THE EFFECTS OF CLOFAZIMINE ON *MYCOBACTERIUM SMEGMATIS* BIOFILM FORMATION

BY

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Submitted in fulfilment of the requirements for the degree of

Masters of Science

In

The department of Immunology

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Pretoria

June 2013

CHAPTER 1: LITERATURE REVIEW

1.1 BACKGROUND

1.1.1 History and epidemiology of tuberculosis

In 1982, Robert Koch identified the most microbial pathogen, *Mycobacterium tuberculosis* (*M. tuberculosis*), which causes tuberculosis (TB) in humans, a devastating disease of humankind (Koch, 1882; 1932; Kaufmann and Winau, 2005). In the past, about 1 000 years ago, the organism was established as being a pathogen for both animals and humans, and was transmitted during domestication of animals (Smith et al., 2009).

The disease is still a major health concern, globally, and affects mainly humans. To date, there are about nine million new cases of TB reported annually, with close to two million people dying from the disease (Figures 1.1 and 1.2). It is the second highest cause of mortality due to infection, following human immunodeficiency virus/ acquired immunodeficiency syndrome (HIV/AIDS) (Dolin et al., 2010).

Of the TB cases reported worldwide, approximately 30% are in Africa, with the greatest burden in sub-Saharan Africa, mainly South Africa, and the remainder in South East Asia (Figure 1.1) (Dye, 2005; WHO, 2010). Most of these cases are young adults (between 15-59 years), with latent disease (88%) and co-infected with HIV (70%) (WHO, 2006; Kumar et al., 2007).

In most circumstances, TB is curable. About 90% of people with drug-susceptible TB can be cured within six months of treatment, using combinations of first-line drugs (Lopez et al., 2006). Despite this, approximately 0.4-0.5 million cases (WHO, 2010; 2011) of multidrug-resistant tuberculosis (MDR-TB) and extensively-drug resistant tuberculosis (XDR-TB) cases are reported, every year. Treatment of these cases is challenging as it requires the lengthy use of second-line drugs for approximately two years, which is costly and is associated with severe side effects. In addition, the cure rate is usually low, ranging from 50-70% (WHO, 2011).

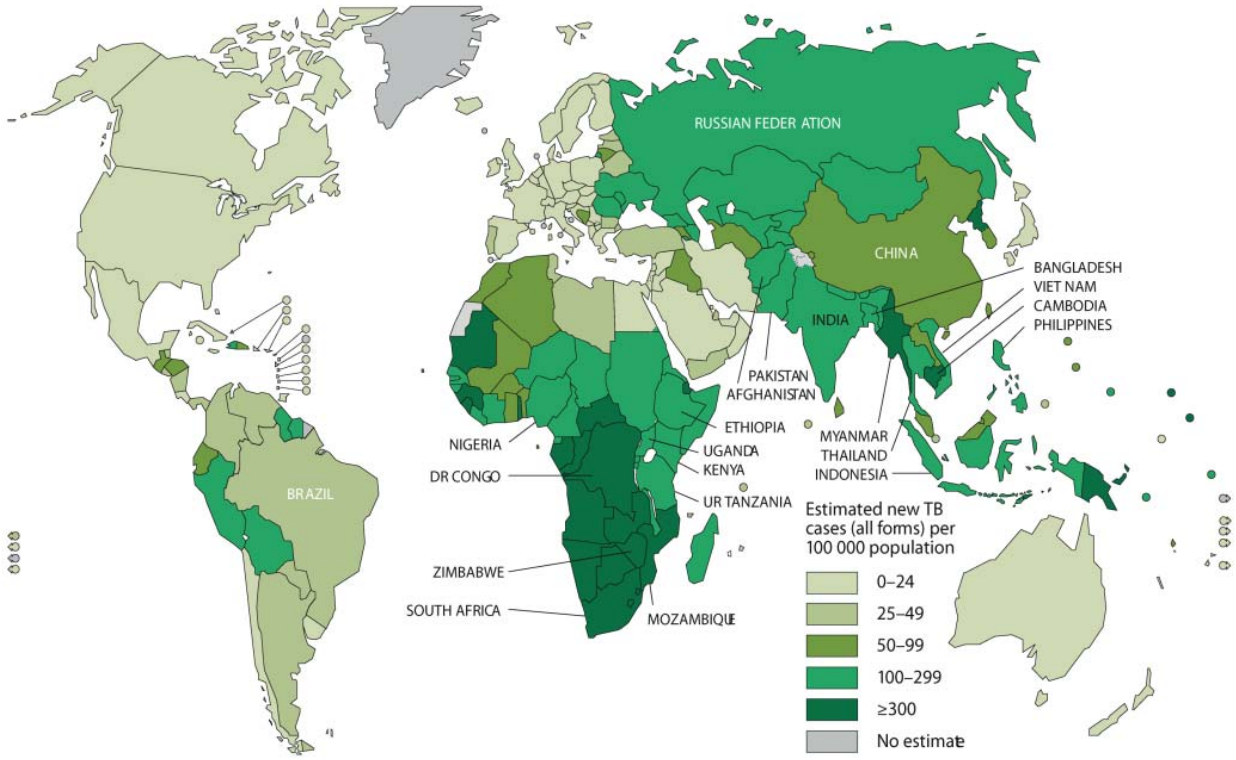


Figure 1.1: Estimated tuberculosis incidence rates in 2010 (Source: WHO, 2010).

1.1.2 Pathogenesis of *Mycobacterium tuberculosis* infection

The pathogenesis of tuberculosis is as illustrated in Figure 1.2. *M. tuberculosis* bacilli are inhaled as aerosols and enter the lungs where they infect the alveolar macrophages. Here, the bacteria bind to phagocytic receptors of the macrophages, dendritic cells and monocytes from the bloodstream and are phagocytosed. The organisms survive and replicate within macrophages and to some extent in dendritic cells (Ahmad, 2011). Immune mediators, such as interferon gamma (IFN-γ), activate macrophages to kill the intracellular pathogen by activating the production of reactive nitrogen and oxygen intermediates (RNI and ROI) (Chan and Flynn, 2004), and inducing phagolysosome formation (Chackerian et al., 2002; MacMicking et al., 2003). Cluster of differentiation (CD) 4^+ and $CD8^+$ T cells are also activated to potentiate the control of *M. tuberculosis* infection (Flynn and Chan, 2001; Flynn, 2004; Kahnert et al., 2006).

In most infected individuals, granulomas develop and wall off the infectious foci, preventing the bacilli from replicating and spreading. The granuloma is made up of infected macrophages which are surrounded by lipid-loaded macrophages, monocytes, and lymphocytes enclosed within endothelial cells (Zarht, 2003; Gomez and McKinney, 2004). This structure supports the bacteria with fatty acids, as a source of energy, allowing it to persist inside the host for decades (Korbel et

al., 2008). In addition, regulatory cells and cytokines such as interleukin (IL)-10 and transforming growth factor-beta (TGF-β), control the immune response to avoid immune pathology. During this stage of protective immunity, called latency, the bacteria reduce their metabolic rate and switch to a slow- or non-replicating state (Jindani et al., 2003). There are also no clinical symptoms in infected individuals during this stage of bacterial infection (Lin and Flynn, 2010).

In most immune-competent TB-infected people, the latent stage can be lifelong (Weis et al., 2002). This may be ended by immunocompromising conditions in the host, which will allow the bacilli to reactivate and grow. During this stage, the infectious foci enlarge, becoming caseous and may eventually break open, releasing the bacteria into the circulation, as well as into the alveoli. These bacteria can be transmitted to other recipient hosts through coughing and sneezing, leading to further transmission of the disease (Hu et al., 2006; Ahmad, 2011).

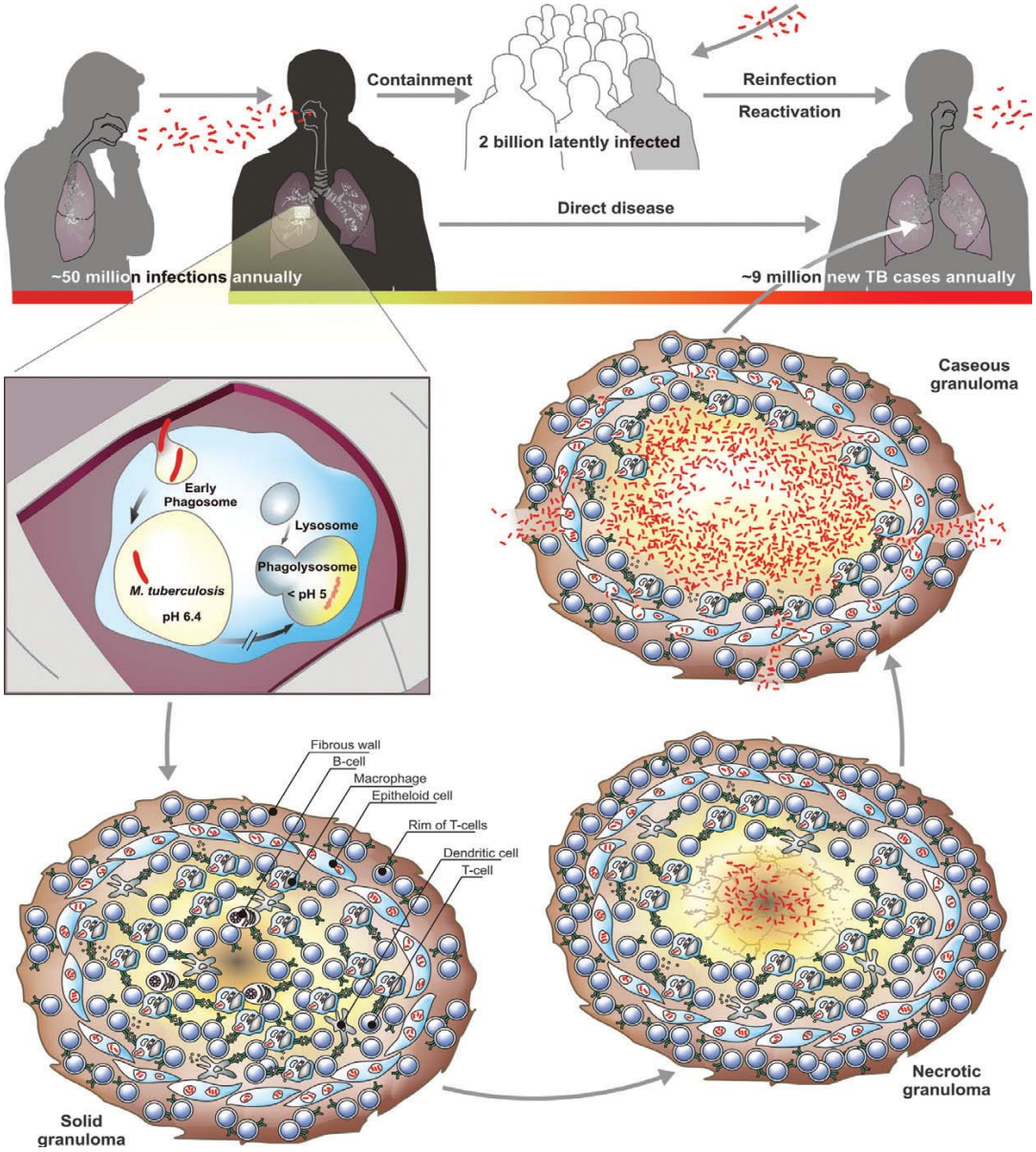


Figure 1.2: Transmission and pathology of tuberculosis (Source: Gengenbacher et al., 2012).

1.1.3 Survival strategy of *Mycobacterium tuberculosis* within the host

1.1.3.1 Intracellular survival in macrophages

Macrophages act as first-line defence cells against *M. tuberculosis* (Nathan and Shiloh, 2000; Liu and Modlin, 2008; Deretic et al., 2009). They engulf the microorganisms through phagocytosis leading to the formation of phagosomes (Aderem and Underhill, 1999). Upon immunological activation with IFN-γ, the phagosomes acidify and fuse with the lysosomes to form phagolysosomes where the bacteria will be exposed to protons from the vacuolar ATPase, RNI from inducible nitric oxide synthase (iNOS) and ROI from NADPH oxidase (NOX2) as illustrated in Figure 1.3.

Once inside the macrophages, *M. tuberculosis* bacilli arrest phagosomal maturation and prevent the formation of phagolysosomes, thereby avoiding the delivery of microbicidal effectors from the lysosomes into the phagosome (Vergne et al., 2003; Walburger et al., 2004; Vergne et al., 2005; Robinson et al., 2007; Axelrod et al., 2008; Katti et al., 2008; Ehrt and Schnappinger, 2009; Russell et al., 2009).

Several mechanisms are used by the bacteria to prevent phagolysosomal fusion. Bacteria may alter membrane proteins or bind the tryptophan/aspartate-containing coat proteins, which lead to the blockage of fusion of phagosome to the lysosome (Sturgill-Koszycki et al., 1994; Van Crevel et al., 2002; Chopra et al., 2003; Russell, 2007). Another mechanism involves the production of ammonia, which increases phagosomal pH, thereby delaying its maturation. The increase in pH also decreases the effectiveness of lysosomal enzymes as many of them require acidic pH for their optimal activity (Bruns et al., 2012).

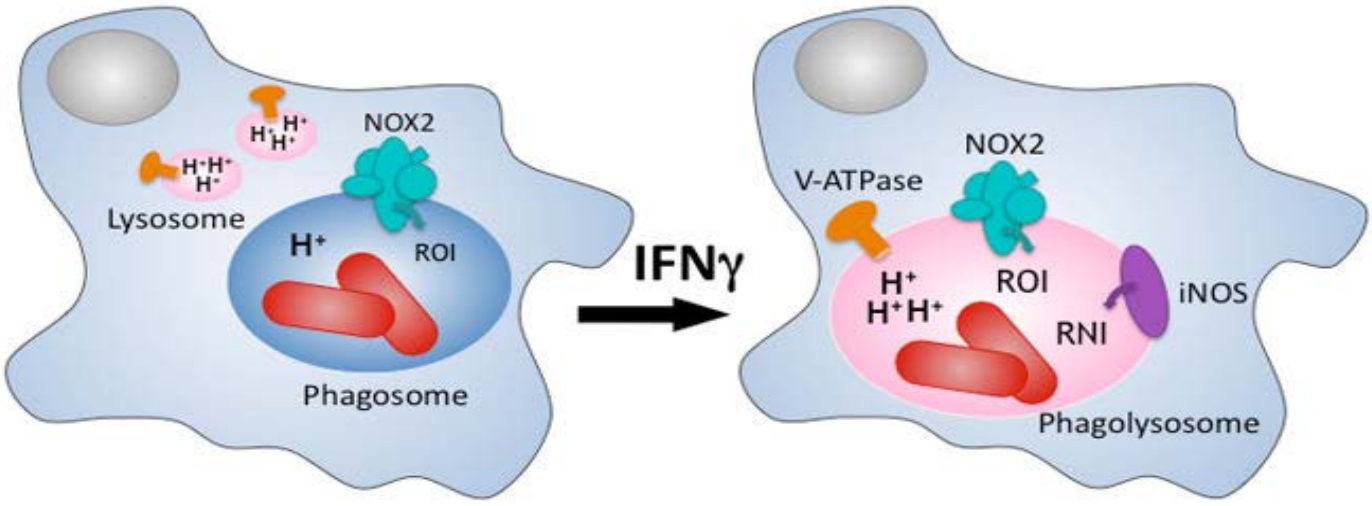


Figure 1.3: *Mycobacterium tuberculosis* inside the macrophage. (Source: Ehrt and Schnappinger, 2009).

