulphur atom and one or two nitro groups on the aromatic structure were required to inhibit bacterial growth *in vitro* (Makarov, Manina, Mikusova, Möllmann, Ryabova, Saint-Joanis, Dhar, Pasca, Buroni, Lucarelli, Milano, de Rossi, Belanova, Bobovska, Dianiskova, Kordulakova, Sala, Fullam, Schneider, Mckinney, Brodin, Chridtope, Waddell, Butcher, Albrethsen, Rosenkrands, Brosch, Nandi, Bharath, Gaonkar, Shandil, Balasubramanian, Balganesh, Tyagi, Grosset, Riccardi & Cole 2012).

2.11.2.3 Pyrroles (LL3858)

The mycobacterial target of LL3858 is not yet known. Since LL3858 is active against *M. tuberculosis* strains that are resistant to available anti-TB drugs, the target probably differs from the targets of the currently used drugs (Deidda, Lampis, Fioravanti, Biava, Porretta, Zanetti & Pompei 2011). Experiments conducted on mice and dogs showed that the compound is well absorbed, with levels in serum above the MIC and better half-life and maximum plasma concentration (C_{max}) than those showed by isoniazid. In search for compounds against mycobacteria and fungi, several pyrroles derivatives have been developed. LL3858 is being investigated in phase I clinical trials. A fixed–dose combination called LL3848, containing LL3858 and standard first line anti-TB drugs is also being developed (Ragno, Marshall, di Santo, Costi, Massa, Rompei & Artico 2012).

2.11.2.4 ATP Synthase Inhibitor FAS20013 (FASgene)

FAS20013 is a novel compound identified by Fasgen. It belongs to the class of ß-sulphonylcarboxamides. Fasgen claims that “FAS20013 will kill more organisms in a 4-hour exposure than isoniazid or rifampicin can during a 12- to 14-days exposure. The compound is very effective in killing MDR-TB organisms that are resistant to multiple drugs currently in use. A series of recent laboratory experiments indicate the superior effect of FAS20013 compared to current drugs in terms of its ability to "sterilize" TB lesions and kill latent TB (Jones, Parrish, Houston, Stapon, Bansal & Dick 2011). Therapeutic evaluation of FAS20013 has repeatedly shown its effectiveness in mice, and appears to have no serious side effects. The compound is up

to 100% bioavailable when administered orally. To date no dose limiting toxicity has been encountered, even when doses 10 times the effective dose were administered." The compound is thought to act through inhibition of ATP synthase. However, available scientific publications assessing the efficacy of this compound are of poor quality (Parrish, Kuhajda, Heine, Bishal & Dick 1999).

2.11.2.5 Translocase I inhibitor (Sequella Inc.)

Sequella is developing a series of translocase inhibitors for the potential treatment of tuberculosis. The compounds specifically inhibit mycobacterial translocase I, an enzyme required for bacterial cell wall synthesis. Preclinical evaluation of the compounds is planned (http://www.sequella.com/pipeline/translocaseinhibitor.asp).

2.11.2.6 Isocitrate Lyase inhibitors

The isocitrate lyase (ICL) enzyme has been shown to be essential for long-term persistence of *M. tuberculosis* in mice but not required for bacilli viability in normal culture or hypoxic conditions. McKinney and collaborators have recently shown that inhibition of ICL 1 and ICL 2, the two isomers of isocitrate lyase present in *M. tuberculosis*, blocks growth and survival of *M. tuberculosis* bacteria in macrophages and in mice at early and late stages of infection. The absence of ICL in mammals should facilitate the development of glyoxylate cycle inhibitors as new drugs for the treatment for tuberculosis. Such a new drug is expected to be able to kill persistent bacteria and therefore have sterilizing activity and shorten treatment time (Mckinney *et al.* 2012).

2.12 CHALLENGES OF DEVELOPING NEW ANTI-TB DRUGS

The rapid development of new anti-TB drugs has been hampered by several obstacles. First of all, the TB drug market is associated with insufficient profit opportunity or investment return to instigate pharmaceutical industries to develop new drugs. The cost of developing a new drug is estimated at R115 to R240 million.

A second challenge in TB drug development is the difficulty to identify new compounds with activity against *M. tuberculosis*. Regimens against TB should kill both the rapidly growing mycobacteria (bacteriocidal activity) and the persisting mycobacteria in lesions (sterilizing

activity) (van den Boogaard, Kibiki, Kisanga, Boeree & Aarnoutse 2012). The molecular mechanisms responsible for mycobacterial dormancy, persistence and drug resistance are not yet fully understood. The deciphering of the mycobacterial genome in 1998 has been of great help in elucidating regulatory mechanisms of metabolic pathways and thereby revealing new drug targets.

The next challenge arises with the evaluation of new compounds, as there are currently no animal models available that predict with accuracy the required treatment duration with newly identified compounds. The guinea pig model is being explored as an alternative for the mouse model since it resembles TB pathology in humans more closely (Koul, Arnoult, Lounis, Guillemont & Andries 2012).

The phase of clinical testing of new anti-TB drugs is time-consuming, as in the current "gold standard" to assess efficacy of anti-TB regimens in phase III clinical trials the relapse rate is 2 years after completing treatment. In phase II clinical trials, the sputum culture conversion rate after 2 months of treatment is used as a surrogate marker for relapse rate. Large sample sizes are needed in phase III clinical trials to compare the effective standard regimen to a new regimen. This contributes to the length of the TB drug development process.

Another challenge is the scarcity of trial sites with sufficient research capacity to conduct clinical trials with large sample sizes (Corbett, Marston, Churchyard & de Kock 2006). Trials should be performed in countries where the TB burden is highest, but the human and infrastructural capacity for performing large, high-quality phase III clinical trials is usually limited in these settings. Despite the challenges of TB drug development, studies are being conducted with higher doses of the rifamycins and several new drug candidates have reached the phase of clinical testing.

2.13 CONCLUSION

For the first time in 40 years, a large number of consortia and pharmaceutical companies have exhibited massive drug discovery efforts to develop new chemical series using either target-based or phenotypic screens. There are at least ten compounds in clinical trials and strategies for the development of new molecules that are ready to fuel the pipeline. Most of them are still in

preclinical testing but one might expect a candidate to rapidly reach the clinic. New targets have been identified and validated with drug-like molecules and the most advanced compound TMC207 might open a bright avenue for TB treatment. The number of pharmaceutical companies involved in TB drug development projects has also increased. However, drug development is a long process especially for TB and it is likely that only one or two new drugs will arrive on the market from these efforts. Bacterial resistance and thus requirement for combinations of molecules tend to suggest that the current development pipeline is not yet sufficiently backed-up to overcome the major unmet medical needs in TB treatment. Efforts are still eagerly needed if we want to soon have a chance to win the battle against endemic disease (Villemagne *et al.* 2012).

CHAPTER 3

SECOND LITERATURE REVIEW

3.1 HISTORY OF MEDICINAL PLANTS

Drugs based on plants extracts have been used worldwide for the treatment of several diseases from ancient times. A great interest in phytomedicine and natural product structures are screened in order to measure their pharmacological activity. It has been estimated that less than 1 – 10% of the large diversity of 250.000 – 500.000 plant species on earth have been studied chemically and pharmacologically for their medicinal properties (Farnsworth 1991; Verpoorte 2000). This is especially true for the tropical flora, as at date only 1% of the species in these habitats have been studied for their pharmaceutical potential (Gurib-Fakim 2006). Tropical forests and many other tropical ecosystems are rich sources of a diversity of plant-derived chemical compounds, both because of the high species diversity but also because of the "eternal summer" which forces the plant to consistently produce chemical defense compounds against herbivores and pathogens as well as against other plant species. Plants in tropical rainforest also have to compete for space and light and this forces species to develop more efficient means of using energy and nutrients as well as to allocate resources for secondary compound production. For these reasons a greater portion of the tropical plant species contains secondary compounds which are potentially useful as models for/as medicines (Wood-Sheldon, Balick & Laird 1997).

Plant derived compounds have been, and are still, important as such or as lead compounds for medicines. Fifty percent of the prescription products in various countries in Europe and the US are either natural products or natural product derivatives (Cordell 2002; Newman, Cragg & Snader 2003). To date about 50 drugs have come from tropical plants (Gurib- Fakim 2006). Plants continue to be a potent source of lead compounds.

Although combinational techniques have been used for the optimization of a number of recently approved agents, these methods have not been able to identify a *de novo* combinational compound (Newman *et al.* 2003). Examples of successful medicines derived from natural product leads include most antibiotics, the acethylcholine esterase (ACE) inhibitors, many anticancer agents, the immunosuppressants, cyclosporine and rapamycin and the antiparasitic avermectins (Harvey & Waterman 1998).

3.2 THE IMPORTANCE OF NATURE IN THE DISCOVERY OF TB TREATMENT

The beginning of TB chemotherapy well illustrated the importance of nature in the fight against diseases. The first drug discovered to treat TB was streptomycin (Fig. 5), an aminoglycoside isolated from the actinobacterium *Streptomyces griseus*, identified in 1943 by Selman Abraham Waksman and his student Albert Schatz at Rutgers University (Kingston 2004). This discovery saved countless lives and due to the importance of this natural product for mankind, Waksman was awarded the Nobel Prize for medicine in 1952. After the streptomycin discovery, in the period known as the golden era of TB research (1940–70), several synthetic drugs were introduced into the market. However, nature still played a crucial role in drug discovery against TB. For example, other aminoglicosides such as kanamycin from *Streptomyces capreolus*, the semisynthetic amikacin produced from kanamycin A and capreomycin from *Streptomyces kanamyceticus*, as well as D-cycloserine from *Streptomyces* spp., are used nowadays in TB treatment as second-line drugs (Souza 2006).