1.1.3.2 Granuloma

As described in Section 1.1.2, the granuloma is an aggregate of cells, composed predominantly of macrophages and other immune cells (Geijtenbeek et al., 2003; Peters and Ernst 2003; Tailleux et al., 2003; Cosma et al., 2004). Inside the granuloma, unfavourable conditions, such as nutrient limitation and low oxygen tension, trigger metabolic downshift of the organisms into dormancy, resulting in non-replicating and persistent organisms. During this stage, these organisms use fatty acids as a source of energy, allowing them to persist and survive until favourable conditions occur.

There are three types of granulomas in human TB and these are clearly illustrated in Figure 1.2, viz: (i) solid, which contains bacteria; (ii) necrotic, observed during early stages of active TB and; (iii) caseous, found during the end-stage or severe TB. The three stages can coexist during active TB and provide diverse micro-environments for adaptation of the pathogen (Gengenbacher and Kaufmann, 2012). In *in vitro* setting, the microbial population consisting of non-replicating persistent organisms and environmental conditions such as limited oxygen and nutrient supply, have been found in biofilms of several bacteria including mycobacteria (Ojha et al., 2008; 2010).

1.2 MICROBIAL BIOFILMS

Microbial biofilms are communities of microorganisms, embedded in a matrix of extracellular polymeric substances (EPS) (Rodney et al., 2002). The organisms produce these substances by attaching to surfaces in an irreversible manner, using protein filaments, and initiating their synthesis, which ultimately surround them (Rodney et al., 2002).

In these matrices, the organisms live close to one another and are protected from hostile environments (Proal, 2008), such as external toxic chemical gradients, ultra violet (UV) irradiation, and pH and temperature fluxes, as well as nutrient limitation, fluid flow and desiccation (Hall-Stoodley and Stoodley, 2005). These environments, which are acidic, anoxic and nutrient-depleted zones, allow the bacteria to adjust their physiological and metabolic activities, altering their growth rates (Hall-Stoodley and Stoodley, 2005), thereby entering into stationary or dormant phases (Walters et al., 2003; Ojha et al., 2010).

In developed countries, more than 60% of infectious diseases are due to biofilm formation (Li and Yu-mei, 2011). The most common bacterial infections that has been associated with biofilm include P*seudomonas aeruginosa* (*P. aeruginosa*) in cystic fibrosis pneumonia (Singh et al., 2000), *Escherichia coli* (*E. coli*) in urinary tract infections (Anderson et al., 2004), *M. tuberculosis* in human TB (Ojha et al., 2008), and *Streptococcus mutans* (*S. mutans*) on tooth surfaces, as well as

other species such as *Vibrio, Salmonella, Listeria and Staphylococcus* (Waldvogel and Bisno, 2000; Stoodley et al., 2002; Hall-Stoodley et al., 2004; Hall-Stoodley and Stoodley, 2005). In some cases, these infections are also found on medical devices such as sutures, shunts, contact lenses, ventilators, dental units, water lines catheters and medical implants (Costerton et al., 1999). Treatment in these cases usually involves the removal of the affected devices and the use of cocktails of antibiotics (Donlan, 2001; Costerton et al., 2003; Hall-Stoodley and Stoodley, 2005).

1.2.1 Stages involved during biofilm development

The process for the formation of microbial biofilms has been described for both pathogenic and non-pathogenic organisms, and begins with reversible surface attachment, followed by permanent surface attachment, and lastly matrix synthesis as shown in Figure 1.4 (O'Toole et al., 2000; Phillips et al., 2008).

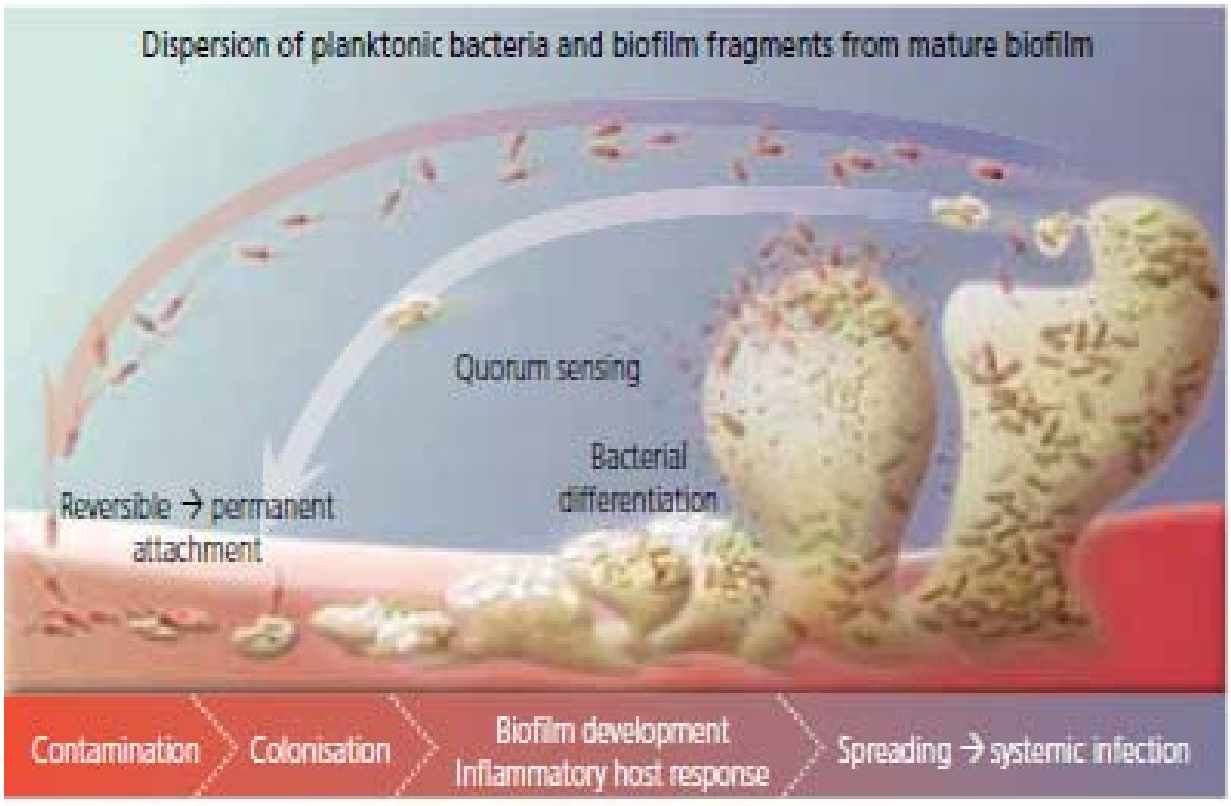


Figure 1.4: Stages of biofilm development (Source: Phillips et al., 2008)

1.2.1.1 Stage one: reversible surface attachment

During the reversible attachment stage, most bacteria are free-floating single cells, growing in a planktonic phase. When biofilm develops, filaments extrude from these bacteria and initiate attachment of bacteria to the surface, by anchoring the organisms to the location (Stoodley et al.,

2002; Zambro and Kolter, 2005; Flemming et al., 2007). In the host, during infection, the organisms use adhesion molecules to colonise different sites of the body (Li and Yu-mei, 2011).

1.2.1.2 Stage two: permanent surface attachment

After initial attachment to the surfaces, bacteria multiply, differentiate and become firmly attached to one another (Donlan and Costerton, 2002). Organisms achieve these processes by communicating with each other via quorum sensing (QS) mechanisms as shown in Figure 1.5. During QS, bacteria release small signaling molecules or chemicals into the surrounding environments, which are detected by other bacteria, ultimately regulating their behaviour, such as cell differentiation and development of biofilm structures (Spigelman and Ma, 2004; Li and Yu-mei, 2011). High accumulation of the signaling molecules favours activation of polymer secretion, while disruption of cell-to-cell communication allows cells to be in a planktonic state (Hammer, 2000; Singh, 2000; Parsek and Greenberg, 2005; Waters and Bassler, 2005; Sakuragi and Kolter, 2007; Li and Yu-mei, 2011).

Factors that affect QS include surface attachment (Dunne, 2002), extracellular polymer production (Davies et al., 1998; Hammer, 2000; Sakuragi and Kolter, 2007), bio-surfactant synthesis (Schuster, 2006), sporulation (Grossman, 1995), competence (Li et al., 2001), bioluminescence (Engebrecht and Silverman, 1984; Miller and Bassler, 2001), secretion of nutrient-sequestering compounds and virulence factors (Williams et al., 2000; Miller et al., 2002; Hentzer, 2003).

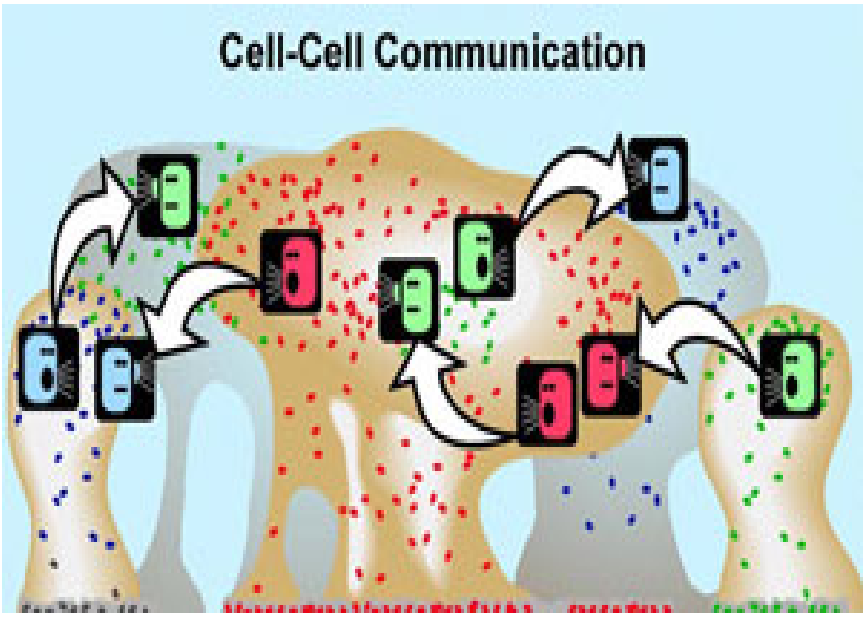


Figure 1.5: Cell-to-cell communication of sessile organisms during quorum sensing (Source: Singh et al., 2000).

1.2.1.3 Stage three: sliming protective matrix/ biofilm

Once the cells are firmly attached, they begin to secrete EPS (Sutherland, 2001), which helps with further attachment of the bacteria to the surface and to one another (Donlan and Costerton, 2002; Stoodley and Stoodley, 2009). This is a protective matrix or slime. The composition and structure of EPS are discussed in detail in section 1.2.2.1.

As the biofilm matures, the microorganisms and their associated EPS are released into the bulk fluid bathing the biofilm (Hunt, 2004). These organisms detach either as individuals (planktonic) or aggregates (Characklis et al., 1990), using shear stress mechanisms, which occur during changes in direction or rate of flow, resulting in increased cell erosion (Characklis et al., 1990; Davies et al., 1998; Islam et al., 2012).

1.2.2 Biofilm structure

1.2.2.1 Extracellular polymeric substances

The primary matrix material of biofilm is composed of EPS, which makes approximately 50-90% of the total organic carbon of this structure (Flemming et al., 2000). The composition of the EPS matrices vary between organisms, and in most bacteria, they are constituted predominantly of exopolysaccharides (secreted polysaccharides), lipids, proteins and deoxyribonucleic acid (DNA) (Sutherland, 2001; Branda et al., 2005; Zambrano and Kolter, 2005; Flemming et al., 2007; Bardouniotis et al., 2008; Stoodley and Stoodley, 2009; Ojha et al., 2010). Non-cellular materials may also be found in the matrix of the biofilm such as mineral crystals, metal ions, divalent cations, corrosion particles, clay or silt particles, or blood components, depending on the environment in which the biofilm has developed (Flemming et al., 2000; Rodney, 2002).

EPS contains 1,3- or 1,4-linked hexose residues, which are responsible for rigidity, solubility and deformation of the biofilm. The biofilm is highly hydrated due to incorporation of water into its structure (Sutherland, 2001).

Production of EPS is usually affected by the nutrient status of the growth medium, rate of bacterial growth and age of the culture. It is favoured by excess availability of carbon, limitation of nitrogen, potassium, or phosphate and slow growth rate, which occur in aging cultures (Sutherland, 2001).

1.2.2.2 Microcolonies

As bacteria grow, they attach to each other and to the surface, forming tiny, independent mushroom-shaped communities at a narrow base, attaining the sessile stage (Stoodley et al., 2002;

Vlamakis et al., 2008). Most bacteria prefer to grow in this state in biofilm rather than as single planktonic cells. These communities contain thousands of compatible bacteria within and are referred to as microcolonies. Different microcolonies may contain varying combinations of bacterial populations. Each bacterial microcolony uses QS to communicate with other microcolonies, and utilize their individual abilities and skills to maximize each other's survival (Xavier and Foster, 2007; Hibbing et al., 2010).

Bacteria within the microcolony are able to survive in either aerobic or anaerobic conditions (Nield-Gehrig and Willmann, 2003; Teal et al., 2006; Stewart and Franklin, 2008). They are also tolerant to stress, by altering their metabolism and attaining a dormant state (McNeill and Hamilton, 2003; Fux et al., 2005; Ojha et al., 2010).

1.2.2.3 Fluid Channels

Fluid channels are interconnected, water-filled channels, which facilitate movement of bacterial metabolites, nutrients, oxygen, enzymes and waste products within the biofilm (Nield-Gehrig and Willmann, 2003). Their poor penetration efficiency of the channels may lead to limited nutrients and other metabolites supplied to the bacteria, also allowing the cells to alter their metabolism into a stationary phase-like dormant state as occurs in the microcolony environment (Stewart and Costerton, 2001; Walters et al., 2003; Borriello et al., 2004; Fux et al., 2004; Borriello et al., 2006; Shah et al., 2006).

1.2.3 Mechanism of biofilm resistance to antimicrobial agents

Most bacterial biofilms are resistant to antimicrobial agents. This resistance is mediated by several mechanisms (Li and Yu-mei, 2011) as described below.