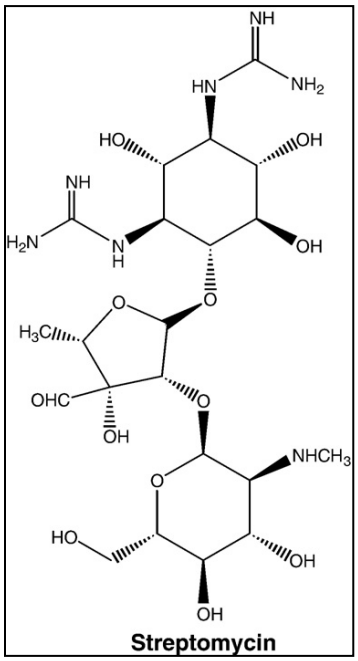

Figure 5: Structure of streptomycin

Another outstanding example of the importance of mother nature in modern TB chemotherapy is the semisynthetic compound, rifampicin or rifampin (Fig. 6). This compound belongs to the

family of ansamycin antibiotics called rifamycins, which were isolated for the first time in 1959 by Sensi and co-workers at Lepetit SA in Milan, Italy (Floss & Yu 2005).

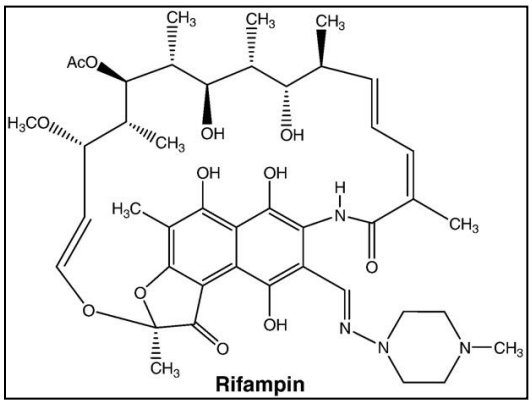

Figure 6: Structure of rifampin

The fermentation broth of *Streptomyces mediterranei* on addition of diethylbarbituric acid produced rifamycin B as a major product, which can be transformed into rifamycin SV by chemical or biochemical means. Rifamycin SV has a much better activity against Gram-positive bacteria than does rifamycin B, the first compound of this class to be used clinically. Due to this promising perspective, several synthetic modifications were produced by the Lepetit group, leading to the discovery of rifampin. This semisynthetic compound, which was clinically available in 1966, had a tremendous impact on TB treatment, being responsible for the reduction of the duration of therapy, from 12 to 6 months, when combined with other drugs, as well as from 9 to 2 or 3 months in latent infections (Souza 2005).

Due to the importance of rifampin in TB treatment and the advent of MDR-TB against this drug, other semisynthetic rifampin analogues have been synthesized and evaluated by other pharmaceutical companies. The rifampin analogues, rifapentine and rifabutin, were introduced for clinical use. Rifapentine was developed by Hoechst Marion Roussel under the trade name Priftin and approved by the FDA in 1998, and possesses more favorable pharmacokinetics than does rifampin (Floss 2005).

Rifabutin (Souza 2006) is manufactured by Adria Laboratories, Columbus, Ohio, under the brand name Mycobutin, and was the first rifamycin derivative to gain approval by FDA for the

prevention of *Mycobacterium avium* complex (MAC) disease in people with advanced HIV infection.

3.3 PROMISING NATURAL PRODUCTS IN CLINICAL TRIALS

3.3.1 Pleuromutilin

Pleuromutilin is a natural product under development by the TB Global Alliance (Fig. 7) (Floss 2006), who are also investigating several other promising analogues in the search for new drugs against MDR-TB. Pleuromutilin is a diterpene possessing a fused 5-6-8 tricyclic skeleton with 8 stereogenic centers.

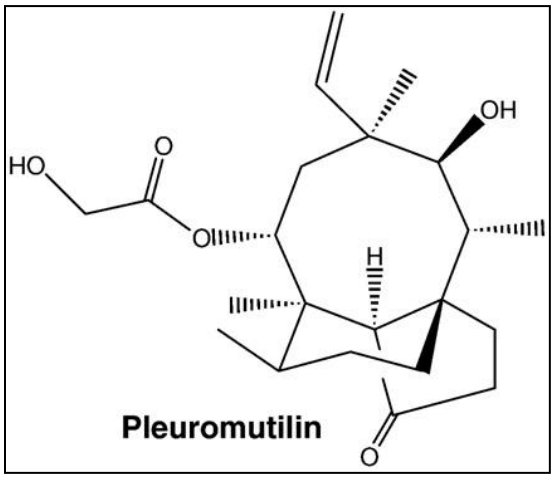

Figure 7: Structure of Pleuromutilin

This natural product has been isolated from *Pleurotus mutilus, Pleurotus passeckerianus, Drosophilia* substrata, and others species of basidiomycetes, and was identified for the first time in 1951 by Kavanagh and co-workers (Fig. 7) (Kavanagh, Herve & Robbins 2006). Due to its important biological activity as an antibiotic against different drug resistant Gram-positive strains and mycoplasmas several studies were carried out to identify its mechanism of action. This was identified as the inhibition of bacterial protein synthesis by interaction with the prokaryotic ribosome. One advantage of pleuromutilin as a TB-drug is its unique mechanism of action, which seems not to have any specific cross-resistance with other antibacterial classes, and then has a promising perspective against MDR-TB (Birch, Cameron Holzapfel & Richards 1963).

3.3.2 Erythromycin

Another natural product under development by the TB Global Alliance is the well known macrolide antibiotic erythromycin (Fig. 8) (Hudson, Imamura, Gutteride, Kanyok & Nunn 2009), which is usually used in patients having an allergy to penicillin antibiotics. This 14-membered lactone, isolated from a strain of the actinomycete *Saccharopolyspora* at the beginning of the 1940's, possesses ten asymmetric centers and two carbohydrates (L-cladinose and D-desoamine). Franzblau and co-workers (Franzblau, Case, Inui, Wang, Cho, Fischer & 2005) evaluated several macrolides of different sizes *in vitro* and *in vivo*. They also investigated the structure–activity relationships and made important contributions to the development of new anti-TB macrolides.

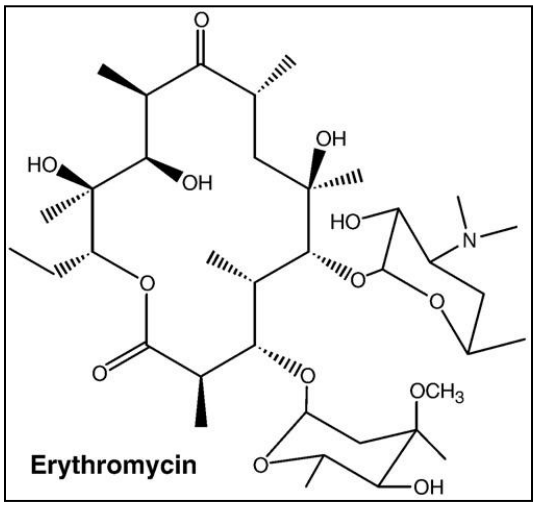

Figure 8: Structure of erythromycin

3.3.3 Pacidamycin and Caprazamycin

The mycobacterial cell wall is a very important component surrounding the cell membrane, and provides support and protection. Peptidoglycan and arabinogalactan are two important polysaccharides contained in the mycobacterial cell walls. These are produced by the phospho-N-acetylmuramyl-pentapeptide translocase (translocase I), enzyme responsible for catalyzing the first step in the biosynthesis of the cell wall (Souza *et al*. 2008). Due to the importance of this enzyme for the survival of the bacterium, it is an interesting target for new drugs. In this context, it is worth mentioning the pacidamycin (Boojamra, Lemoine, Blai, Vernier, Stei, Magon,

Chamberland, Hecker & Lee 2003) and caprazamycin families (Igarashi, Takahashi, Shitara, Nakamura, Naganawa, Miyake & Akamatsu 2005), isolated from *Streptomyces coeruleorubidus* and the culture broth of *Streptomyces* sp. MK730-62F2, respectively.

3.3.4 Capuramycin

Another promising natural product able to efficiently inhibit translocase I inhibitors is capuramycin, isolated in 1986 by Yamaguchi and co-workers (Yamaguchi, Sato, Yoshida, Takada, Itoh, Seto & Otake 1986) from the culture of *S. griseus* 446-S3. Capuramycin possesses a uracil nucleoside and a caprolactam subunit. Several analogues of capuramycin have been synthesized and evaluated against MDR-TB. One of these is (RS-118641), identified by Koga and co-workers (Koga, Fukuoka, Doi, Harasaki, Inoue, Hotoda, Kakuti, Muranatsu, Yammer, Hoshi & Hirota 2004), and synthesized at the Lead Discovery Research Laboratories of Sankyo Co. Ltd. (Tokyo, Japan); it is currently under Phase I study.

3.3.5 Cerulenin

Cerulenin is a natural product isolated from the fungus *Cephalosporium caerulens*. It was isolated for the first time from the culture broth of the fungal strain KF-140 by Omura and coworkers in 1967 and was identified as an antimicrobial agent (Sano, Nomura, Kamio, Omura & Hata 1967). The activity of cerulenin against *M. tuberculosis* (D'Agnolo, Rosenfeld, Awaya, Omura & Vagelos 1973; Omura 1976) is based on the inhibition of the biosynthesis of fatty acids, which are important for its survival. These acids help in the fight against hydrophobic drugs and dehydration, and also allow this bacterium to be more effective in the host's immune system by growing inside macrophages. The target of this natural product for inhibition of the bacterial growth is the enzyme fatty acid synthase (FAS). This enzyme is known as type I FAS, present in animals, fungi and some bacteria (eukaryotic cells) and type II FAS, present in plants, some bacteria and mitochondria (prokaryotic cells) (Souza, Ferreira, Pinheiro, Saraiva, Almeida & Valle 2008). In both enzymes, cerulenin is able to irreversibly block β-ketoacyl synthase, which is a condensing enzyme necessary for the production of fatty acids (Mani & Townsend 1997). An important study of the antimycobacterial activity of cerulenin, as well as its effects on lipid biosynthesis, was made by Parrish and co-workers (Parrish *et al*. 1999), who evaluated the growth of different species of mycobacteria, such as *M. tuberculosis*, *M. bovis* BCG, *M. kansasi*,

M. smegmatis, *M. avium-intracellulare* complex (MAC), *M. chelonei*, *M. fortuitum* and *M. gordonae* with MICs ranging from 1.5 to 12.5mg/L.