One of the mechanisms of biofilm resistance to antibiotics involves the EPS, which constitutes the most protective/ barrier component of the biofilm. The EPS may retard diffusion of antibiotics through the biofilm, by binding directly to the antimicrobial agent and deactivating it, thereby preventing access to the bacteria within (Stewart, 1998). Some antibiotics may diffuse slowly through the matrix, resulting in inadequate concentrations to kill the microorganisms (Hoyle, 1992). Other antibiotics are able to penetrate readily, but are inactive against the sessile dormant population within the biofilm (Stewart and Raquepas, 1995; Donlan, 2000; 2002).

The second mechanism of biofilm resistance to antimicrobial agents involves the fluid channels. These structures may contribute to antibiotic resistance by selectively permitting nutrient inflow and

preventing penetration of antibiotic molecules as they are mostly larger than nutrients (Davies et al., 1993; De Beer et al., 1994; Costerton et al., 1995; Islam et al., 2012). In addition, due to their poor penetration, which may affect nutrients and other metabolite transportation, the organisms in the biofilm may alter their metabolism to a dormant state, and become resistant to antibiotics.

Thirdly in addition to the dormant state, most sessile organisms in the biofilm are resistant to antibiotics, due to the environmental conditions, such as low pH and oxygen levels, which are unfavourable to activities of most antibiotics (Fux et al., 2004; Borriello et al., 2006; Shah et al., 2006).

1.2.4 Mycobacterial biofilms

Several mycobacterial species such as *Mycobacterium avium* complex (MAC) (Carter et al., 2003), *Mycobacterium fortuitum* (Hall-Stoodley and Lappin-Scott, 1998), *Mycobacterium marinum* (Hall-Stoodley et al., 2006), *Mycobacterium smegmatis* (Recht et al., 2000), *Mycobacterium ulcerans* and *M. tuberculosis* (Ojha et al., 2008) have been shown to produce biofilm. In some of these organisms such as *M. ulcerans* (chronic skin ulcers), and *M. avium* subspecies *paratuberculosis* (Crohn's disease), biofilm formation has been associated with pathogenesis of disease (Marsollier et al., 2005; Wu et al., 2009).

When grown in detergent-free liquid medium, these organisms form pellicles with clumping or cording at the air-interfaces (Hunter et al., 2006; Ojha et al., 2008). The cord formation has been associated with virulence factors in *M. tuberculosis* (Middlebrook et al., 1947) and *M. marinum* (Clay et al., 2008; Tobin et al., 2010). In TB patients, bacterial populations similar to those grown in biofilm, have been isolated from granuloma lesions. Inside cavities of these lesions, air-interface pellicles, which surround bacteria, have been formed (Hunter et al., 2006).

While most bacterial biofilms are composed of proteins, polysaccharides, lipids and DNA, those of mycobacteria are composed mainly of lipids, including free mycolic acids (FM) (Ojha and Hatfull, 2007). In *M. smegmatis* the FM are formed by the hydrolysis of the trehalose dimycolate (TDM) by the TDM-specific esterase *Msmeg_1529*. Other precursors involved in FM synthesis which are found in this organism include GroEL1, which regulates biofilm maturation by interacting with the B-keto acyl-ACP synthatase (KasA) enzyme, a component of the multi-enzyme complex type II fatty acid synthatase (FAS II) involved in mycolic acid synthesis (Ojha et al., 2005).

These lipids are major cell envelope constituents (40-60% of the cell dry weight), which play an important role in its structure and function (Besra and Brennan, 1997; Barry et al., 1998; Daffe and Draper, 1998), including among others, penetration of antibiotics (Ojha et al., 2005; 2008; 2010). Other lipids such as glycopeptidolipids, play a role in initialising attachment during biofilm formation (Recht et al., 2000).

The mycobacterial EPS, like those of other bacteria, are also responsible for biofilm rigidity and resistance to antibiotics, due to poor penetration (Ojha et al., 2005; 2008). In addition, the microbial subpopulation residing in this environment consists of sessile drug-tolerant persisters, which require high concentrations of primary anti-TB drugs to be eliminated (Hunter, 2006; Lenearts et al., 2007; Ojha et al., 2008; 2010).

1.3 TUBERCULOSIS CHEMOTHERAPY

1.3.1 History of tuberculosis chemotherapy

Before chemotherapy, the prevalence of TB was beginning to decline due to improvements of sanitation and living conditions. However, the decline became significant after the introduction of anti-TB chemotherapy in the 1950s (Ryan, 1993).

The first anti-TB drug to be discovered was streptomycin (SM), isolated from the actinobacterium *Streptomyces griseus* (Schatz et al., 1944). It was initially used as a monotherapy and acquired resistance, rapidly. Several anti-TB agents such as isoniazid (INH: isonicotinylhydrazine) (Chorine, 1945), pyrazinamide (PZA) (Malone et al., 1952), ethambutol (EMB) (Thomas et al., 1961) and rifampicin (RIF) (Maggi et al., 1965) were later synthesised. To date many anti-TB agents have been developed and those most commonly used during anti-TB chemotherapy are as shown in Table 1.

These antibiotics have different antibacterial activities being either bacteriostatic such as EMB and p-aminosalicylic acid (PAS) or bactericidal (sterilizing), which include INH, RIF, SM, PZA and fluoroquinolones (FQ). Some of these antibiotics are bacteriostatic under certain conditions and bactericidal in different environments (Zhang, 2005). For instance, PZA is bacteriostatic against replicating bacilli but lethal to non-replicating, dormant organisms found in acidic environments such as the granuloma (Zhang and Mitchison, 2003).

The anti-TB drugs are categorised as either first-line (INH, RIF, PZA, EMB, and SM) or second-line drugs (kanamycin, amikacin, capreomycin, cycloserine, PAS, ethionamide, thiacetazone, and

FQ) (Zhang, 2005). The first-line agents are mostly used in the treatment of drug-sensitive isolates, while the second-line drugs are included with the first-line agents for the treatment of MDR- and XDR-TB cases.

Table 1.1: Commonly used Anti-tuberculosis drugs

Current tuberculosis drugs and their targets

Drugs	MIC (μg/ml)	Mechanisms of action	Targets	Genes involved in resistance*
Isoniazid	0.01–0.20	Inhibition of cell wall mycolic acid synthesis	Enoyl acyl carrier protein reductase (InhA)	*katG, inhA*
Rifampin	0.05–0.50	Inhibition of RNA synthesis	RNA polymerase, β subunit	*rpoB*
Pyrazinamide	20–100 (pH 5.5 or 6.0)	Depletion of membrane energy	Membrane energy metabolism	*pncA*
Ethambutol	1–5	Inhibition of cell wall arabinogalactan synthesis	Arabinosyl transferase	*embCAB*
Streptomycin	2–8	Inhibition of protein synthesis	Ribosomal S12 protein and 16S rRNA	*rpsL, rrs*
Kanamycin	1–8	Inhibition of protein synthesis	16S rRNA	*rrs*
Capreomycin	4	Inhibition of protein synthesis	16S rRNA, 50S ribosome, rRNA methyltransferase (TlyA)	*rrs, tlyA*[b]
Fluoroquinolones	0.2–4.0	Inhibition of DNA synthesis	DNA gyrase	*gyrA, gyrB*
Ethionamide	0.6–2.5	Inhibition of mycolic acid synthesis	Acyl carrier protein reductase (InhA)	*inhA, etaA/ethA*
PAS	1–8	Inhibition of folate pathway and mycobactin synthesis?	thymidylate synthase (ThyA)?	*thyA*[c]

*KatG, PncA, EtaA/EthA are enzymes involved in the activation of the prodrugs isoniazid, pyrazinamide and ethionamide, respectively.

(Source: Maus et al., 2005)

1.3.2 The current tuberculosis chemotherapy

Current TB chemotherapy consists of two phases. The first is the intensive phase involving four drugs, viz. INH, RIF, PZA and EMB, taken daily for two months. This is followed by the continuation, less-intensive phase, consisting of only INH and RIF, given daily for the remaining four to seven months (WHO, 2003; Department of Health, 2009).

In TB patients' lesions, there are at least four different subpopulations of bacteria as shown in Figure 1.6. These are:
(i) actively-growing bacilli (A), which are sensitive to either INH, RIF or EMB;
(ii) bacteria with spurts of metabolism (B), which are mostly targeted by RIF;
(iii) dormant organisms (C), that reside in an acidic environment, killed by PZA; and
(iv) the persisters (D), which are not killed by any currently-available anti-TB drugs (Mitchison, 1979; Zhang et al., 2005).

The use of RIF and PZA in anti-TB chemotherapy was aimed at reducing the bacillary loads in lesions as both are sterilizing drugs. However, dormant bacilli or persisters, which are tolerant to

both drugs, remain in the lesion. Due to the presence of these organisms, chemotherapy is usually extended from 6-9 months to minimize relapse and reoccurrence of the disease caused by reactivation of this population, which can occur upon the host immune suppression (Spigelman and Ma, 2004).

Despite this effort, treatment is not always effective and allows for the selection of drug-resistant traits, due to ineffective activity of antibiotics against dormant or non-replicating cells, residing in the lesions. In these necrotic tissues, drugs such as SM are unable to penetrate, while some of those that do penetrate, are unable to kill this bacterial population (Canetti, 1955; Jindani et al., 2003; Zhang et al., 2005; Spigelman and Ma, 2004).

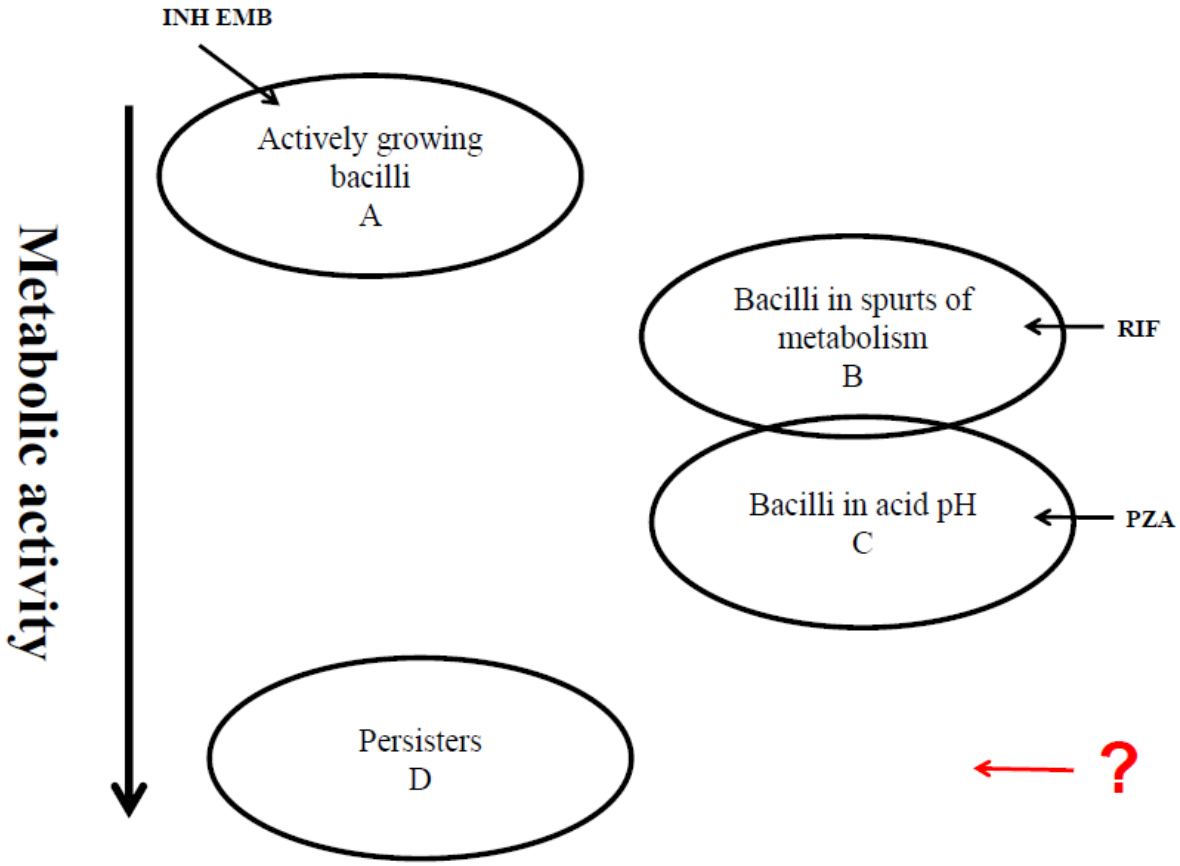


Figure 1.6: Anti-tuberculosis drugs targeting distinct *Mycobacterium tuberculosis* subpopulation. (Source: Zhang et al., 2005).

1.3.3 Targets and mode of action of current anti-tuberculosis drugs

Due to the extensive number of anti-TB agents available, only the first-line and some of the second-line agents will be discussed in this work.

1.3.3.1 Isoniazid

INH is a prodrug and its molecular structure is shown in Figure 1.7. This antibiotic requires activation by *M. tuberculosis* catalase-peroxidase (KatG) to generate a range of ROI and RNI species, which in turn attack multiple targets in the tubercle bacillus (Zhang et al., 1992). It binds to the enoyl acyl carrier protein (ACP) reductase (InhA) of the fatty acid synthetase II (FAS II) pathway, found on the mycobacterial cell wall (Winder and Collins, 1970; Banerjee et al., 1994; Timmins and Deretic, 2006). The ROI and RNI species react with nicotinamide adenine dinucleotide (NADH) to form INH-NADH adducts, which inhibit the InhA enzyme (Broussy et al., 2003). In most INH-resistant isolates, resistance to INH is associated with KatG mutations (Lee et al., 2001).

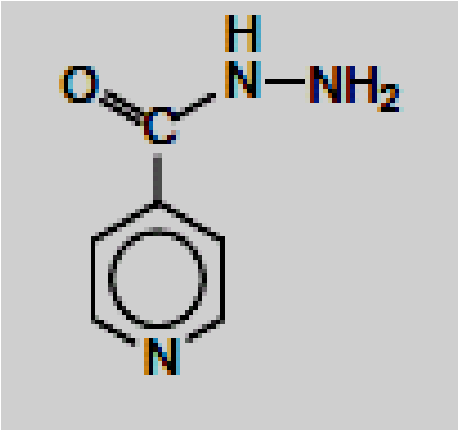


Figure 1.7: The molecular structure of isoniazid (Source: Berstein et al., 1952).

1.3.3.2 Rifampicin

RIF as shown in Figure 1.8 is a derivative of the rifamycin group of antibiotics. The drug is active on both actively-growing and non-replicating bacilli. Its inclusion in TB chemotherapy has resulted in reduction of treatment length from 12 - 18 months to 9 months (Mitchison, 1985).

It acts by inhibiting RNA synthesis by binding to the bacterial DNA-dependent RNA polymerase β-subunit encoded by the *rpoB* gene (Aristoff et al., 2010). About 96% of RIF-resistant *M. tuberculosis* isolates have mutations at the 81-bp region of this gene (Telenti et al., 1993). It also causes cross-resistance with other rifamycins such as rifabutin and rifapentine (Williams et al., 1998).

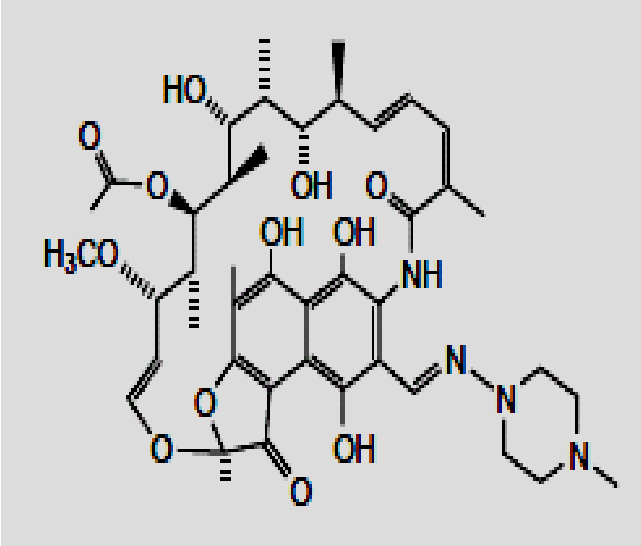


Figure 1.8: The molecular structure of rifampicin (Source: Maggi et al., 1966).

1.3.3.3 Ethambutol

EMB Figure: 1.9 (*S*,*S*_)-2,2_(ethylenediimino)di-1-butanol) is a bacteriostatic antibiotic acting against actively-growing *M. tuberculosis* bacilli. Its mechanism of action involves targeting the mycobacterial cell wall, specifically arabinogalactan (Takayama and Kilburn, 1989) and lipoarabinomannan (Wolucka et al., 1994; Mikusov et al., 1995; Younosian et al., 2005) synthesis, by targeting the arabinosyltransferase enzyme. The arabinosyltransferase enzyme is encoded by the *embCAB* operon (Telenti et al., 1997) with its mutations being responsible for about 65% of EMB-resistant *M. tuberculosis* clinical isolates.

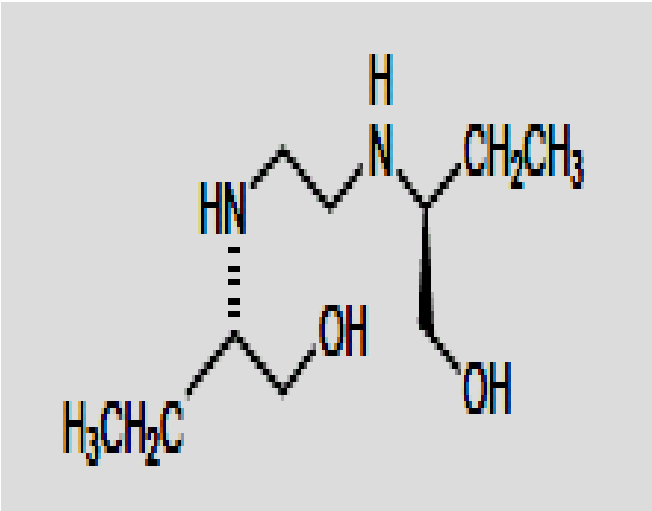


Figure 1.9: The molecular structure of ethambutol (Source: Thomas et al., 1961).

1.3.3.4 Pyrazinamide

PZA is an inactive prodrug that shares the same structural features with nicotinamide (Figure 1.10). Due to its high sterilizing activity *in vivo* (Mitchison, 1985; Fox et al., 1999), its inclusion in the

chemotherapy of TB has led to the shortening of the duration of anti-TB chemotherapy from 18-6 months. Its activity against the young TB bacteria, growing under normal culture conditions near neutral pH is poor (Tarshis and Weed, 1953) but it is active against bacilli from old cultures residing in acidic and hypoxic environments (Zhang et al., 2002; Wade and Zhang, 2004).

PZA is converted into pyrazinoic acid (POA) by PZase/nicotinamidase when in an acidic pH and targets membrane energy metabolism. The acidic pH leads to low membrane potential providing easier diffusion and accumulation of POA through bacterial membrane. The PZase enzyme is encoded by the *pncA* gene (Scorpio et al., 1996) and its mutation results in resistance to PZA in most isolates (Scorpio et al., 1997; Zhang et al., 1999; Zhang et al., 2003).

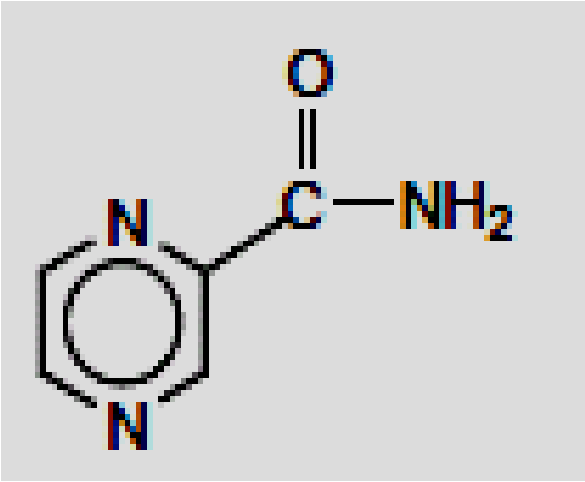


Figure 1.10: The molecular structure of pyrazinamide (Source: Malone et al., 1952).

1.3.3.5 Fluoroquinolones

FQ (Figure 1.11) are broad-spectrum antimicrobial agents. Several of these have been evaluated for their anti-TB activities and are being used as second-line drugs for the treatment of MDR- and XDR-TB strains (Grimaldo et al., 2001). They act by inhibiting DNA synthesis by targeting the DNA gyrase (Andersson and MacGowan, 2003).

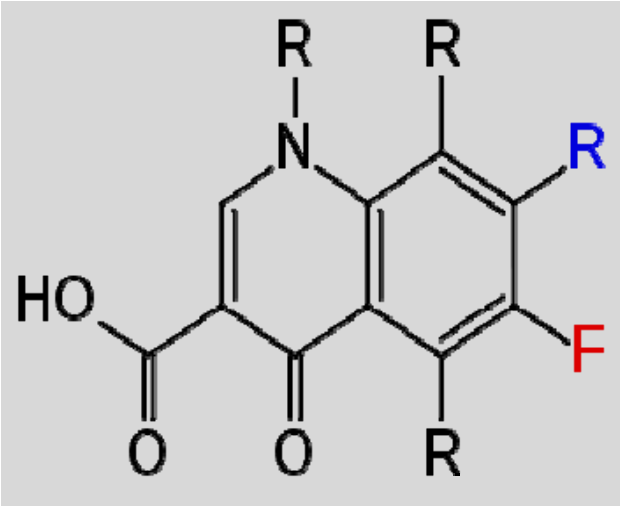


Figure 1.11: The molecular structure of quinolones (Source: Deitz et al., 1963).

Based on the current scenario of lack of the available effective anti-TB agents that act on dormant and persistent organisms including the M/XDR-TB strains, as well as those that provide shorter treatment, resulting in few or no side effects, the search for new anti-TB drugs continues (WHO, 2011).

1.4 CLOFAZIMINE

1.4.1 Background of clofazimine

Clofazimine is a riminophenazine antibiotic, developed initially in 1954 for the treatment of *M. tuberculosis* infections in humans (Barry et al., 1957). The riminophenazines are a group of active phenazine compounds, wherein an 'R' substituent has been included in the imino part of the molecule as shown in Figure 1.12. These compounds were derived from lichens, which were important sources of anti-TB agents in the past. Among these derivatives, was a compound named diploicin, obtained from the lichen *Buellia canescens*, from which an anilinoaposafranine compound, which was highly active against *M. tuberculosis*, was synthesised. The anilinoaposafranine compound was further modified with the R-substitution at the imino group (NH), leading to those compounds being referred as 'rimino' compounds. Of these, the rimino compound named 2-chloro-anilino-5-p-chlorophenyl-3,5-dihydro-3-isopropyl iminophenazine, was found to be highly active against *M. tuberculosis*. The compound was initially called B663 and was later named lamprene or clofazimine.

Clofazimine was developed as a potential candidate drug for the treatment of TB, as it accumulated preferentially inside the cells of the mononuclear phagocyte system (MPS), previously called the reticuloendothelial system (RES) and also had a long half-live. Despite these impressive *in vitro*

activities, the drug failed to demonstrate positive results in experimental TB when it was tested against TB in the guinea pig model, which was the standard animal model for TB at that time (Reddy et al., 1999). Moreover, it also demonstrated negative activity in experimental simian TB, which was a standard requirement for the usefulness of any drug in clinical TB during those days (Barry et al., 1960).

Due to the above-mentioned poor *in vivo* activity against *M. tuberculosis* in higher animals (Reddy et al., 1999), its use in the chemotherapy of TB patients was discontinued, despite findings from subsequent studies in guinea pig and monkey models that showed that failure of the drug was due to poor absorption by the oral route and also that the drug showed high activity in hamsters and mice, where it was well absorbed by the oral route. For instance, in mice receiving a daily dose of 20 mg/kg mean plasma concentrations were approximately 0.55 mg/L (Dooley et al., 2012). However, the drug was subsequently used for the treatment of *Mycobacterium leprae* and MAC infections since 1969, when new drugs for the treatment of these infections where needed (Reddy et al., 1999; Phillip et al., 2012).

1.4.2 Structure of clofazimine

Clofazimine is a substituted riminophenazine antibiotic, and its chemical name is 3-(p-chloroanilino)-10-(p-chlorophenyl)-2, 10-dihydro-2-isopropyliminophenazine. Its structure is as illustrated in Figure 1.13. Like all riminophenazines, its structure consists of a phenazine nucleus with an alkylimino (R-imino) group at position 2 and phenyl substituents at positions 3 and 10 of the phenazine nucleus (Figure 1.12 and 1.13).

Several analogues have been prepared using substitutions at positions R1, R2 and R3 (Figure 1.12). Generally, compounds with an R1 substitution with chlorine or methoxy or ethoxy groups have increased antibacterial activity and an increased capacity to release superoxide (O_2^-) and arachidonic acid (AA) from neutrophils (Franzblau and O'Sullivan, 1988; Jagannath et al., 1995). On the other hand, compounds with substitution at the R2 positions, such as clofazimine, have higher anti-TB activity but have increased lipophilicity, fat retention and many gastrointestinal side effects. The last compounds with substitutions at the R3 position, have been designed to further increase their antimicrobial activity. In all these analogues, viz. R1, R2 and R3, an imino group has been demonstrated to be essential for activity (Reddy et al., 1999). Recently, it has been shown by Liu and co-workers (2012) that the central tricyclic phenazine system and the aromatic rings are also important for anti-TB activity.

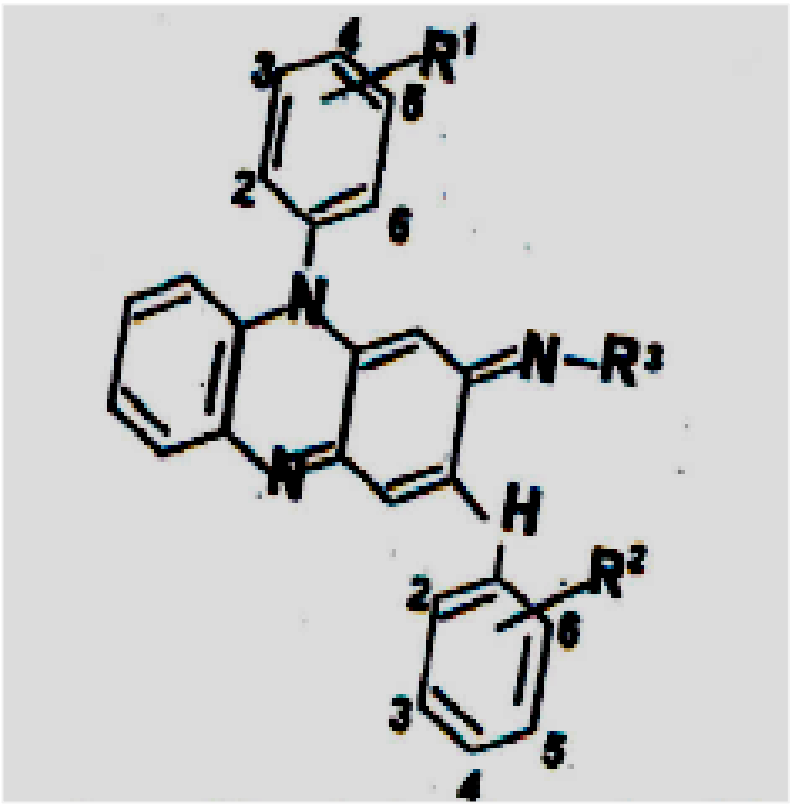


Figure 1.12: The chemical structure of riminophenazine (Van Rensburg et al., 2000).

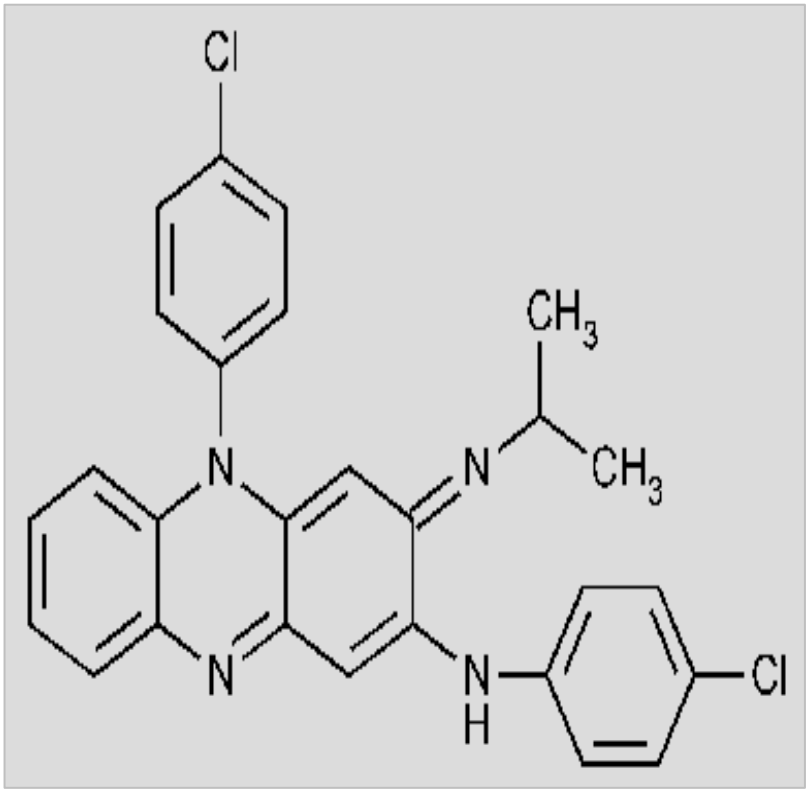


Figure 1.13: The molecular structure of clofazimine (Source: Arbiser et al., 1995).