3.4 ROLE OF TRADITIONAL MEDICINE AND PLANTS IN DRUG DISCOVERY

It is estimated today that plant materials are present in, or have provided the models for 50% of Western drugs. Many commercially proven drugs used in modern medicine were initially used in crude form in traditional or folk healing practices, or for other purposes that suggested potentially useful biological activity. The primary benefits of using plant-derived medicines are that they are relatively safer than synthetic alternatives, offering greater therapeutic benefits and more affordable treatment. Much of the exploration and utilization of natural products as antimicrobials arise from microbial sources. It was the discovery of penicillin that led to later discoveries of antibiotics such as streptomycin, aureomycin and chloromycetin (Iwu, Duncan & Okunji 1999). Though most of the clinically used antibiotics are produced by soil microorganisms or fungi, higher plants have also been a source of antibiotics. Medicinal plants are rich in a large variety of secondary metabolites of antimicrobial properties such as saponines, tannins, alkaloids, alkenyl phenols, glycoalkaloids, flavonoids, sesquiterpenes lactones, terpenoids and phorbol esters (Tiwari & Singh 2004; Lewis & Ausubel 2006).

A survey of plant-derived pure compounds used as drugs in countries hosting WHO-Traditional Medicine centers indicated that, of 122 compounds indentified, 80% were used for the same or related ethnomedical purposes and were derived from only ninety-four (94) plant species (Fabricant & Farnsworth 2001). Some relevant examples are Khellin, from *Ammi visnaga* (L) Lark, which led to the development of Chromolynas, a bronchodilator; galegine, from *Galega officinais* L., which was the model for the synthesis of metformin and other antidiabetic drugs. Papaverine from *Papaver somniferum* which formed the basis for verapamil used in the treatment of hypertension. The plant is better known as being the source of painkillers such as morphine and codeine, but probably the best example of ethnomedicine's role in guiding drug discovery and development of antimalarial drugs, particularly quinine and artemisinin (Buss & Waigh 2004).

The isolation of the antimalarial drug quinine, from the bark of *Cinchona* species, was reported in 1820 by the French pharmacists, Caventou and Pelletier. The bark had long been used by

indigenous groups in the Amazon region for the treatment of fevers, and was introduced in Europe for the treatment of malaria (Wongsrichanalai, Pickard, Wernsdorfer & Meshnick 2008). Quinine formed the basis for the synthesis of the commonly used antimalarial drugs, chloroquine and metfloquine which largely replaced quinine in the mid 20th century, but with emergence of resistance to both these drugs in many tropical regions another plant long used in the treatment of fevers in Traditional Chinese Medicine (TCM), *Artemisia annua*, gained prominence. This discovery in 1971 by Chinese scientists using data from ancient texts in TCM provided an exciting new natural product lead compound, now known as Artemisinin. Artemisinin analogues are now used for the treatment of malaria in many countries (Cardelina II 2002).

Other significant drugs developed from traditional medicinal plants include the antihypertensive agent, reserpine, isolated from *Rauwolfia serpentina* used in *Ayurvedic* medicine for the treatment of snakebite and other ailments (Kapoor 2010); ephedrine, from *Ephedra sinica*, a plant long used in traditional Chinese medicine, and the basis for the synthesis of the anti-asthma agents (Buss & Waigh 2004).

3.5 MEDICINAL PLANTS WITH ANTIMYCOBACTERIAL ACTIVITY

Published work available on antimycobacterial activity of South African plants largely relies on ethnobotanical leads for selection of plants to investigate. The majority of research has been carried out using fast-growing saprophytic *Mycobacterium* species as test microorganisms. An inter-institutional South African collaboration, the Novel Drug Development Platform (www.sahealthinfo.org/noveldrug/novelpamphlet.htm) aims to develop new medicines derived from plants effective against tuberculosis and other diseases. Out of approximately 330 acetone extracts of plants selected as representatives of most of the families of plants occurring in South Africa, 63 provided MIC values lower than 0.1mg/ml against *Mycobacterium smegmatis*. This figure of 19% of plant extracts screened thus far with MIC < 0.1mg/ml is extremely promising, and work is continuing on these plant species, testing them against other mycobacterial species and isolating the active compounds. More collections of representative species of the families of South African plants are being made on an ongoing basis, extending the variety of plant genera tested for antimycobacterial and other biological activities.

Further work was undertaken on the antimycobacterial effects of the genus *Euclea*. This species occurs in different habitats, such as coastal and inland forest and bushveld. It is widely present in tropical and subtropical Africa as well as on the east coast of South Africa. The root bark is used by Zulus to treat bronchitis, pleurisy and chronic asthma (Van Wyk & Van Wyk 1997). An ethanol extract of *Euclea natalesis* was the source of two new compounds, octahydroeuclein and 20 (29)-lupene3β-isoferulate (Weigenand, Hussein, Lall & Meyer 2004). The known compounds shinanolone, lupeol and betulin were also isolated, and of all these compounds, only shinanolone was active against *Mycobacterium tuberculosis* with an MIC of 6.25µg/ml (Weigenand *et al*. 2004). Diospyrin was also active against *M. tuberculosis* (Lall & Meyer 2001), and a 2-aminoacetate derivative of dimethylether-diosporyrin was shown to have enhanced antimycobaterial effects (Lall, Das Sarma, Hazra & Meyer 2003).

Several naphthoquinones and triterpenes were isolated from a chloroform extract of *Euclea natalensis* roots and evaluated for activity against *Mycobacterium tuberculosis* (Lall, Meyer, Wang, Bapela, Van Rensburg, Fourie & Franzblau 2005). The chloroform crude extract, diospyrin and 7-methyljuglone displayed MIC values of 8.0 and 0.5µg/ml against drug sensitive *M. tuberculosis*, and 7-methyljuglone activities against a selection 1.25µg/ml (Lall *et al*. 2005). Interestingly, 7- methyljuglone presented superior intracellular inhibition of *M. tuberculosis* strains ranged J774.1 macrophages when compared to the standard anti-TB drugs streptomycin and ethambutol (Lall *et al*. 2005). Following up on this promising lead, Bapela, Lall, Fourie, Franzblau and Van Rensburg (2006) studied the activity of 7-methyljuglone in combination with anti-TB drugs. It was concluded that 7-methyljuglone synergistically enhanced the activity of isoniazid and rifampicin against *M. tuberculosis* both extracellularly and intracellularly (Bapela, Lall, Fourie, Franzblau, & van Rensburg 2006).

Another naphquinone, neodiospyrin, was isolated for the first time from *Euclea natalensis* roots, together with other known compounds (Van der Kooy, Meyer & Lall 2006) of the six naphquinones isolated, the MIC values of diospyrin (8.0µg/ml), isodiospyrin (10.0µg/ml), 7methyljuglone (0.5µg/ml) and neodiospyrin (10.0µg/ml) compared well to those of the anti-TB drugs ethambutol, isoniazid and rifampicin. Mamegakinone and shinanolone were the least active of the isolated compounds with MIC values of 100µg/ml (Van der Kooy, Meyer. & Lall 2006).

A series of derivatives of the naphquinone 7-methyljuglone was prepared and evaluated for antitubercular activity (Mahapatra, Mativandlela, Houghton, Binneman, Fourie, Hamilton, Meyer, Van der Kooy, Houghton & Lall 2007). The yield of naturally occurring 7-methyljuglone from *Euclea natalensis* is a mere 0.03% (Lall *et al*. 2005), so ability to synthesize the compound was a prerequisite to enable further development of a potential antimycobacaterial product. Mahapatra *et al.* (2007) investigated the activity of 7-methyljuglone and several derivatives as subversive substrates for mycothiol disulfide reductase, but found no direct correlation between this antimycobacterial activity against *M. tuberculosis*. Reasons put forward for this include the possibility that the compounds could non-specifically react with many biological targets, for example other disulfide reductase enzymes (Mahapatra *et al*. 2007).

A herbal treatment with a long and interesting history of use in South Africa, and more recently in Europe where it was introduced, is *umckaloabo*, comprising the roots of two *Pelargonium* species, *Pelargonium sidoides* and *Pelargonium reniforme* (Taylor, Maalim & Coleman 2005; Bladt & Wagner 2007). These species contain many secondary metabolites, including flavonoids, coumarins, phenolic acid derivatives, tannins and phytosterols (Kolodziej 2000). Extracts and constituents of these two species showed antibacterial activity against Gram–negative and Gram-positive bacteria (Kayser & Kolodziej 1997). Using bio-assay guided fractionation of n-hexane extracts of both *Pelargonium reniforme* and *Pelargonium sidoides*, mixtures of straight chain fatty acids with activity against rapidly growing *mycobacteria* were identified (Seidel & Taylor 2004). The test organisms included *Mycobacterium aurum*, *Mycobacterium smegmatis*, *Mycobacterium fortuitum*, *Mycobacterium abscessus* and *Mycobacterium phlei*. All saturated compounds showed no antimycobacterial activity, but unsaturated compounds were active in relation to their degree of unsaturation, their chain length and bacterial species tested (Seidel & Taylor 2004). Linoleic acid was the most potent compound, with MIC of 2µg/l against *Mycobacterium aurum* (Seidel & Taylor 2004). Mativandlela, Lall & Meyer (2006) reported mild antitubercular activity of organic solvent extracts of the roots of *Pelargonium reniforme* against *Mycobacterium tuberculosis* using BACTEC method. It is highly probable that an important component of the antimycobacterial activity exhibited by *umckaloabo* may involve immune stimulating or modulating properties (Kayser, Kolodziej. & Kiderlen 2001; Bladt & Wagner 2007), and Mativandlela *et al.* (2006)

also drew a similar conclusion. This concept requires further investigation not only with regard to *Pelargonium* species but also for others with reputed anti-TB efficacy.

The antimycobacterial efficacy of *Helichrysum caepititium* acetone and water extracts were investigated using the agar plate method against a drug-sensitive strain of *M. tuberculosis*. The acetone extract was relatively active with inhibitory activity at 0.5mg/ml, and the MIC was determined with a radiometric method to be 0.1µg/ml. Another *Helichrysum* species, *Helichrysum melanacme* was the subject of antitubercular activity investigations performed by Lall *et al*. (2006). It was reported that two chalcones isolated from the ethanol extract of *Helichrysum melanacme* shoots had MIC values of 0.05µg/ml against *M. tuberculosis*. The ethanol extract presented MIC of 0.5µg/ml.

Ethanol extracts of seven ethnobotanically chosen medicinal plants were tested against *M. smegmatis* and *M. tuberculosis* (Mativandlela *et al*. 2008). The extracts were also tested for cytotoxicity against Vero monkey kidney cell line to determine selectivity. A flavone was isolated from *Galena Africana*, namely 5, 7, 2´-trihydroxyflavone (Mativandlela, Meyer, Hussein, Houghton, Hamilton & Lall 2008).

One such hydrophobic compound with antimycobacterial activity is *(E)*-phytol, with an MIC of 2µg/ml (Rajab, Cantrell, Franzblau & Fischer 1998). The authors found that a free hydroxy group and overall lipophylicity were the two most important structural characteristics responsible for *in vitro* antituberculosis activity of derivatives of this compound. Similarly, the activity of compounds from *Ferula communis* and synthesized derivatives was dependent on the degree of lipophilicity (Appendino, Mercalle, Fuzzati, Arnoldi, Stavri, Gibbons, Ballero & Mafia 2004), but the authors concluded that a hydroxyl or acetoxyl group caused a reduction in inhibitory activity, while large lipophilic groups at the same position were well tolerated.

Table 2. Plants used in South Africa for treating possible TB-related diseases (respiratory or chest complaints and coughing) (Mcgaw *et al*. 2008)

Family	Species	Use	Plant part used	Potentially bioactive compounds	Screened for antimycobacterial activity
Acanthaceae	*Justicia flava* (Vahl) Vahl [syn.*Adhatoda flava* (Forssk.) Nees]	The alkaloids lycorine and tazettine (Watt & Breyer-Brandwijk 1962)	Leaves, roots	Lignans, hydrocarbons, β-sitosterol, stigmasterol, campesterol, β-sitosterol-β-d-glucoside, salicylic acid, podophyllotoxin-type justicinol, helioxanthin, (+)-isolariciresinol, docosanoic acid (Hutchings, Scott, Lewis, & Cunningham 1996)	N/A
Adiantaceae	*Adiantum capillus-veneris* L.	Cough suppressant, respiratory problems (Hutchings, Scott, Lewis, & Cunningham. 1996)	Whole plant, leaves	Gallic, tannic and quinic acids, terpenoids, glycosides (Hutchings, Scott, Lewis & Cunningham 1996)	N/A
Agavaceae	*Agave* L. sp.	Chest pains, coughing (Pujol 1990)	Shoots and roots	Gallic, tannic and quinic acids, terpenoids, glycosides (Hutchings, Scott, Lewis & Cunningham 1996)	N/A
Aizoaceae	*Carpobrotus* L. spp.	TB, infections (Watt & Breyer-Brandwijk 1962; Springfield, Amabeoku, Weitz, Mabusela & Johnson 2003; Springfield & Weitz 2006)	Leaf juice	Tannins, malic acid and citric acid (Watt & Breyer-Brandwijk 1962). Flavonoids, hydrolyzable tannins, phytosterols, aromatic acids in *Carpobrotus mellei*(Springfield & Weitz 2006)	Ethyl acetate extracts of *Carpobrotus mellei*leaves MIC = 15mg/ml, water extract MIC = 30mg/ml (*Mycobacterium smegmatis*) (Springfield & Weitz 2006).*Carpobrotus muirii* and*Carpobrotus quadrifidis* active (*Mycobacterium smegmatis*) in disc diffusion and bioautography (Springfield, Amabeoku, Weitz, Mabusela & Johnson 2003)

Table 2 Continued. Plants used in South Africa for treating possible TB-related diseases (respiratory or chest complaints and coughing) (Mcgaw et al. 2008)

Family	Species	Use	Plant part used	Potentially bioactive compounds	Screened for antimycobacterial activity
Aizoaceae	*Galenia africana*L	Chest pains, TB (Mativandlela, Meyer, Hussein, Houghton, Hamilton & Lall 2008)	Leaves	5,7,2′-Trihydroxyflavone (Mativandlela, Meyer, Hussein, Houghton, Hamilton & Lall 2008)	Ethanol extract of leaves MIC = 0.78mg/ml (*Mycobacterium smegmatis*), MIC = 1.2mg/ml (*Mycobacterium tuberculosis*); 5,7,2′-trihydroxyflavone MIC = 0.031 and 0.10 mg/ml (*Mycobacterium smegmatis* and *Mycobacterium tuberculosis*, respectively) (Mativandlela, Meyer, Hussein, Houghton, Hamilton & Lall 2008)
Alliaceae	*Agapanthus africanus* (L.)	Chest troubles, coughs (Watt & Breyer-Brandwijk 1962)	Roots	Sitosterol, yuccagenin, agapanthagenin, steroid spirostan sapogenins (Hutchings, Scott, Lewis & Cunningham 1996)	N/A
Alliaceae	*Tulbaghia alliacea*L.	TB symptoms (Bamuamba, Gammon, Meyers, Dijoux-franca& Scott 2008)	Rhizomes	N/A	

3.6 ANTIMICROBIAL ACTIVITY OF NATURAL PRODUCTS

Most plants contain several compounds with antimicrobial properties for protection against aggressor agents, especially microorganisms. Active compounds found in some plants have antiseptic action; for example, thyme has thymol and carvacrol, clove has eugenol and isoeugenol, and oregano has carvacrol and terpinenol-4. In some cases, terpenes from essences that are soluble in water have higher antibacterial power than others (Knobloch, Pauli & Iberl 1989). Mechanisms of actions of natural compounds are related to disintegration of cytoplasmic membrane, destabilization of the proton motive force (PMF), electron flow, active transport and coagulation of the cell content. Not all action mechanisms work on specific targets, and some sites may be affected due to other mechanisms (Burt 2004).

Important characteristics responsible for the antimicrobial action of essential oils include hydrophobic components that allow the participation of lipids from the bacterial cell membrane, which disturb cell structures and make them more permeable (Sikkema, de Bont & Poolman). Chemical compounds from essential oils also act on cytoplasmic membrane proteins (Knobloch, Pauli & Iberl 1989). Cyclic hydrocarbons act on ATPases, enzymes known to be located at the cytoplasmic membrane and surrounded by lipid molecules. In addition, lipid hydrocarbons may distort the lipid-protein interaction, and the direct interaction of lipophilic compounds with hydrophobic parts of the protein is also possible (Sikkema, de Bont & Poolman 1994). Some essential oils stimulate the growth of pseudo-mycelia, evidencing that they may act on enzymes involved in the synthesis of bacterium structural components (Conner & Beuchat 1984).

3.6.1 Several compounds and their mechanisms of action on microorganisms

3.6.1.1 Carvacrol and thymol

The structure of thymol is similar to that of carvacrol; however, they differ as to the location of the hydroxyl group in the phenolic ring. Both substances seem to make the membrane permeable (Lambert, Skandamis, Coote & Nychas). Their structure disintegrates the external membrane of gram-negative bacteria, releasing lipopolysaccharides (LPS) and increasing the permeability of the cytoplasmic membrane to ATP. The presence of magnesium chloride does not influence this action, suggesting a chelating mechanism of different cations on the external membrane (Helander, Alakomi, Latva-kala, Mattila-Sandholm, Pol, Smid, Gorris & von Wright 1998).

3.6.1.2 Eugenol

Different concentrations of eugenol may inhibit the production of amylase and protease by *Bacillus cereus*. Furthermore, cell wall degradation and cell lysis were also reported (Thoroski, Blank & Biliaderis 1989).

3.6.1.3 p-Cymene

A precursor of carvacrol, this hydrophobic compound provokes greater swelling of the cytoplasmic membrane compared to carvacrol (Ultee, Bennik & Moezelaar 2002).

3.6.1.4 Carvone

When tested at concentrations higher than its minimum inhibitory concentration, carvone dissipates gradient pH and cell membrane potential. The growth of *E. coli, Streptococcus thermophilus* and *Lactococcus lactis* may decrease according to the concentrations of carvone, suggesting that it acts by disturbing the general metabolic status of the cell (Oosterhaven, Poolman & Smid 1995).

3.6.1.5 Cinnamaldehyde

Cinnamaldehyde is known to inhibit *E. coli* and *Salmonella typhimurium* growth at concentrations similar to those of carvacrol and thymol. However, it neither disintegrates the outer membrane nor weakens the intracellular ATP. Its carbonyl group has affinity for proteins, preventing the action of decarboxylase amino acids on *E. aerogenes* (Wendakoon & Sakaguchi 1995).