1.4.3 Physical properties of clofazimine

The chemical structure of clofazimine is $C_{27}H_{22}C_{12}N_4$. It has a molecular weight of 473.14 and a melting point of 210-212 °C. It is a basic hydrophobic drug and is readily soluble in acidified aqueous and organic solvent solutions such as benzene, chloroform, and partially soluble in ethanol and methanol, while poorly soluble in acetone and ethyl acetate and insoluble in water (Hernandez-Valdepena et al., 2009).

It exists in a charged form and is deep-red to orange in colour at physiological pH. As the pH drops the colour becomes dark-red and the aqueous solubility increases (Reddy et al., 1999). Its colour may also change at different temperatures. *In vivo*, the cellular pH is insufficiently low to change its colour, but the drug is seen in different colours due to perhaps the ability of the body to reduce it to various extents (Reddy et al., 1999).

1.4.4 Antimicrobial activities of clofazimine

Clofazimine has high antimicrobial activities against several Gram-positive bacteria, including *Staphylococcus aureus*, *Streptococcus pneumoniae* and Enterococcus species. However, it has shown no activity when tested against Gram-negative bacteria (De Bruyn et al., 1996; Oliva et al., 2004; Huygens et al., 2005). In addition to high antimicrobial activity for Gram-positive bacteria, it has also shown low emergence of drug resistance, as no clofazimine-resistant isolate has been identified from Gram-positive bacteria to date. However, despite these positive effects, clofazimine lacks the ability to exhibit a post-antibiotic effect (PAE) and is less synergistic when in combination with other antibiotics in the treament of these Gram-positive bacteria (Oliva et al., 2004), although exceptions have been reported (see below).

Among mycobacterial species, it has demonstrated mostly inhibitory activities, against both pathogenic and non-pathogenic strains, and both rapidly- and slow-growing organisms. Its minimum inhibitory concentration (MIC) against rapidly-growing organisms such *as Mycobacterium chelonae*, *Mycobacterium abscessus* and *M. fortuitum* has been very low, and has ranged from 0.5-1mg/L. Different from the simple Gram-positive bacteria, it has shown some synergistic activity when used in combination with agents such as amikacin against the fast-growing mycobacteria (Shen et al., 2010).

Clofazimine has demonstrated high antimycobacterial activity against slow-growing organisms, which include MAC, *Mycobacterium bovis* (Peters et al., 2000; Griffith et al., 2007) and *M. tuberculosis*. Its MIC against laboratory strains, H37Rv and clinical isolates including drug-

sensitive and resistant isolates such as MDR- and XDR-TB, have been very low, ranging from 0.06-4 mg/L (Jagannath et al., 1995; Cholo et al., 2006; Gui et al., 2011). Apart from low MIC, which has been demonstrated on actively-growing bacilli, clofazimine has shown potent inhibitory activity against hypoxic, non-replicating *M. tuberculosis* bacilli, which it exerts when the level of oxygen in the culture medium is reduced (Grant et al., 2012). As with Gram-positive bacteria, it has also demonstrated a very low emergence of drug resistance. To date, only one study has reported clofazimine-resistant isolates, when MDR- and XDR-TB patients were surveyed in Samara region in Russia, and 2.9% of the 251 MDR-/XDR-TB strains evaluated, were reported to be clofazimine-resistant (Balabanova et al., 2005). It has also demonstrated synergistic activity when used in combination with drugs such as PAS, prothionamide, clarithromycin and capreomycin against *M. tuberculosis* clinical isolates (Lu et al., 2010).

This drug has also demonstrated impressive antimicrobial activity in intracellular TB infectious models. When tested against H37Rv strain, in human-monocyte-derived macrophages, it exhibited a 2-log reduction in lethal activity at 2.5 mg/L (Verma et al., 2013). Other studies have suggested its activity in macrophages to include reversing the inhibitory effect of *M. tuberculosis*-derived 25 kDa protein on phagocytic functions (Wadee et al., 1988; 1995). These antimicrobial activities in phagocytes may be mediated by its ability to enhance the phagocytic and microbicidal activities of human and murine phagocytes by sensitizing the cells to hyperreact to various stimuli, leading to increased production of ROS with antimicrobial potential (Van Rensburg et al., 1993).

In vivo, clofazimine has demonstrated contrasting activities. Studies conducted in murine model of experimental TB against H37Rv and MDR-TB strains have showed that a very low concentration of the drug, with MIC ranging from 0.12-1.92 mg/L, is required for it to achieve its antimicrobial activity (Lu et al., 2008). However, in higher animals such as humans, the drug has not been effective. In one study conducted in humans, there was no difference in treatment outcome in MDR- and XDR-TB patients who received treatment in the absence or presence of clofazimine. In these patients, the drug failed to improve bacterial conversion, which is an indicator of treatment success (Xu et al., 2012).

1.4.5 Mechanism of antimicrobial action of clofazimine

The exact mechanisms of clofazimine-mediated antimicrobial activity remain unclear. However several mechanisms have been proposed which include targeting the bacterial respiratory chain and ion transporters, as illustrated in Figure 1.14.

Because of its high lipophilic nature and redox potential (20.18 V at pH 7), it was originally proposed that intracellular redox cycling was likely to contribute to the antimicrobial activity of clofazimine, by a mechanism involving oxidation of reduced clofazimine, leading to generation of the antimicrobial ROS O_2^- and hydrogen peroxide (H_2O_2) (Barry et al.,1957). However, this mechanism was not supported by Van Rensburg and coworkers (1992), when they failed to establish any association of catalase positivity/negativity with the degree of susceptibility of the bacteria to clofazimine. Moreover, susceptibility of Gram-positive facultative anaerobic bacteria was shown to be elevated under strictly anaerobic conditions. On the other hand, about 11 different species of Gram-negative bacteria, which were known to be susceptible to the antimicrobial actions of redox cycling agents, were found to be uniformly resistant to clofazimine (Imlay and Fridovich, 1992; Malone et al., 2008). Despite these findings, that exclude redox cycling mechanisms, Yano and coworkers (2011) showed data in favour of this mechanism, where they have illustrated that clofazimine is reduced and later oxidized by reduced NADH, leading to the generation of O_2^- and H_2O_2, when it is added to isolated membrane fractions from *M. smegmatis* in the presence of the terminal cytochrome respiratory chain inhibitor, potassium cyanide (KCN) and the oxidizable co-factor NADH (Cholo et al., 2012).

Apart from the redox cycling mechanism, the most favoured and reported mechanism of clofazimine-mediated antimicrobial activity seems to be associated with membrane distabilization (De Bruyn et al., 1996; Steel et al., 1999; Matlola et al., 2001; Oliva et al., 2004; O'Neill et al., 2004; Cholo et al., 2006; Shen et al., 2010). Previously, in Gram-positive bacteria, it has been demonstrated that the mechanism of antimicrobial activities of clofazimine, begins with the increased release of lysophospholipid, due to the activity of phospholipase A_2 (PLA_2) enzyme hydrolysing membranous phospholipids (Van Rensburg et al., 1992; De Bruyn et al., 1996; Steel et al., 1999, Matlola et al., 2001). However, this proposed mechanism was excluded for mycobacteria, especially for *M. tuberculosis*, when the genes encoding for the phospholipase A enzymes (PLA) could not be sequenced, but those that coded for four phospholipase C proteins were identified (Cole et al., 1998). Furthermore, the *M. tuberculosis plc*-deficient mutant when compared with wild-type (WT) *M. tuberculosis* strain (H37Rv) displayed a similar level of susceptibility to clofazimine, illustrating the absence of phospholipase involvement in the membrane-associated clofazimine activity (Bopape et al., 2004; Cholo et al., 2006).

In both Gram-positive and mycobacterial species, clofazimine resulted in the inhibition of the uptake of K^+ by the bacteria. In Gram-positive bacteria, the inhibition in the uptake of K^+ was one of the earliest detectable indicators of membrane dysfunction, occurring within minutes of exposure

of *M. tuberculosis* to clofazimine, which was dose-related, and coincided with membrane hydrolysis phospholipid hydrolysis, mentioned above (Steel et al., 1999; Matlola et al., 2001). In *M. tuberculosis*, the exact clofazimine-mediated inhibitory mechanism of the uptake of K^+ still need to be determined as has demonstrated to be non-phospholipase-dependent (Bopape et al., 2004).

K^+ is the most abundant intracellular cation in most cells, as it is necessary to sustain diverse, essential cellular processes, which include the resting membrane potential, active transport of nutrients, various enzyme activities involved in cellular metabolism, and biosynthesis of macromolecules. In *M. tuberculosis*, two major, structurally distinct K^+ transporters, namely, the constitutively operative Trk A/B systems and the inducible Kdp system, are utilized by the bacteria (Cole et al., 1998). Both systems are strongly inhibited following exposure of *M. tuberculosis* to clofazimine and it therefore seems unlikely that this agent functions as a primary, selective inhibitor of these K^+ transporters. Alternatively, clofazimine may simply be a membrane-destabilizing agent, dismantling membrane architecture both directly and via lysophospholipids, with consequent dysfunction of bacterial systems such as K^+ transporters (Van Rensburg et al., 1992; Steel et al., 1999; Matlola et al., 2001; Cholo et al., 2012). Other effects that may be associated with membrane-damaging activities include inhibition of DNA, RNA and protein synthesis (Oliva et al., 2004).

On the other hand, the non-susceptibility of Gram-negative bacteria to clofazimine may be due to the poor penetration of the outer membrane by this agent and/or differences in the inner membrane phospholipid compositions of Gram-negative and Gram-positive bacteria. These insights into the mechanisms of antimicrobial activity of clofazimine, have therefore identified novel targets/strategies for future development of antibiotics (Epand et al., 2008; Epand et al., 2011).

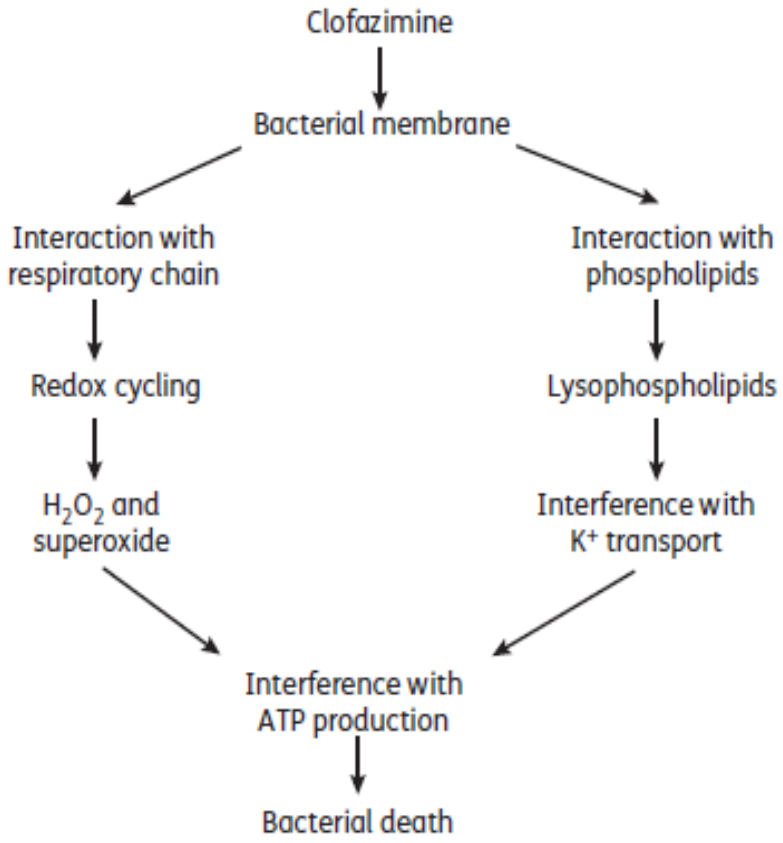


Figure 1.14: Proposed mechanism of the membrane-targeted anti- mycobacterial activity of clofazimine. These include the respiratory chain and ion transporters (Source: Cholo et al., 2012).

1.4.6 Anti-inflammatory activities of clofazimine

In addition to its primary antimicrobial activity, clofazimine also possesses anti-inflammatory and immunosuppressive properties. Based on the immunosuppressive effects, the drug has been proposed to be useful in the treatment of auto-immune disorders such as multiple sclerosis, systematic lupus erythematosis, Melkersson–Rosenthal syndrome, necrobiosis lipoidica, granuloma annulare and psoriasis (Anderson and Smit, 1993; Van Rensburg et al., 1993; Cholo et al., 2012).

Although the drug is relatively non-toxic and non-myelosuppressive, it has been reported to possess direct anti-neoplastic activity. This activity may be achieved by increasing the generation of tumoricidal ROS by phagocytes. The direct anti-proliferative as well as the pro-oxidative mechanisms of anti-neoplastic activity are dependent on activation of PLA_2 in both tumour and phagocytic cells, respectively (Van Rensburg et al., 1993; Van Rensburg et al., 1994; Durandt et al., 1996).

In mycobacterial infection, the secondary immunosuppressive properties of clofazimine may be detrimental or beneficial. In patient with severe TB, with advanced immunosuppression due to AIDS, clofazimine may suppress cell-mediated immunity and interefere with the efficacy of other antimicrobial agents. These adverse effects may be reduced if clofazimine is administered later during chemotherapy, based on the findings that it has a greater inhibitory effect on slow-growing persisters (http://www.newtbdrugs.org). On the other hand, the immunosuppressive activity of clofazimine may be useful in controlling the adverse effects of therapy-associated recovery of cell-mediated immunity as has been reported to occur in leprosy patients (Imkamp, 1973; Dutta, 1980; Imkamp, 1981; Anderson, 1983). This may also benefit the HIV-infected patients on dual highly active antiretroviral therapy (HAART)/anti-TB therapy who are vulnerable to the development of immune reconstitution inflammatory syndrome (IRIS).

1.4.7 Mechanism of the anti-inflammatory action of clofazimine

The anti-inflammatory-mechanism of action of clofazimine includes suppression of neutrophil, lymphocyte and macrophage activities, by inhibiting the inflammatory and the proliferative responses of these cells (Krajewska and Anderson, 1993; Prinsloo et al., 1995; Sano et al., 2004; Cholo et al., 2012).

The most sensitive cells to the immunomodulatory actions of clofazimine are the T lymphocytes. Clofazimine, at peak serum concentrations attained during the chemotherapy of leprosy, has been reported to cause significant suppression of the mitogen and antigen-driven proliferative responses of isolated T lymphocytes *in vitro* (Anderson and Smit, 1993; Ren et al., 2008) by targeting the plasma membrane K^+ transport, where it inhibits the sodium (Na^+),K^+-exchanger, Na^+,K^+-ATPase, (Segal and Lichtman, 1976) and the potassium channel, Kv1.3 (Ren et al., 2008; Cholo et al., 2012). Na^+,K^+- exchanger, which operates by exchanging three Na^+ ions for two K^+ ions, is responsible for the uptake of K^+ in eukaryotic cells. The cation is required by the cells for sustaining multiple activities as mentioned for microbial cells in section 1.4.5.