3.7 *IN VITRO* ASSAYS FOR EVALUATION OF ANTI-TUBERCULAR ACTIVITY

Anti-mycobacterial activity of plant extracts is usually done by culturing mycobacteria in ranging types of Agar and broth based media (Newton, Lau & Wright 2000). These methods are: agar well/disc diffusion method, macro and micro dilution method and micro plate alamar blue assay. The Agar well/disc diffusion method is one of the most commonly used methods (Newton *et al.* 2000).

In this method, wells or micro discs are impregnated with the drug extract and placed on to the inoculated medium; zones of inhibition are then measured after a period of incubation 47 (Parish & Stoker 1998). The major disadvantage with such a method is that hydrophilic compounds may not diffuse thus can be missed owing to the fact that mycobacteria cell wall is lipophilic (Gautam, Saklani & Jachak 2007; Connel & Nikaido 1994). Secondly, these are non quantitative methods but only indicative of whether there is activity or not (Newton *et al*. 2000).
In the macro and micro dilution method known concentrations of the extracts are tested on the bacteria in agar media (Pauli, Case, Inuli, Wang, Cho, Fischer & Franzblau 2005). The method allows for quantification and determination of minimum Inhibitory concentration (Gautam *et al*. 2007). The medium is supplemented with oleic acid, albumin, dextrose and catalase (OADC supplement Difco) (Pauli *et al*. 2005). The major disadvantage with the method is that it requires at least 18 days to visibly detect growth of the colonies (Gautam *et al*. 2007).

Micro broth dilution method is used in the determination of MIC. In this method serial dilutions of the extract are placed in different test tubes containing inoculated broth (Suffredin, Sander, Goncalves, Reis, Gales, Varella & Younes 2004). Quantification is done by visualizing turbidity in the test tube. This poses a disadvantage of misinterpretation due to the tendency of mycobacteria to clump and also crude extracts may impart some turbidity to the medium (Gautam *et al*. 2007).

The use of a redox indicator dye (alamar blue) makes this test not only rapid but also sensitive (Gautam *et al*. 2007). Micro plate alamar blue assay can be read visually without necessarily using instrumentation (Franzblau *et al*. 1998).The reduced form of the dye can also be quantified calorimetrically by measuring absorbance at 570nm (Pauli *et al*. 2005)

CHAPTER 4

4. 1 METHODS

4.1.1 Test compounds

Forty synthetic compounds were obtained from the Council for Scientific and Industrial Research (CSIR) for screening at South African Medical Research Council (SAMRC). The compounds were firstly screened against H37Rv, a reference TB strain. Compounds with MICs below 10µg/ml (MVB 282/61215, 61223, 61270 & SMJ 5 belong to the class of pre ring closed compounds1,3-substituted amines-4,6-dinitrobenzene compounds and MVB 282/61271 is clofazamine analogue) were further tested against clinical sensitive TB strains and drug resistant TB strains. Their toxicity levels and synergistic activity were further determined.

4.1.2 Colorimetric alamar blue assay

Alamar blue assay (Collins & Franzblau 1997) involves the addition of alamar blue dye to microplates containing test compounds and cultures of *M. tuberculosis*. After an incubation period of 5 to 6 days, the growth of *M. tuberculosis* was observed as a change in colour of the alamar blue solution from blue to pink due to reduction of the dye. MICs of each compound tested were determined by a change in colour in the wells.

4.1.3 Test isolates

Three resistant clinical isolates (MDR, Pre-XDR & XDR) and two drug-susceptible (1 clinical isolate and the H37Rv) were studied. The strains were grown for three weeks on Lowenstein-Jensen (LJ) slant.

4.1.4 Preparation of the bacterial inocula

A colony of *M. tuberculosis* was removed from a 3 week old Löwenstein- Jensen slant. The bacterium was transferred into a glass tube containing sterile beads and 5ml of saline. The contents were vortexed for 1-5 minutes to remove the clumps and then left in a biosafety cabinet (BSCIII) to settle for 5 minutes. The top supernatant was transferred to a sterile glass tube. The turbidity was adjusted by comparing to a McFarland number 1 standard by adding saline ($\approx 10^7$ cfu /ml).

4.1.5 Preparation of the inocula stock

A small colony was removed from a 3 week old Lowenstein-Jensen slant using a sterile plastic disposable inoculating loop and deposited into a test tube containing glass beads and then vortexed for 2 minutes to remove clumps. A 7H9 medium containing 15% glycerol was added to the tube and vortexed again for 2 minutes. The contents were allowed to settle for 15 to 30 minutes. Before removing the supernatant with a pipette and transferring it to the empty tube the optical density of the bacterial suspension was measured using a spectrophotometer at a wavelength of 600 nanometer. The bacterial suspension was then diluted to obtain a bacterial inoculum of 1×10^{7} - 1×10^{8} cfu/ml. The bacterial inocula were divided into aliquots of 500µl and stored at -70 °C until use.

4.1.6 Preparation of the inocula for antimycobacterial testing

An aliquot of 500µl of frozen *M. tuberculosis* was thawed at room temperature and the bacterial cells were declumped using a pipette. The bacterial suspension was further diluted to 1:25 in Middlebrook 7H9 supplemented with 0.2% of glycerol, 0.1% casitone and 10% oleic albumin dextrose catalase (OADC).

4.1.7 Preparation of drug/sample dilutions

Prior to the experiment: Test compounds and INH /RIF were dissolved in 100% DMSO and water to a final concentration of 10mg/ml. A 1:2 dilution of the drug/sample was made in a 96 well plate and mixed well.

4.1.8 Antimycobacterial testing by the colorimetric microplate alamar blue assay

A 96 well, sterile, transparent microtiter plate was used to perform MABA. The outer perimeter wells were filled with 200µl of sterile water to prevent dehydration during incubation. Into each well was dispensed 98µl of 7H9 broth except for the control wells which were dispensed with 100µl (rows B to G and columns 2 to 11). A volume of 2µl of the drug/sample was added into each well (column 2, row B to G), the control wells were drug free. The individual MIC's for INH and RIF were determined for each isolate using the following ranges of drug concentrations: 1 to 0.00390µg/ml. The final drug concentrations tested were 10 to 0.039µg/ml. A volume of

100µl of the inocula was added to make the total volume in these wells 200µl. The plates were covered and sealed with parafilm and incubated at 37 ºC.

After 7 days of incubation, 50µl of freshly prepared 1:1 mixture of alamar blue dye and 10% Tween 80 was added to the control wells (B 11) only. The plates were resealed with parafilm and re-incubated for 24hrs at 37 ºC. At 24hr incubation, if there was a colour change from blue to pink in B 11 wells, 50µl of 1:1 mixture of alamar blue dye and 10% of Tween 80 was added to the rest of the wells. The plates were resealed with parafilm and were incubated for an additional 24 hours at 37 ºC, and the colours of all the wells were recorded. The results were read manually by colour determination in the wells. A colour change from blue to pink was interpreted as bacterial growth (Fig. 9). MIC was defined as the lowest concentration of the drug that showed no colour change. Duplicate wells were used per concentration of the test compound in 96 well plates and each experiment was repeated at least 3 times.

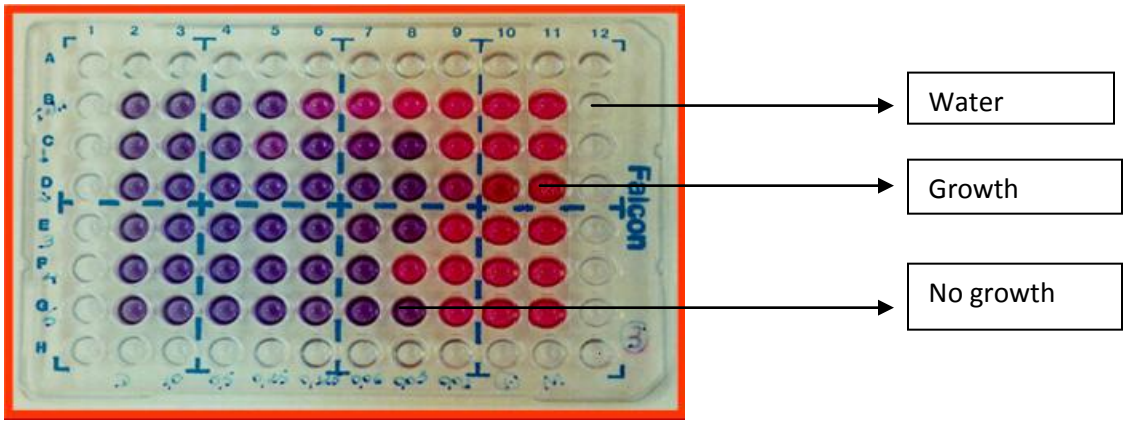

Figure 9: Microtitre plate for Alamar Blue Assay

4.2 CYTOTOXICITY

4.2.1 Preparation of cells for the assay

The C2C12 mouse adherent myoblast cell line was provided to the Medical Research Council by the University of Pretoria, Pharmacology department. In a 15ml conical tube the cells were centrifuged at 200g for 5 minutes. The supernatant was removed and the pellet was resuspended with 1ml of 2% Dulbecco's modified Eagles medium (DMEM) supplemented with fetal calf serum.

4.2.2 Counting viable cells using tryptan blue

A 180µl solution of tryptan blue was transferred to a sterile 5ml test tube**.** The solution was added with 20µl of the cell suspension mixed thoroughly. The contents were allowed to stand for 5 to 15 minutes to allow viable and non-viable cells to take up the dye. With a cover slip in place, a pipette was used to transfer a small amount of the tryptan Blue-cell suspension mixture to both chambers of the hemacytometer (Fig. 10). The hemacytometer was placed on the microscope for cell counting.

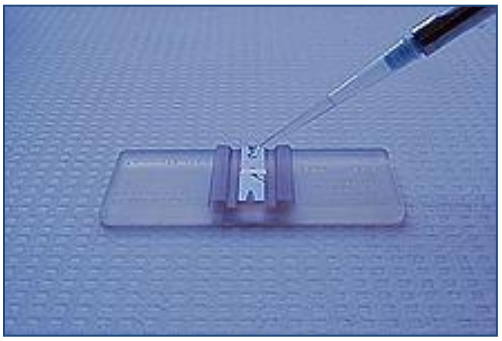

Figure 10: Hemocytometer (www.wikipedia.org/wiki/Hemocytometer)

Starting with chamber 1 of the hemacytometer, all the cells were counted in the 1mm center square and four 1mm corner squares (Fig. 11)

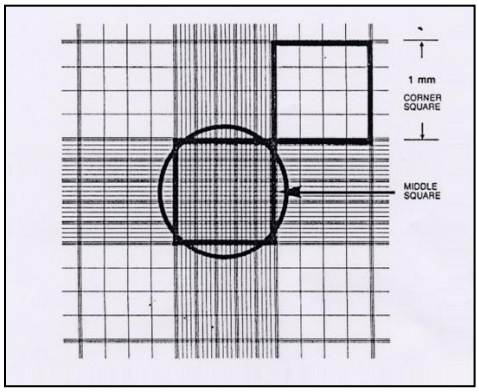

Figure 11: Appearance of the haemocytometer grid visualised under the microscope (http://www.phe-culturecollections.org.uk/technical/ccp/cellcounting.aspx)

To calculate the total volume of cells that can be made up, the following calculation was used:

Equation 1:

$$\text{Cells per ml} = \frac{\text{the average of cellls counted per square} \times \text{the dilution factor } (10) \times 10^4}{\text{Cell concetration } (12.5) \times 10^4} \ldots.1$$

4.2.3 Plating of cells

In a sterile 96 well microtiter plate, the blank wells were filled with 80µl of 2% DMEM medium. The rest of the wells (controls and for test compounds) were filled with 80µl of cell suspension with medium. The plates were kept in a flouro tub containing a wet paper towel to prevent evaporation of the plates during incubation. The plates were incubated for 24hrs at 37 ºC. Following plating and a 24 hr recovery to allow cells to resume exponential growth, the blank and control wells were filled with 80µl of culture medium. The vehicle controls (DMSO and water) were filled with 80µl of culture medium containing DMSO and water. Test compound wells were filled with culture medium containing the test drug. Isoniazid was used as positive control. Each drug concentration was done in triplicates. The plates were incubated for 72hrs at 37 ºC. After 72hr incubation the cells were fixed by means of a protein precipitation with 30% trichloroacetic acid at 4 ºC for 24hrs. The cells were then washed with tap water five times and plates were kept in the oven to dry.

4.2.4 Sulforhodamine B (SRB) Assay

The assay relies on the ability of SRB to bind to protein components of cells that have been fixed to tissue-culture plates by trichloroacetic acid (TCA). SRB is a bright-pink aminoxanthene dye with two sulfonic groups that bind to basic amino-acid residues under mild acidic conditions, and dissociate under basic conditions. The fixed dye is solubilized and is measured photometrically at 540nm wavelength with a reference filter of 690nm. The optimal density (OD) values correlate with total protein content and therefore with cell number.

Each well of the dried plates was filled with 100µl of SRB stain and the plates were allowed to stand for 30 minutes on a bench. After 30 minutes the plates were subsequently washed 4 times

with 1% acetic acid to remove the unbound stain. The plates were air dried and bound protein stain was solubilised with 200µl of 10mmol/l of unbuffered tris base [tris (hydroxyl-methyl) aminomethane]. The optical density was read using a plate reader at 630nm (Fig. 12). The experiment was repeated three times to confirm results.

Figure 12: A microplate reader with 96-well in a sample drawer

4.3 SYNERGITIC TESTING

4.3.1 Preparation of the inocula for antimycobacterial testing

An aliquot of 500µl of frozen *M. tuberculosis* was thawed at room temperature and the bacterial cells were declumped using a pipette. The bacterial suspension was further diluted to 1:25 in Middlebrook 7H9 supplemented with 0.2% of glycerol, 0.1% casitone and 10% albumin-dextrose.

4.3.2 Preparation of drug/sample dilutions

Prior to the experiment: Test compounds and INH /RIF were dissolved in 100% DMSO and water to a final concentration of 10mg/mL. A volume of 50µl of RIF/INH was combined with 50 µl of the test compound in the first well of sterile 96 well plates. A 1:2 dilution of the combined drugs was made in a 96 well plate and mixed well.

4.3.3 Combined drug action by the colorimetric alamar blue assay

Two different combinations of synthetic compounds in combination with INH and RIF were tested for possible synergistic activity against the drug resistant and drug susceptible *M.*

tuberculosis strains. Combination 1 included the six compounds in a two-drug combination with the first line drug INH. Combination 2 included all six compounds in combination with RIF.

A 96 well, sterile, transparent microtiter plate was used to perform MABA. The outer perimeter wells were filled with 200µl of sterile water to prevent dehydration during incubation. Into each well was dispensed 98µl of 7H9 broth except for the control wells which were dispensed with 100µl. A volume of 2µl of the drug/sample was added into each well; the control wells were drug free. The individual MIC's for INH and RIF were determined for each isolate using the following ranges of drug concentrations: 1 to 0.00390. The final drug concentrations tested were 10 to 0.039µg/ml. A volume of 100µl of the inoculum was added to each well. The plates were covered and sealed with parafilm and incubated at 37 ºC. After 7 days of incubation, 50µl of freshly prepared 1:1 mixture of alamar blue dye and 10% Tween 80 were added to the control wells only and the plates were resealed with parafilm and re-incubated for 24hrs at 37 ºC. After 24hrs incubation, if there was a colour change from blue to pink, 50µl of 1:1 mixture of alamar blue dye and 10% of Tween 80 was added to the rest of the wells. A colour change from blue to pink was interpreted as bacterial growth. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of the drug that showed no colour change. Duplicate wells were used per concentration of the test compound in 96 well plates and each experiment was repeated at least 3 times.

The activity of two drug combinations was evaluated at sub –MIC levels (below original MIC values). Analysis of the drug combination data was achieved by calculating the fractional inhibitory concentration (FIC) index as follows:

Equation 2:

$$FIC = (MIC_{a\ combination} / MIC_{a\ alone}) + (MIC_{b\ combination} / MIC_{b\ alone}) \quad \ldots\ldots 2$$

FIC- Fractional inhibitory concentration

MIC- Minimum inhibitory concentration

≤- less than or equal to, ≥- greater than or equal to

= equal to

The FIC was interpreted as: FIC $\leq$ 0.5, synergistic activity; FIC=1 indifference/additive activity; FIC $\geq$2 or more, antagonistic activity. Subscripts a sand b represented two different compounds.

CHAPTER 5

5.1 RESULTS AND INTERPRETATION

Table 3: MICs and antibacterial activity of identified active compounds (MVB 282/61271, SMJ 15, MVB 282/61215, MVB 282/61270, MVB 282/661280 and MVB 282/61223) against both drug sensitive and multi-drug strain *M. tuberculosis*

Test Compound Code	**MIC(µg/ml) of drug sensitive & multidrug resistant strain**				
	[a]H37Rv	**[b]BAS 1109/12**	**[c]SZ-47/11**	**[d]SZ -105/11**	**[e]SZ-94/11**
MVB 288/61271	0.781	0.390	0.781	0.390	0.390
SMJ 15	6.25	0.390	0.781	0.781	3.125
MVB 282/61215	3.125	0.781	0.781	0.781	0.781
MVB 282/61270	6.25	3.125	3.125	3.125	1.5625
MVB 282/61280	0.390	0.390	0.781	0.390	0.390
MVB 282/61223	6.25	0.781	0.781	0.781	0.781
INH	0.25	>100	>100	>100	>100
RIF	0.5	>100	>100	>100	>100

a-Reference strain, b- clinical sensitive strain, c- MDR strain, d- Pre-XDR strain and e- XDR strain

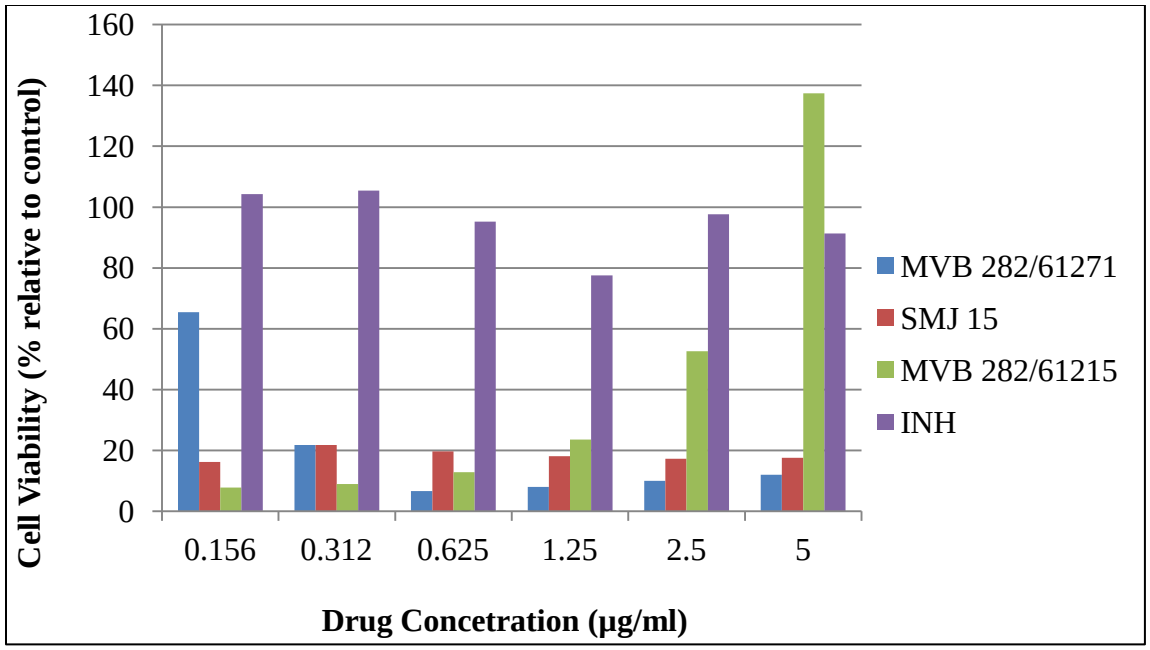

Figure 13: Cytotoxicity of MVB 282/61271, SMJ 15 & MVB 282/61215 on C2C12 cell line in comparison with INH by SRB assay