The clofazimine-mediated decrease in T lymphocyte Na^+,K^+-ATPase activity was associated with increased generation of AA, which may be a result of membrane phospholipids hydrolysis (Anderson and Smit, 1993), by activation of PLA_2, and/or direct hydrolysis of the ester linkage at the C2 position of the glycerol backbone of membrane phospholipids (Baciu et al., 2006), resulting in the formation of lysophospholipids (Cholo et al., 2012).

The Kv 1.3 potassium channel (Ren et al., 2008) is a voltage-gated, delayed rectifier K^+ ion channel that regulates membrane potential and Ca^{2+} signalling in effector memory T cells (Airmid, 2007). Clofazimine-mediated inhibiton of this channel results in the interference with K^+ efflux and failure of the efficient membrane repolarization response necessary to drive Ca^{2+} influx (Ren et al., 2008).

From effect on K^+ transportation, clofazimine may suppress T cell proliferation by promoting the release of anti-proliferative ROS and E-series prostaglandins (PGs), especially PGE_2, from neutrophils and monocytes (Zeis and Anderson, 1986), via the production of AA from membrane phospholipid hydrolysis (Anderson et al., 1988; Fukutomi et al., 2011).

1.4.8 Pharmacokinetic properties of clofazimine

Because of its lipophilic nature, the absorption of orally administered clofazimine in humans is usually low, resulting in low peak serum concentrations. However, absorption improves when the drug is taken with food (Reddy et al., 1999). For instance, mean serum concentrations of approximately 0.24 mg/L have been achievable only after a month of daily intake of 50 mg intake (Venkatesan et al., 2007), which is an insufficient concentration for activity against *M. tuberculosis*.

Once absorbed, the drug is distributed primarily into fatty tissues, as well as cells of the MPS, such as macrophages. In leprosy patients receiving clofazimine, concentrations as high as 5.3 mg/g have been found in fat tissues, and approximately 1 mg/g in tissues such as bile, gall bladder, kidney, pancreas, skin, liver, spleen, lymph nodes, eyes and lung. As mentioned earlier, it also has a long half-life of 70 days (Dooley et al., 2012).

1.4.9 Metabolism

After oral administration, clofazimine is absorbed and stored in the MPS and in fat tissues. In addition to its long half-life, it is excreted slowly and in an unchanged form. Small amounts of the drug are excreted in faeces with the remainder being excreted in urine, sputum, sweat and breast milk (Phillip et al., 2012).

To date, clofazimine is reported to be metabolised into only three compounds, viz. metabolite I (3-hydroxyanilino-10-(p-chlorophenyl)-2, 10-dihydro-2-isopropylinimino-phenazine), metabolite II (3-(B-D-glucopyranosiluronic acid)-2-isopropylinimino phenazine), and metabolite III (3-(p-chloroanilino)-10-(pchlorophenyl)-4-(B-D-glucopyranosiluronic acid)-2-isopropyliminophenazine). These have been identified in human urine with no evidence of their biological activities (Feng et al., 1989; Holdiness et al., 1989; Reddy et al., 1999). Metabolite I is thought to undergo hydrolytic

dehalogenation, and sulphate conjugation and 4-hydroxylation to metabolite II, while metabolite III undergoes glucuronic acid conjugation (Feng et al., 1989). All three metabolites occur at very low concentrations, totalling less than 1% of the drug (0.2%, 0.25% and 0.2% for the three metabolites, respectively) (Venkatesan, 1989; Reddy et al., 1999).

1.4.10 Adverse effects

Generally, clofazimine is a well-tolerated drug when it is administered in dosages no greater than 100 mg daily. The most consistent adverse reactions are usually dose-related and are reversible when clofazimine is discontinued.

Due to its physicochemical and pharmacokinetic properties, which include its accumulation in fat tissues and long half-life, its administration leads to unwelcome skin discoloration (Phillip et al., 2012). In most patients, it presents as orange to pink discoloration of the skin. Recently, a 45-year old leprosy patient receiving treatment, had his grey hair darkened, as a result of clofazimine in the treatment regimen. This persisted for eight months after completing multibacillary multidrug therapy (Phillip et al., 2012). Clofazimine is also present in body fluids such as tears and saliva and may result in staining of the cornea, conjunctiva, and sputum (Jadhav et al., 2004). However, staining of the conjuctiva by the drug has not been found to affect vision.

Upon prolonged treatment, clofazimine accumulates in higher doses, affecting the internal organs. Crystal deposition of clofazimine in the small bowel mucosa has resulted in abdominal pain, nausea and diarrhoea. In some cases, accumulation may be fatal resulting in severe enteropathy, which occurs mostly after several months of therapy with high doses of the drug (>100 mg daily) (Belaube et al., 1983; Freerksen and Seydel, 1992). About 20% of neonatal mortality has been reported in pregnant women with leprosy receiving clofazimine (Arbiser et al., 1995). In rare cases, AIDS patients taking clofazimine develop bull's-eye, a condition characterised by brownish-red discoloration of cornea and conjunctiva, caused by crystalline clofazimine depositing in these tissues (Barot et al., 2011).

1.4.11 Analogues

Due to its poor pharmacokinetic properties, which include high lipophilicity, poor oral absorption, low serum concentration and high skin pigmentation, as mentioned previously, clofazimine was discontinued from the chemotherapy of TB. Several analogues, which are less lipophilic and less skin-pigmenting, while retaining their anti-mycobacterial efficacies, have been developed. These include compounds such as B746, B4157 and tetramethylpiperidine (TMP)-substituted phenazines,

which have demonstrated higher anti-TB activities against rapidly-replicating drug-sensitive and drug-resistant clinical isolates and are less skin pigmenting than clofazimine (Reddy et al., 1999; Van Rensburg et al., 2000; Matlola et al., 2001; Huygens et al., 2005). In addition, most of these analogues have been found to possess superior intracellular activity against clinical isolates than clofazimine (Van Rensburg et al., 2000).

Other analogues which have been recently formulated by the Global Alliance TB Drug Development group, in China, have shown comparable *in vitro* activities against intracellular and non-replicating *M. tuberculosis* bacilli to clofazimine, in addition to enhanced activities against drug-sensitive and drug-resistant strains (Lu et al., 2011; Liu et al., 2012). Another analogue, KS6 which has been synthesised by Yano and co-workers (2011), has demonstrated improved solubility relative to clofazimine but had comparable antimicrobial activity to clofazimine.

1.4.12 Spray-dried formulation

Several strategies have been developed to improve the dissolution and absorption properties of clofazimine, including formulation into solid dispersions.

Encapsulation of certain antibiotics in liposomes has been shown to improve the efficacy of these agents against intracellular bacilli and in animal models of experimental TB (Adams et al., 1999). For instance, liposomal clofazimine has shown increased activity relative to native clofazimine in mice infected with *M. tuberculosis* or MAC, resulting in a significant reduction in the number of viable bacteria in lung, liver and spleen. In addition, this liposomal preparation showed improved solubility and presented with less side effects in murine model of infected TB (Mehta, 1996; Kansal et al., 1997; Cholo et al., 2012).

Other alternative formulation strategies include spray-drying, resulting in nano-particle formulations. This approach improves solubility, and increases the dissolution rate and oral availability of poorly-soluble drugs. With this technique, the drug, dispersed in water, is ground by shear forces, i.e high pressure homogenization, to particles with mean diameter in the nanometre range (100-1000 nm). This process dries the droplets of their volatile substance and leaves non-volatile components in the form of dry particles, with particle size, morphology, density and volatile content controlled by drying process parameters. The result yields drug particles in the amorphous state, usually with increased water solubility. By spray-drying using appropriate conditions and excipients, large porous particles are formed with geometric diameter (d_{geo}) >5 um and density *p* <0.1 g/cm3, with ideal aerodynamic properties such as diameter of 1-5 µm for delivery to the

alveolar region of the lungs (Cholo et al., 2012). This may enhance the bioavailability of drugs as the nano-suspension will reach its target passively.

This formulation has demonstrated beneficial results in a murine model of experimental infection. Several anti-TB drugs, which include capreomycin, para-aminosalicylic acid, PA-824 and rifampicin, have been formulated as dry powder microparticles for pulmonary delivery. Studies in animal models of experimental TB have demonstrated that direct delivery of these agents to the lungs resulted in high local concentrations and reduced bacterial burden compared with the same treatment delivered via other routes (Cholo et al., 2012). Treatment of C57BL/6 mice infected with the MAC strain TMC 724, with nano-particle clofazimine showed similar results to those with a liposomal formulation in reducing bacterial loads in the liver, spleen and lungs (Peters et al., 2000). In another study, clofazimine nano-particle formulation has also been evaluated for anti-TB activity in a murine model of experimental TB infection. The nano-particle formulation resulted in a higher efficacy than the native drug by reducing colony forming unit per millilitre (cfu/mL) in the lungs by as much as 2.6-$\log_{10}$ relative to a 0.7-$\log_{10}$ reduction (Verma et al., 2013).

Nano-particle suspensions offer a promising advantage to drug formulation, especially of poorly soluble drugs such as riminophenazines, as they are easy to prepare and to lyophilize and can be stored for extended periods (Peters et al., 2000). In the current study, the aforementioned clofazimine nano-particle (Verma et al., 2013) was compared with its native formulation with respect to antimycobacterial activity against *M. smegmatis* during biofilm formation. *M. smegmatis* has been used in this study as a surrogate organism for *M. tuberculosis*.

1.5 HYPOTHESIS

M. smegmatis biofilms are sensitive to the antimycobacterial activities of clofazimine.

1.6 AIM

To compare the antimycobacterial activities of native and spray-dried formulations of clofazimine, as well as effects on biofilm formation and penetration using *M. smegmatis* as a surrogate for *M. tuberculosis*.

1.6.1. OBJECTIVES

The objectives are as follows:

- To induce *M. smegmatis* biofilm *in vitro* using synthetic medium.
- To determine the antimycobacterial activity of native and spray-dried preparations of clofazimine against sessile biofilm-producing *M. smegmatis* population using colony-counting procedures.
- To determine the antimycobacterial activity of native and spray-dried preparations of clofazimine against sessile biofilm-encased *M. smegmatis* population using colony-counting procedures.
- To determine the effects of native and spray-dried formulations of clofazimine on *M. smegmatis* biofilm formation.
- To determine the effects of clofazimine on the lipid composition of *M. smegmatis* biofilm using the thin-layer chromatography (TLC) analysis.

Diagram of experimental design

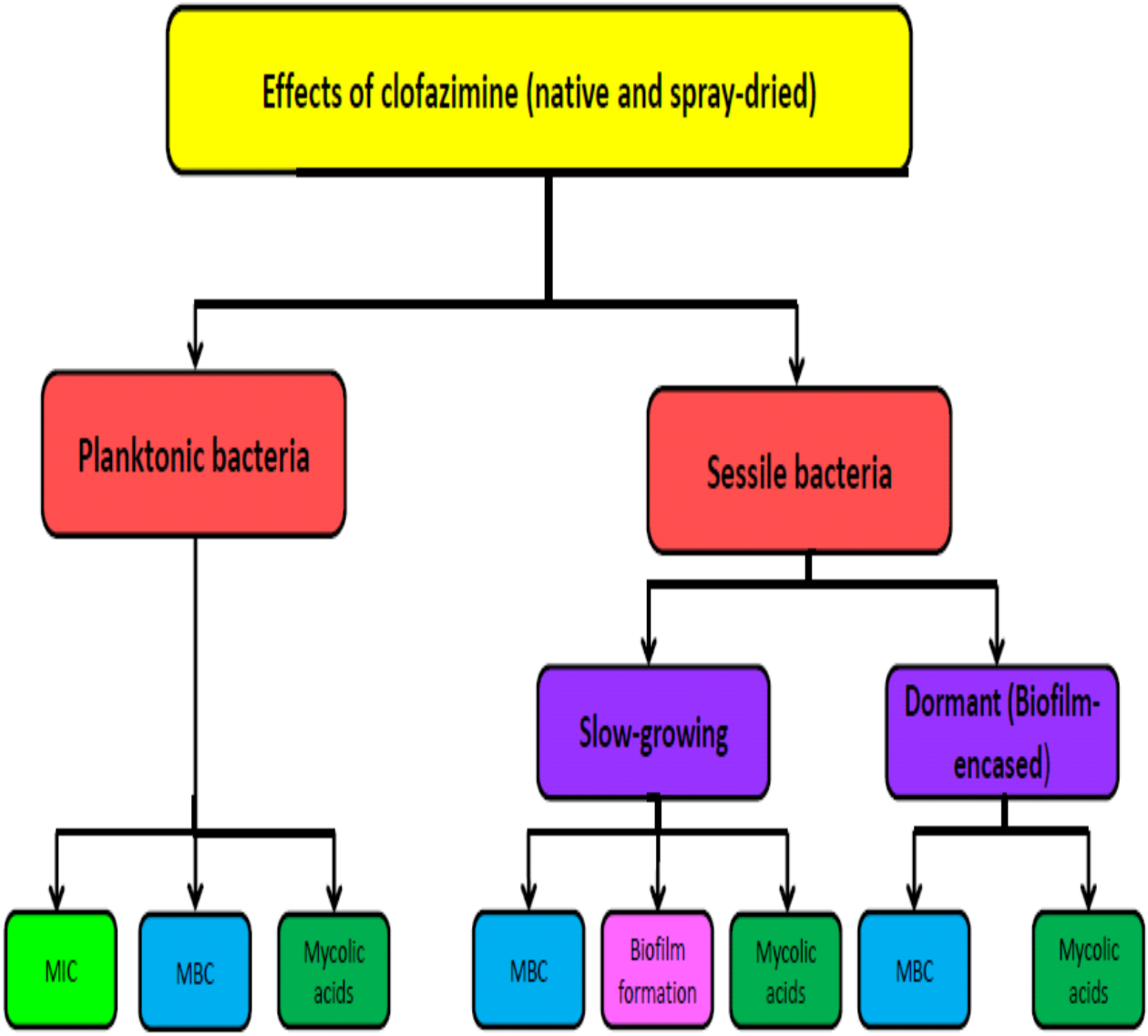


Figure 1.15: Summary of designed experiments.

CHAPTER 2

2.1 *MYCOBACTERIUM SMEGMATIS*

M. smegmatis is a fast-growing non-pathogenic mycobacterium, which was originally isolated from humans but is mostly found in soil and water (Ettiene et al., 2005). It is classified as a saprophytic species that is not dependent on living in animal cells. It is 3.0-5.0 µm long (Gordon and Smith, 1953) and is readily cultivated in most synthetic media with a generation time of 3-4 hours, forming white to non-pigmented creamy/yellowish colonies on a solid medium. The bacteria also vary in texture, being smooth, flat and glistening or coarsely-folded or finely-wrinkled (Gordon and Smith, 1953; Husson, 1998).