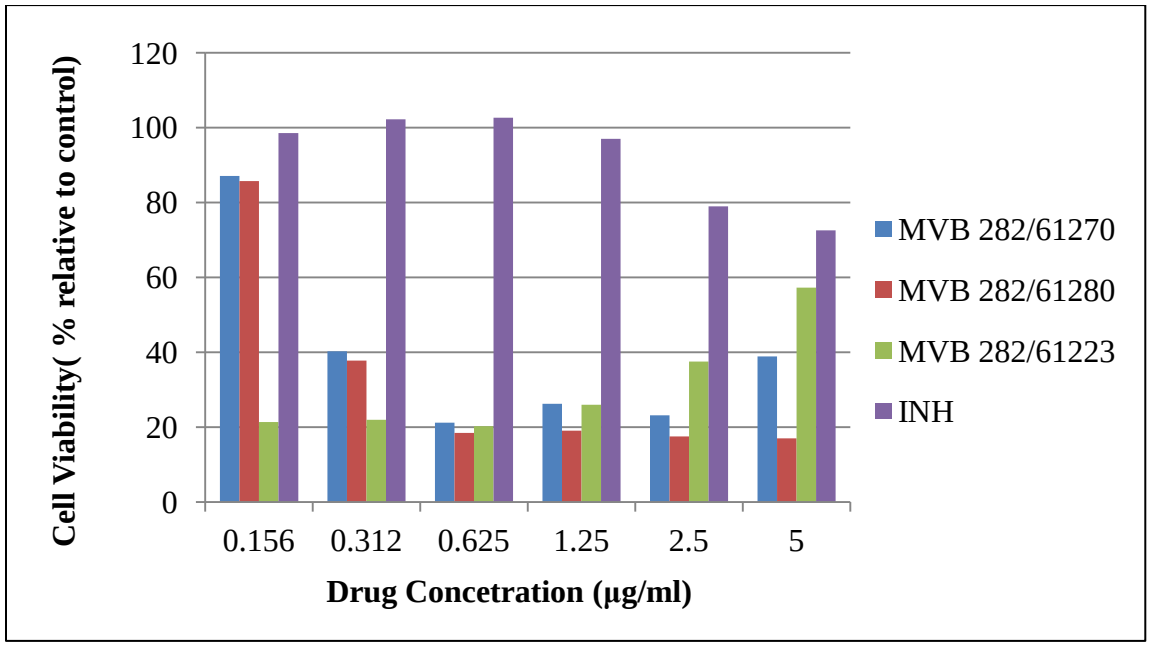

Figure 14: Cytotoxicity of MVB 282/61270, MVB 282/61280&MVB 282/61223 on C2C12 cell line in Comparison with INH determined by SRB assay

Table 4: Synergistic activity of MVB 288/61271, SMJ 15, MVB 282/61215, MVB 282/61270, MVB 282/61280&MVB 282/61223 with existing antituberculous drugs (INH) against drug sensitive *M. tuberculosis* strains using the colorimetric alamar blue assay

Drug combination	**MIC(µg/ml) and FIC of compound combinations against drug-sensitive *M. tuberculosis* strains**			
	H37Rv		**BAS 1109/12**	
	MIC	**FIC**	**MIC**	**FIC**
MVB 61271+INH	0.039	0.21	0.039	0.25
SMJ 15 +INH	0.039	0.17	0.039	0.25
MVB 61215 +INH	0.039	0.17	0.039	0.21
MVB 61270 +INH	0.039	0.17	0.039	0.17
MVB 61280 +INH	0.039	0.26	0.039	0.26
MVB 61223 +INH	0.039	0.17	0.039	0.21

Table 5: Synergistic activity of MVB 288/61271, SMJ 15, MVB 282/61215, MVB 282/61270, MVB 282/61280&MVB 282/61223 with existing antituberculous drugs (INH) against drug-resistant *M. tuberculosis* strains using the colorimetric alamar blue assay.

Drug Combination	**MIC(µg/ml) and FIC of compound combinations against drug resistant *M. tuberculosis* strains**					
	SZ-47/11		**SZ-105/11**		**SZ-94/11**	
	MIC	**FIC**	**MIC**	**FIC**	**MIC**	**FIC**
MVB 61271+INH	0.156	0.82	0.078	0.51	0.078	0.51
SMJ 15 +INH	0.156	1.65	0.156	0.82	0.078	0.33
MVB 61215 +INH	0.3125	4.50	0.3125	1.65	0.3125	1.65
MVB 61270 +INH	0.625	2.70	0.3125	2.25	0.625	2.90
MVB 61280 +INH	0.039	0.21	0.078	0.51	0.0390	0.26
MVB 61223 +INH	0.625	3.30	0.0625	3.30	0.156	0.82

Table 6: Synergistic activity of MVB 288/61271, SMJ 15, MVB 282/61215, MVB 282/61270, MVB 282/61280 & MVB 282/61223 with existing antituberculous drugs (RIF) against drug-sensitive *M. tuberculosis* strains using the colorimetric alamar blue assay

Drug Combination	**MIC(µg/ml) and FIC of compound combinations against drug-sensitive *M. tuberculosis* strains**			
	H37Rv		**BAS 1109/12**	
	MIC	**FIC**	**MIC**	**FIC**
MVB 61271+ RIF	0.039	0.13	0.039	0.18
SMJ 15 + RIF	0.039	0.09	0.039	0.18
MVB 61215 + RIF	0.039	0.09	0.039	0.13
MVB 61270 + RIF	0.039	0.09	0.039	0.09
MVB 61280 + RIF	0.039	0.18	0.039	0.18
MVB 61223 + RIF	0.039	0.09	0.039	0.13

Table 7: Synergistic activity of MVB 288/61271, SMJ 15, MVB 282/61215, MVB 282/61270, MVB 282/61280&MVB 282/61223 with existing antituberculous drugs (RIF) against drug-sensitive *M. tuberculosis* strains using the colorimetric alamar blue assay

Drug Combination	**MIC(µg/ml) and FIC of compound combinations against drug -resistant *M. tuberculosis* strains**					
	SZ-47/11		**SZ-105/11**		**SZ-94/11**	
	MIC	**FIC**	**MIC**	**FIC**	**MIC**	**FIC**
MVB 61271+RIF	0.039	0.13	0.078	0.36	0.039	0.18
SMJ 15 +RIF	0.039	0.13	0.039	0.09	0.039	0.09
MVB 61215 +RIF	0.039	0.13	0.3125	0.13	0.039	0.26
MVB 61270 +RIF	0.039	0.09	0.625	0.09	0.078	0.21
MVB 61280 +RIF	0.039	0.13	0.039	0.18	0.039	0.18
MVB 61223 +RIF	0.039	0.13	0.156	0.13	0.078	0.26

CHAPTER 6

6.1 DISCUSSION

After several decades, pharmaceutical companies and research scientists are showing renewed interest in finding improved drugs to treat tuberculosis. In developing countries, the drugs now available are no longer effective. The development of new drugs with antimycobacterial activity against multidrug-resistant mycobacteria is a top priority for health care. Such drugs must be well tolerated, amenable to the intermittent short course treatment of large population groups and above all, of low cost. Screening of medicinal plants used in indigenous communities is one way of achieving this goal. In this study the antimycobacterial activity of synthetic compounds isolated from medicinal plants against five strains of *M. tuberculosis* by colorimetric alamar blue assay was evaluated.

The MICs of RIF, INH and the synthetic compounds were determined by colorimetric alamar blue assay against all the five strains of *M. tuberculosis* (SZ-47/11, SZ-105/11, SZ-94/11, H37Rv and BAS- 1109/11). The alamar blue assay results were available in 8 days. Forty (40) synthetic compounds were screened, only six compounds showed antimycobacterial activity with MICs below 10µg/ml. The results of synthetic compounds (table 3) showed that MVB 282/61271 and MVB 282/61280 are the most active compounds exhibiting MIC values of 0.781µg/ml and 0.390µg/ml against drug sensitive strains and 0.781µg/ml and 0.390µg/ml against drug-resistant strains, respectively.

MVB 282/ 61215 and MVB 282/61270 are the second most active compounds exhibiting MIC value of 6.25µg/ml against drug-sensitive strain and 0.390µg/ml against drug-resistant strain. SMJ 15 and MVB 282/61270 are the least active compounds; they exhibited MIC value of 6.25µg/ml against drug-sensitive strains and 3.125µg/ml and 1.5625µg/ml against drug-resistant strains. MVB 282/61270 and SMJ 15 had a higher MIC against multidrug-resistant strains as compared to drug-sensitive strains; this might be because multidrug-resistant strains have efflux pumps, requiring lower concentrations of compounds to kill them. Although the MIC of these compounds is higher than those of the control RIF and INH, it is still comparable than some of

the drugs (eg. Ethambutol, kanamycin and ofloxocin). The screening results showed the dosage is dependent on the bactericidal effects of the compounds

The emergence of multidrug-resistant strains of *M. tuberculosis* poses a serious threat in tuberculosis control. Resistance to RIF is almost always associated with MDR, hence RIF was chosen as the control. The colorimetric MIC obtained in the study for RIF-susceptible and INH strains of *M. tuberculosis* are in close agreement with the MICs for susceptible strains (≤ 0.5 & ≤ 0.25) reported by Yajko, Madej, Lancaster, Sanders, Cawthon, Gee, Babst and Hadley (1995) and Franzblau Witzig, Mclaughlin, Torres, Madico, Hernandez. Degnan, Cook, Guimper, Ferguson and Gilman (1998).

Sulforhodamine (SRB) cytotoxicity assay is a rapid and less cost-effective tool to help choose optimal candidates, those samples with low toxicity and high antimycobacterial activity, similar to a therapeutic dose, and to exclude any too toxic samples to test at their antimycobacterial concentration for ensuring intracellular assays. The isolated compounds were evaluated *in vitro* for their inhibitory ability against the growth of C2C12 cell line. The results obtained in (Fig. 13) indicated that the cytotoxicity effects of the three compounds on C2C12 cells demonstrated marginal toxicity except for MVB 282/61215 which showed a high toxicity at the lowest concentration of 0.156µg/ml with over 100% viable cells at the highest concentration (5µg/ml). MVB 282/61271 had the highest percentage cell viability (65%) at the lowest concentration as compared to SMJ 15 and MVB 282/61215. The little toxicity is most likely due to lack of protein binding phenols. MVB 282/61215 was not toxic to C2C12 at the highest concentration with more than 50% of the cells still viable at this concentration. Further studies, including *in vivo* experiments and toxicity tests are necessary to gain a full understanding of the effectiveness and possible toxic nature of these compounds. Isoniazid had a different effect on the cells with more than 80% of the cells still viable at all concentrations.

At the highest concentration to lowest concentration, compounds MVB 282/61270 and MVB 282/61280 (Fig. 14) had a lower percentage of cell survival. At 0.156µg/ml concentration, the percentage survival of cells was over 50% which is an expected effect of a non-toxic compound; a highly potent drug evokes a bigger response at low concentrations, while a drug of lower potency evokes a small response at low concentrations. The two compounds have higher

potency. MVB 282/61280 showed a lower toxicity compared to its MIC of 0.390µg/ml. Similar to compound MVB 282/61215, with MVB 282/61223 there was a higher percentage of cell survival at higher drug concentrations than at lower concentrations. This might be because toxic substances in low concentrations sometimes stimulate cell activity. Since cells have functions to protect themselves from exposure of toxic substances, enzymatic activity of cells may increase at the initial stage. Then, the cells start to die after a certain concentration. It might also be because the two compounds are enzyme inhibitors, which means molecules bind to enzymes and decrease their activity. Since blocking an enzyme's activity can kill a pathogen or correct a metabolic imbalance, many drugs are enzyme inhibitors. A medicinal enzyme inhibitor is often judged by its specificity (its lack of binding to other proteins) and its potency. A high specificity and potency ensures that a drug will have few side effects and thus low toxicity.

The study of the combined effect of the different drugs used in the treatment of TB could be the first step for predicting the efficacy of drug combinations. In this study the *in vitro* drug combinations in the treatment of TB involving two drug combination has been evaluated. The INH combination (table 4) with six compounds showed synergism in the drug susceptible clinical isolate and H37Rv with fractional inhibitory concentration (FIC) ≤ 0.5. The MIC in combination in the clinical isolate and H37Rv reduced significantly to 0.039µg/ml. MVB 282/61280, exhibited synergistic antimycobacterial activity against drug resistant strains with FICs 0.21, 0.51 and 0.26 respectively. Another synergistic effect was seen on MVB 282/6127 and SMJ 15 on Pre-XDR and XDR isolates (table 5). Their FICs were 0.51 and 0.33 respectively. Most MICs in INH combination in drug resistant isolates decreased to concentrations approaching susceptible rates suggesting that the action of combination could overcome the resistance in the INH- resistance isolates. Most compounds in combination with INH on drug resistant isolates reflected additive and antagonistic activity.

The best synergistic result is reflected in the combination of all the six compounds (MVB 282/61271, SMJ 15, MVB 282/61215, MVB 282/61270, MVB 282/61280 and MVB 282/61223) in combination with RIF with FICs ≤ 0.5 (tables 6 & 7). Most MICs in combination decreased as compared to MICs' individual compounds. This synergistic activity of these compounds implies that if a patient were to take Rifampicin prescribed for the treatment of TB and combined the

regime with either of the synthetic compounds tested; other possible benefits such as reduced length of RIF's treatment and decreased toxicity due to lower intake concentrations of one of the compounds could subsist.

By combining components of indigenous medicinal plants that either have a direct or an indirect influence on mycobacterial survival, it may be possible to provide relatively inexpensive therapeutic formulations for treatment of mycobacterial infections, particularly in regions where cost considerations are of paramount importance. Testing the cytotoxicity of this combination should be the next step to shed some light on this possibility. It would be of interest to determine if the synergistic effect could be demonstrated in an animal model of infection. If proven effective and relatively non-toxic to humans, addition of one the drug to INH or RIF may provide an alternative regimen for prophylactic or preventive treatment of persons likely to be infected with single drug-resistant strains of *M. tuberculosis*.

All the six compounds in combination with RIF inhibited both drug-resistant strains of *M. tuberculosis* (MDR& XDR) at an MIC of 0.039µg/ml. This activity indicates a mechanism of action different to INH, RIF, STR and EMB. These drugs target cell wall synthesis (INH, EMB), inhibit gene transcription (RIF), and inhibit protein synthesis (STR) (Zhang 2005). The antimycobacterial mechanism of action of the six compounds has to be investigated in order to confirm this supposition as a different mechanism of action to RIF could hold positive implications on preventing drug resistance as well as targeting strains already resistant to RIF with the use of a drug therapy that combines one of the six compounds with RIF.

Similar to a therapeutic index it is necessary to compare the difference between cytotoxicity and antimycobacterial activity of the tested samples in order to choose the best candidates for possible treatment options. Of all samples MVB 282/61270 and MVB 282/61280 had the broadest difference in toxicity when compared to biological activity, followed by MVB 282/61271. MVB 282/61215 had the narrowest therapeutic range followed by SMJ 15 and MVB 282/61223.

The limitations of the assays used, alamar blue assay is cheaper, it requires fewer reagents to get results comparable to the competition. It also much easier and safer because it is nontoxic,

nonradioactive reagent that is safer for the user, the cells and the environments. It is compatible with fluorescence or absorbance-based instrumentation. Using alamar blue one has to assess whether the compound to be tested does not interfere with alamar blue itself. Alamar blue-based assays have been shown to be powerful and sensitive tools for the identification of growth-inhibitory compounds irrespective of the underlying mode of action and chemical classification. They are widely used for screens with both mammalian cells and microorganism including mycobacterial species.

With SRB assay, the solvent controls allow to detect solvent effects. If the solvent values differ significantly from the growth control values, inhibition values of test compounds are to be interpreted with caution. The method not only allows a large number of samples to be tested within a few days, but also requires only simple equipment and inexpensive reagents. Therefore the SRB assay an efficient and effective method for screening.

6.2 CONCLUSION

The value of this research lies in the scientific verification of the use of many of the class of the compounds studied. There is much potential for future research activities in this field, as investigation of the active principles of other compounds with good biological activity may yield exciting discoveries.

With 40 compounds being screened and only 6 showing antimycobacterial activity this suggests that more lead compounds are needed for the development of new drugs. Certain class of compounds appears to hold promise for the development as anti-TB agents, for example MVB 288/61271 which is a clofazamine analogue has been screened for antimycobacterial activity with encouraging results. Toxicity leads with a different cell line may yield good results.

The two-drug combination tested may be useful against drug–resistant isolates although the combination including RIF showed better efficacy, being of a potential use in drug-susceptible and drug- resistant isolates. The present study of screening compounds for drug development and determining their efficacy in two drug combinations against drug-resistant TB is an interesting approach to better design studies *in vivo* and *ex vivo* models. This will be useful for the design of treatment schedules in resistant cases as well, in the evaluation of treatment regimens including

new drugs. The various aspects of the challenges faced in TB drug discovery are applicable to other infectious agents. The active compounds against *M. tuberculosis* should be explored further for their use in disease control. With synthetic compounds being reintroduced to the field of medicinal plants science, these compounds could also be applied to screening for activity against other pathogens. It is the interplay of co-developed methods on natural products chemical perspective that will improve the chances of treatment success.

There is no doubt that natural products, with their range of interesting chemical structures and powerful antimycobacterial effects are certain to remain important participants in the development of new generations of antimycobacterial drugs. With its rich heritage of plant biodiversity coupled with the high local incidence of TB, South Africa is sure to be a key participant in the search for new antimycobacterial agents, whether as purified compounds or developed extracts, of plant origin.

With the TB structural genomics consortium releasing protein structures of potential drug targets, bioinformatics and molecular modeling may help generate new leads in the future. Increasing understanding of host-pathogen interactions of mycobacteria may also uncover novel targets for future antimicrobials. Researchers working on discovery of antimycobacterials now have a variety of assays and techniques at their disposal capable of identifying interesting compounds/compound classes. The choice of assay for a particular study will be influenced by a variety of factors including the background of the compound to be tested, instrumentation available, and of course the cost. It is reasonable expectations to see a number of new drugs finally reach the market in the near future, after a greater than 45 year gap.

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