Based on its rate of growth and non-pathogenicity, *M. smegmatis* serves as a surrogate for the research analysis of other species of mycobacteria, such as *M. tuberculosis*, *M. avium* subsp. *paratuberculosis* and *M. leprae* (Jacobs, 2000). Most of these species are labour-intensive, time-consuming and slow growers, and may require biosafety level 3 containment as they are pathogenic.

In this study *M. smegmatis* was used as a surrogate for studying the development of mycobacterial biofilm (Ojha et al., 2008), in response to clofazimine and its spray-dried formulation. As mentioned previously, mycobacterial biofilm is highly rich in lipids such as glycolipids and FA, most of which are components of the mycobacterial cell envelope (Ojha et al., 2008; 2010).

2.2 THE CELL WALL STRUCTURE OF MYCOBACTERIA

All mycobacterial species have a similar cell wall structure as illustrated in Figure 2.1 (Jean-Marc and Daniel, 2001). This provides the bacterium with shape, protection and survival in harsh conditions, and is highly impermeable to antibiotics.

The components of the cell wall include cell/plasma membrane, peptidoglycan (PG), arabinogalactan (AG), mycolic acids (MA) and capsular-like materials. Their structure and functions are as follows:

(i) The plasma membrane is a bilayer, surrounded by an inner and an outer layer (Brennan, 2003).

(ii) The PG (murein) is made up of peptides and glycan strands, and maintains the structure and the shape of the bacterial cell wall while providing protection to bacteria from osmotic turgor pressure yet is malleable enough for the bacteria to grow and expand.

(iii) The AG consists of arabinan and galactan, and constitutes the major polysaccharide of the cell wall (McNeil et al., 1987).

(iv) The MA represents the major component of the cell envelope, and plays a crucial role in the structure and function of the mycobacterial cell envelope (Barry et al., 1998; Daffe and Draper, 1998). Its attachment to the AG forms an outer permeability barrier that confers an extremely low fluidity and an exceptionally low permeability of antibiotic penetration through the mycobacterial cell-wall (Jarlier and Nikaido, 1990). In *M. tuberculosis*, the trehalose mycolates, which are some of the MA of this species, have been implicated in numerous biological functions related both to the physiology and virulence of *M. tuberculosis (*Laval et al., 2001).

The lipid part of the outer layer, which is linked to polysaccharides, consists of lipoarabinomannan (LAM), lipomannan, phthiocerol-containing lipids such as phthioceroldimycocerosate, dimycolyltrehalose (cord factor), sulfolipids specific to *M. tuberculosis*, and the phosphatidylinositol mannosides. The LAMs are usually capped at the terminal -Ara residue with either mannose or phosphoinositol residues and are referred to as ManLAMs or PILAMs, respectively. The ManLAMs are commonly found in slow-growing, pathogenic mycobacteria, such as *M. tuberculosis* and *M. leprae* (Chatterjee et al., 1992; Prinzis et al., 1993; Nigou et al., 2003), whereas PILAMs are found in the fast-growing mycobacteria, such as *M. smegmatis* and *M. fortuitum* (Nigou et al., 2003).

The protein component of the cell wall plays a crucial role in many vital processes such as cell-cell interactions, ion and nutrient transport and cell signalling, and participates in the key virulence mechanisms. Some of the proteins are scattered on the mycobacterial surface. These proteins like other structural components discussed above, are also involved in protection, as well as lipid metabolism, and are required for the pathogenicity of mycobacteria, including modification of the host immune response (Chamacho et al., 1999; Dalfe and Ettiene, 1999).

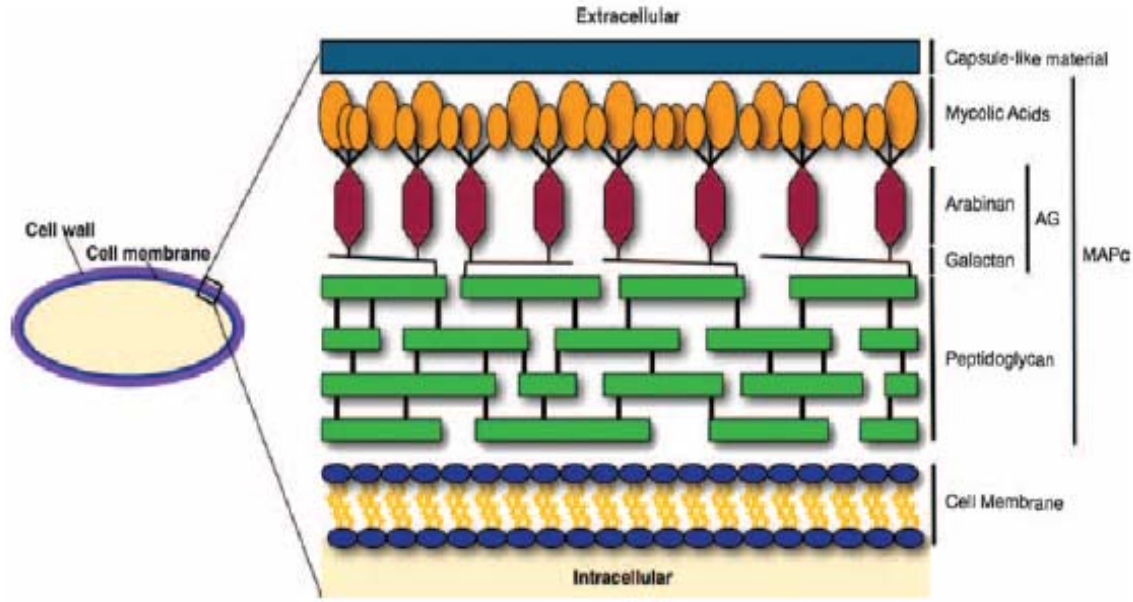


Figure 2.1: The basic components of the mycobacterial cell wall. MAPc, MA-AG-PG complex (Source: Hett and Rubin, 2008).

The laboratory research presented in this study was designed to evaluate the antimycobacterial activities of native and spray-dried formulations of clofazimine on biofilm of *M. smegmatis*.

2.3 MATERIALS AND METHODS

2.3.1 Materials

2.3.1.1 Reagents

Unless otherwise stated reagents were purchased from Sigma-Aldrich Chemicals Co (St Louis, MO, USA). Dimethyl sulfoxide (DMSO) used for dissolving the drug, as well as chemicals used for TLC were purchased from Merck (RSA).

2.3.1.2 Antibiotics

Two drug formulations of clofazimine, viz. native and spray-dried, were used in this study. The native clofazimine (NC) was purchased from Sigma-Aldrich Chemicals Co (St Louis, MO, USA) while the spray-dried clofazimine (SDC) was prepared by Dr WA Germishuizen (Department of Immunology, University of Pretoria and Deltamune (Pty) Ltd., RSA).

Both preparations of clofazimine were dissolved in 100% DMSO and were made to 2 g/L stock solutions. The final working concentrations for both formulations in assays ranged from 0.15-20 mg/L. DMSO was used at a final concentration of 1% in all assays. Solvent controls were included in all experiments.

2.3.1.3 Strains

M. smegmatis (mc^2155) strain was used for all experiments.

2.3.1.4 Culture media

7H9 broth and 7H10 agar base media (Difco), were purchased from Beckton Dickinson (USA) and were prepared according to the manufacturer's instructions.

For 7H9 broth preparation, 4.7 g of broth base powder was dissolved in 900 mL of distilled water. Thereafter 0.2% glycerol and 0.05% Tween 80 were added and the mixture was autoclaved at 121°C for 15 minutes (min). The medium was cooled to 55°C and 10% oleic acid, dextrose, catalase (OADC) was added.

For 7H10 agar preparation, 19 g of the agar base powder was dissolved in 900 mL of distilled water, and thereafter 0.5% glycerol was added. The mixture was autoclaved at 121°C for 15 min, and cooled to 55°C followed by the addition of 10% OADC. About 20 mL of the agar medium was dispensed into 90 mm concord sterile petri dishes and allowed to settle before being used.

Sauton broth medium was prepared as described (Ojha et al., 2008). A mixture of 0.5g potassium dihydrogen phosphate (KH_2PO_4), 0.5g magnesium sulfate ($MgSO_4.7H_2O$), 2g citric acid ($C_6H_8O_7 \cdot H_2O$), 0.05g ferric ammonium nitrate ($FeH_4N_5O_{12}$), and 4g L-asparagine were dissolved in 600 mL of distilled water. Thereafter, 100 mL of 0.1% zinc sulphate ($ZnS0_4$) and 60 mL glycerol were added. The medium was adjusted to pH 6.8 with 10M sodium hydroxide (NaOH) and thereafter autoclaved at 121°C for 15 min. The medium was ready for use.

2.3.2 Methods

2.3.2.1 Preparation of spray-dried clofazimine formulation

The spray-dried formulation of clofazimine was prepared by Dr Andre Germishuizen (Deltammune/Department of Immunology, University of Pretoria). A schematic illustration of the spray drier, together with its components and the spray-drying procedure, are shown in Figure 2.2. The NC was dissolved in ethanol to a final concentration of 1.5 g/L and mixed with L-leucine, followed by spray drying using a Buchi Mini Spray Dryer B-290 (Flawil, Switzerland) with drying air at a pressure of 5-6 bar and a flow rate of 35 L/min. These solutions were then atomized by pumping at a rate of 5-7 mL/min through a 0.7 mm pressure nozzle tip located above the inlet chamber/cylinder. The inlet temperature and flow rates were optimised to result in a minimum moisture content of the dried powder. Initial inlet temperature selection was chosen between 100-

140°C. Spray-dried particles were collected in 6-inch collection vessels located at the bottom of the high performance cyclone.

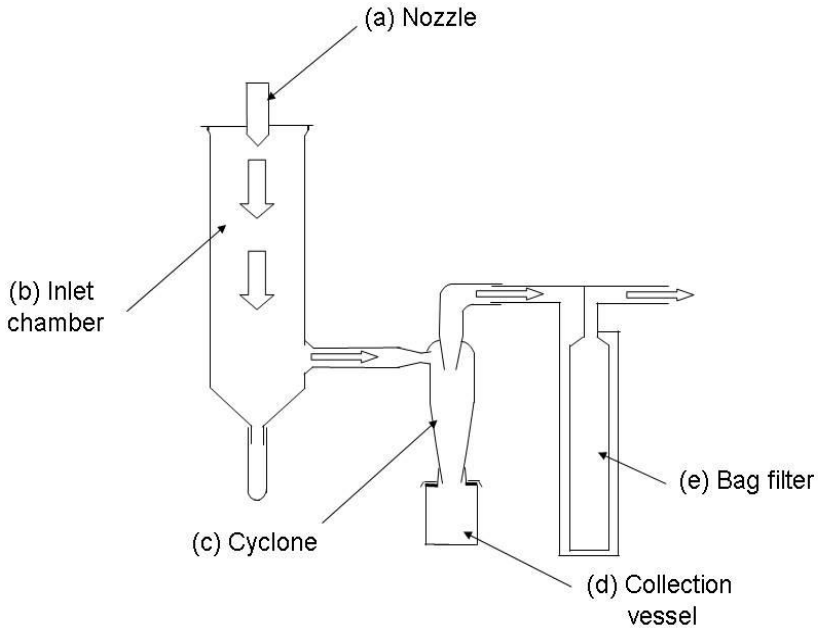


Figure 2.2: A schematic illustration of spray drier and its components (Source: Dr Germishuizen).

2.3.2.2 Preparation of the inoculum

One loopful of *M. smegmatis* cells grown on 7H10 agar plate was scraped and inoculated into a 250 mL Schotts bottle containing 50 mL of 7H9 broth. The culture was placed on a magnetic stirrer and incubated at 37°C for 24 h. The culture was then transferred into a sterile 50 mL tube and centrifuged at 3500 revolution per minute (rpm) for 15 min at 4ºC. The supernatant was discarded and approximately 5 mL 1 mm diameter of glass beads were added to the pellet and vortexed for a 1 min in order to break the clumps. The cells were resuspended in 50 mL of Sauton medium and recentrifuged as above. The supernatant was discarded and the cells resuspended in 20 mL of Sauton medium. The cell suspension was adjusted to an optical density (OD) of 0.6 at 540 nm, which yields approximately 10^7 cfu/mL, using a Helios-γ UV-Vis spectrophotometer (Unicam, UK). This inoculum was used for both planktonic and biofilm assays.

2.3.2.3 Minimum inhibitory concentration determination

The MIC of clofazimine for *M. smegmatis* was determined using the plate-proportion method as described previously (Heifets and Lindholm-Levy, 1987). Briefly, various concentrations of clofazimine ranging from 0.15-5 mg/L were incorporated into 7H10 agar plates. One set of drug-free and all drug-containing plates were inoculated with 10^4 cfu/mL cells, while another set of drug-

free plates, serving as controls, was inoculated with 100x dilution of the inoculum (10^2 cfu/mL). The plates were incubated at 37°C for 72 h to allow for the appearance of colonies. The lowest concentration of the drug that yielded fewer colonies than those that grew on the 100xdiluted controls was regarded as the MIC of that formulation.

2.3.2.4 Minimum bactericidal concentration and time-kill determination of clofazimine formulations

All *M. smegmatis* planktonic and biofilm assays were prepared in 6-well plates in a final volume of 5 mL. A set of drug-free and drug-containing wells were inoculated with bacterial cells to achieve a 10^5 cfu/mL suspension.

2.3.2.4.1 Maximum bactericidal concentration and time-kill determination of clofazimine formulation on planktonic bacilli

Approximately 4900 µL of Sauton media, containing 0.05% Tween 80, was placed into each well. Thereafter, 50 µL of the cells prepared as described in section 2.3.2.2, and varying concentrations of each clofazimine formulation, ranging from 0.15-20 mg/L, were added. The drug-free control system contained 50 µL of 100% DMSO. The plates were mixed and incubated at 37ºC for 24 h. Each well was sampled every 6 h beginning from 0 h and finishing at 24 h, referred to as T_0 (<30 min within inoculation) and T_{24} (stopping time), respectively. These samples were diluted and plated on 7H10 medium and incubated at 37°C in the dark for 72 h, for the appearance of colonies. The number of cfu/mL per assay were counted and the minimum concentration of each clofazimine formulation that yielded a 2-log reduction in the number of colonies as compared to the initial inoculum at T_0 was regarded as the MBC of that formulation.

2.3.2.4.2 Preparation of *Mycobacterium smegmatis* biofilm cultures

Approximately 4950 µL of Sauton media, without 0.05% Tween 80, was placed into each well and 50 µL of the cells prepared as described in section 2.3.2.2, was added. The wells were thoroughly mixed and the plates were wrapped once with parafilm and incubated at 37°C, in the dark without shaking for 5 days. The plates were daily monitored for the development of a white layer on the surface, referred to as biofilm.

2.3.2.4.3 Maximum bactericidal concentration and time-kill determination of clofazimine formulations on biofilm-producing bacilli

Bacterial cultures were prepared as above for planktonic growth in Sauton medium without Tween 80 and various drug concentrations that ranged from 0.15-5 mg/L were added. After thorough

mixing, the plates were wrapped once with parafilm and incubated at 37°C, in the dark without shaking for 5 days. Each day starting from day 0 (day of bacterial and drug inoculation), the number of cfu/mL was determined for each well by adding 100 µL of 0.2% Tween 80 into each set of drug-free and drug-containing wells. The mixtures were incubated at 37°C for 1 h, with frequent shaking, to dissolve the biofilm. The samples were then diluted and plated onto 7H10 agar medium and incubated at 37°C in the dark for 72 h, for the appearance of colonies. The minimum concentration of each clofazimine formulation that yielded a 2-log reduction in the number of cfu/mL compared to that of day 0 was regarded as the MBC of that formulation.

2.3.2.4.4 Maximum bactericidal concentration and time-kill determination of clofazimine formulations on biofilm-encased bacilli

Bacterial cultures were prepared as above in Sauton medium in the absence of drugs. After incubation, parafilm was removed from each plate and both formulations of clofazimine, ranging from 0.15-10 mg/L, were added to all wells. The plates were tilted slightly to distribute the antibiotic evenly in the wells and thereafter rewrapped once with parafilm and incubated at 37°C in the dark for an additional 5 days. The day of addition of clofazimine formulations to the bacterial cultures was regarded as day 0. Each day, 100 µL of 0.2% Tween 80 was added to a set of drug-free and drug treated wells and incubated as above, followed by plating to determine the cfu/mL. The MBC of each formulation was the minimum concentration of the drug that yielded a 2-log reduction in cfu/mL as compared to that at day 0.

2.3.2.5 Biofilm quantification

The effect of clofazimine on biofilm formation was determined. The bacterial cultures treated with the various concentrations of each clofazimine formulation were prepared as described in section 2.3.2.4 and the biofilm was developed for 5 days. The developed biofilm was quantitated using the crystal violet procedure as described (Stepanovic et al., 2004). The supernatant was removed from each well and the biofilm was washed twice with 1.25 mL of distilled water and air-dried at room temperature (RT). Thereafter, 1.25 mL of 1% crystal violet was added to each well, followed by incubation at RT for 30 min. The unbound dye was removed, followed by washing the wells three times with 1.25 mL of distilled water and air dried. The residues were dissolved and the crystal violet was extracted from each well by adding 1.25 mL of 70% ethanol. The samples were diluted and the OD was measured at 570 nm using a Bio-Tech PowerWave$_x$ plate spectrophotometer.

2.3.2.6 Lipid characterization of *Mycobaterium smegmatis* biofilm using a thin-layer chromatography procedure

2.3.2.6.1 Isolation of apolar lipids

Cells were grown planktonically or in biofilm in 6-well plates as described in section 2.3.2.4. After incubation, cells were transferred to 15 mL screw-capped tubes and centrifuged at 3500 rpm for 10 min at RT. The supernatant was removed and the pellet transferred into a 10 mL (16 x 125 mm) borosilicate glass tube and 5 mL of methanol: 0.3% sodium chloride (NaCI) (100:10) was added. This was followed by the addition of 2.5 mL of petroleum ether and the mixture was vortexed for a maximum of 2 min, to separate the layers. The mixture was centrifuged at 3400 rpm, for 1 min at room temperature. The upper petroleum layer, containing the apolar lipids, was removed and transferred into a tablet tube and thereafter dried under nitrogen. The dried extract was dissolved in 200 µL dichloromethane and spotted whole on the silica plate as described below, section 2.3.2.6.2, for chromatography.

2.3.2.6.2 Thin-layer chromatography

In this study a two-dimensional TLC procedure was performed. Each sample, together with the standard control, was spotted onto a 6.6 x 6.6 cm TLC silica gel 60 F_{254} Aluminium plate (Merck, RSA) with the spotting point 1 cm from the left and bottom sides of the plate. A separate silica plate, used for the control, was spotted with a cocktail of 200 µL dichloromethane and 10 µL *M. tuberculosis* mycolic acid standards (2 g/L, dissolved in dichloromethane). The spots were air-dried and the two-dimensional chromatography was performed by first placing the spotted silica plates in a chromatography tank (25 length x 9 breadth x 24 cm height) containing chloroform: methanol (100:80) solvent, which was <0.5 cm below the spotted area. The solvent was allowed to migrate until it reached 1 cm from the top of the silica plate. The plates were removed and air-dried before the second dimension chromatography was performed. For the second dimension, the air-dried silica plates were placed into a second chromatography tank (25 length x 9 breadth x 24 cm height) that contained toluene: acetone (80:20), and was tilted such that the left-hand side was at the bottom of the tank. The solvent was allowed to migrate to the same level as for the first dimension and the plates were removed and air-dried at RT. Each plate was sprayed with 5% molybdophosphoric acid in a fume cupboard, and thereafter air-dried. The plates were then baked at 120°C in a Scientific Series 2000 incubator (Lenton Laboratory and Scientific Equipment, RSA) for 10 min, to allow for the development of dark spots in the silica matrix of the plates. The identity and diameter of the spots were determined using the migration of *M. tuberculosis* mycolic acid standards as reference.

2.3.3 Statistical analysis

In this study the two drug formulations were compared at various concentrations, for their lethal activities against *M. smegmatis* planktonic and biofilm cultures. In order to measure consistency/reliability, each concentration for both formulations, was performed using six replicates.

The rate of killing was plotted as surviving bacteria in cfu/mL versus time in hours and days for planktonic and biofilm, respectively. The time-kill activities for both drug formulations were compared using the two-sample word parameter test (Mann-Whitney test) and a two-way group (time versus drug) comparison was used to investigate the effect of the drugs at varying concentrations for each formulation.

Lastly, the effects of the same varying concentrations of the two clofazimine formulations on the quantities of the biofilm formed at the end of five days were compared also using the Mann-Whitney test.

CHAPTER 3: RESULTS

3.1 Effects of the clofazimine formulations on the growth of planktonic *Mycobacterium smegmatis*

To determine the inhibitory activities of NC and SDC on the growth of *M. smegmatis*, the MIC values for both formulations were determined using a plate-dilution method. For both preparations of clofazimine the MIC was found to be 2.5 mg/L.

To determine the killing activities of both clofazimine formulations on aerobic, actively-growing *M. smegmatis* bacilli, the MBC values for planktonic cultures of *M. smegmatis* were determined. The results are shown in Figure 3.1 for both formulations. Both formulations exhibited comparable activities at each concentration. The control reached a maximum of 10^8 cfu/mL in 24 h. No MBC was established for both formulations which may be due to insolubility of clofazimine at concentrations above 2 mg/L. However, the killing activity of both drugs stabilised at 10 mg/L.

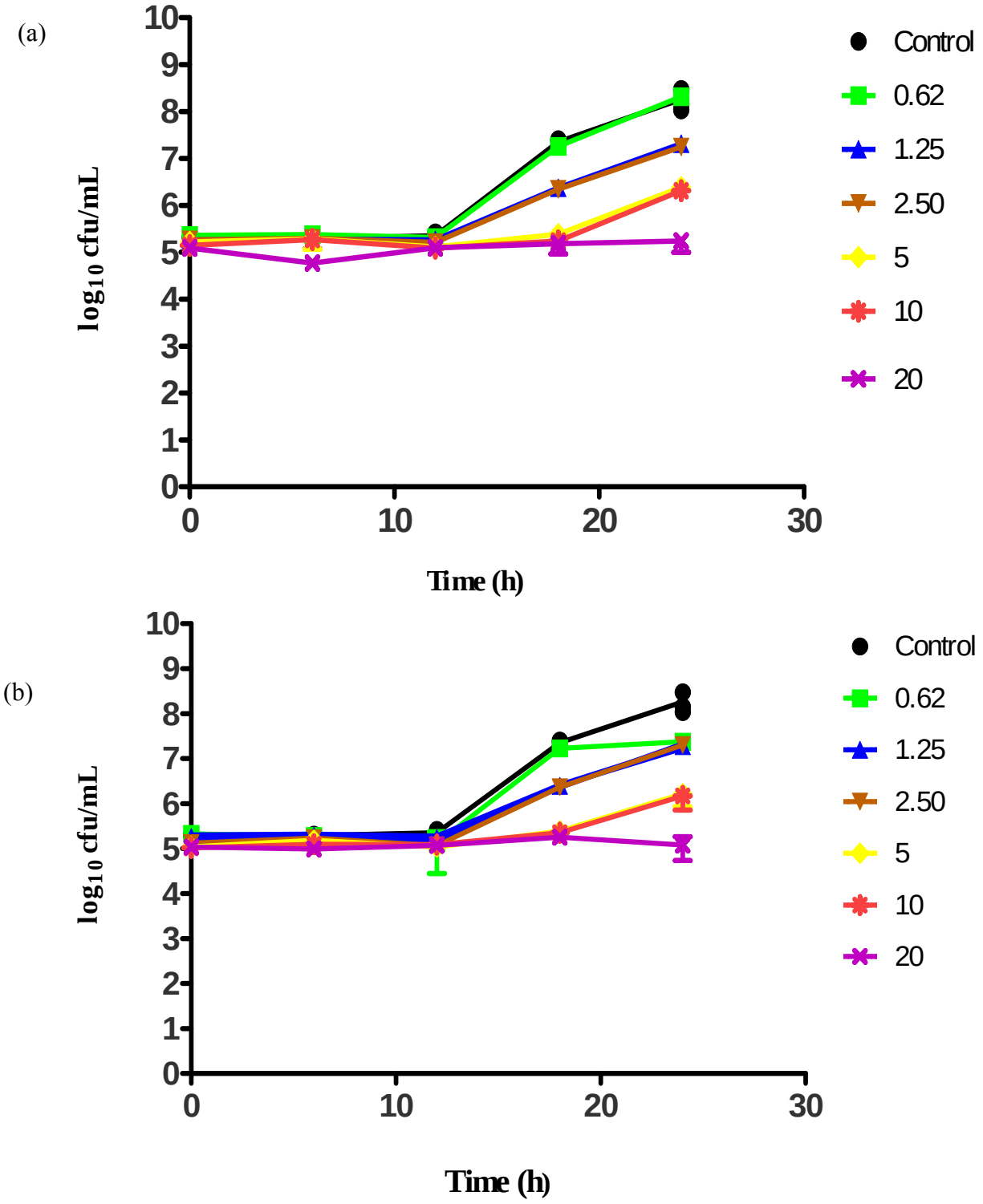


Figure 3.1: The effects of clofazimine on planktonic culture of *Mycobacterium smegmatis*. The rates of bacterial killing of varying concentrations (0.6-20 mg/L) of (a) NC and (b) SDC determined at 6 h time points for 24 h. The results are of three separate experiments performed in triplicate for each concentration of the clofazimine preparations and are presented as the mean values ± SEM.

Figure 3.2: A matured *Mycobacterium smegmatis* biofilm formed in a 6-well plate over 5 days.

3.2 Formation of *Mycobacterium smegmatis* biofilm in a synthetic medium

To determine the effect of clofazimine on *M. smegmatis* biofilm formation, *M. smegmatis* cells were grown in a detergent-free Sauton broth and the culture plates wrapped in parafilm, which increased the level of CO_2, followed by incubation in the dark without shaking, to diminish the level of oxygen in the culture (Recht et al., 2000; Ojha et al., 2008; Kulka et al., 2012). The results are shown in Figure 3.2. Using this medium, biofilm developed and matured over five days. During the development period, within 24 h of incubation, a thin-layer formed on the surface of the culture medium and by day 3, the entire surface of the culture medium was covered with a white matrix, which thickened and became rough or coarse-like, extending to the sides and reaching the top of the plate on the fifth day, representing a matured biofilm. Wrapping the cultures with parafilm also prevented evaporation of the water content from the cultures during long incubation period.

3.3 Effects of the clofazimine formulations on the viability of biofilm-producing *Mycobacterium smegmatis*

The bactericidal effects of clofazimine against slow-replicating biofilm-producing sessile *M. smegmatis* MBCs are shown in Table 3.1 and Figure 3.3. The results indicate that the slowly-growing biofilm-producing bacteria reach a maximum of 10^8 cfu/mL at day 5 (Table 3.1). The MBC values for both formulations were similar, being 2.5 mg/L. The rates of killing of *M. smegmatis* by the two formulations of clofazimine, are shown in Figure 3.3. The MBCs for NC and SPC were achieved on day 5 and 3, respectively.

Table 3.1: Number of cfu/mL of biofilm-producing *Mycobacterium smegmatis* against various concentrations of NC and SDC at day 0 and day 5

NC

Concentration (mg/L)	Incubation (Day 0) Mean ± SEM	Incubation (Day 5) Mean ± SEM
Control	$2.6 \times 10^5 \pm 3.7 \times 10^4$	$2.3 \times 10^8 \pm 7.2 \times 10^6$
0.15	$2 \times 10^5 \pm 1.8 \times 10^4$	$2.5 \times 10^7 \pm 8.2 \times 10^5$
0.31	$1.5 \times 10^5 \pm 3.5 \times 10^4$	$1.4 \times 10^7 \pm 1.1 \times 10^6$
0.62	$1.7 \times 10^5 \pm 7.4 \times 10^3$	$2.7 \times 10^5 \pm 4.3 \times 10^3$
1.25	$1.4 \times 10^5 \pm 2.2 \times 10^4$	$4.7 \times 10^4 \pm 7.2 \times 10^3$
2.5	$1.1 \times 10^5 \pm 1.1 \times 10^4$	$2.6 \times 10^3 \pm 1.2 \times 10^3$
5	$7.1 \times 10^4 \pm 1.7 \times 10^3$	$2 \times 10^3 \pm 5.7 \times 10^2$

SPC

Concentration (mg/L)	Incubation (Day 0) Mean ± SEM	Incubation (Day 5) Mean ± SEM
Control	$1.08 \times 10^5 \pm 2.9 \times 10^4$	$2.3 \times 10^8 \pm 7.2 \times 10^6$
0.15	$1.05 \times 10^5 \pm 2.9 \times 10^4$	$2.2 \times 10^7 \pm 1 \times 10^6$
0.31	$1.03 \times 10^5 \pm 2.9 \times 10^4$	$1.9 \times 10^7 \pm 1 \times 10^6$
0.62	$1.1 \times 10^5 \pm 2.9 \times 10^4$	$2.4 \times 10^5 \pm 1.7 \times 10^4$
1.25	$1.09 \times 10^5 \pm 1.3 \times 10^4$	$6.6 \times 10^4 \pm 3.1 \times 10^4$
2.5	$1.09 \times 10^5 \pm 1.9 \times 10^4$	$2.6 \times 10^3 \pm 8.2 \times 10^2$
5	$1.3 \times 10^5 \pm 2.9 \times 10^3$	$2 \times 10^3 \pm 5.7 \times 10^2$

SEM = standard error of the mean

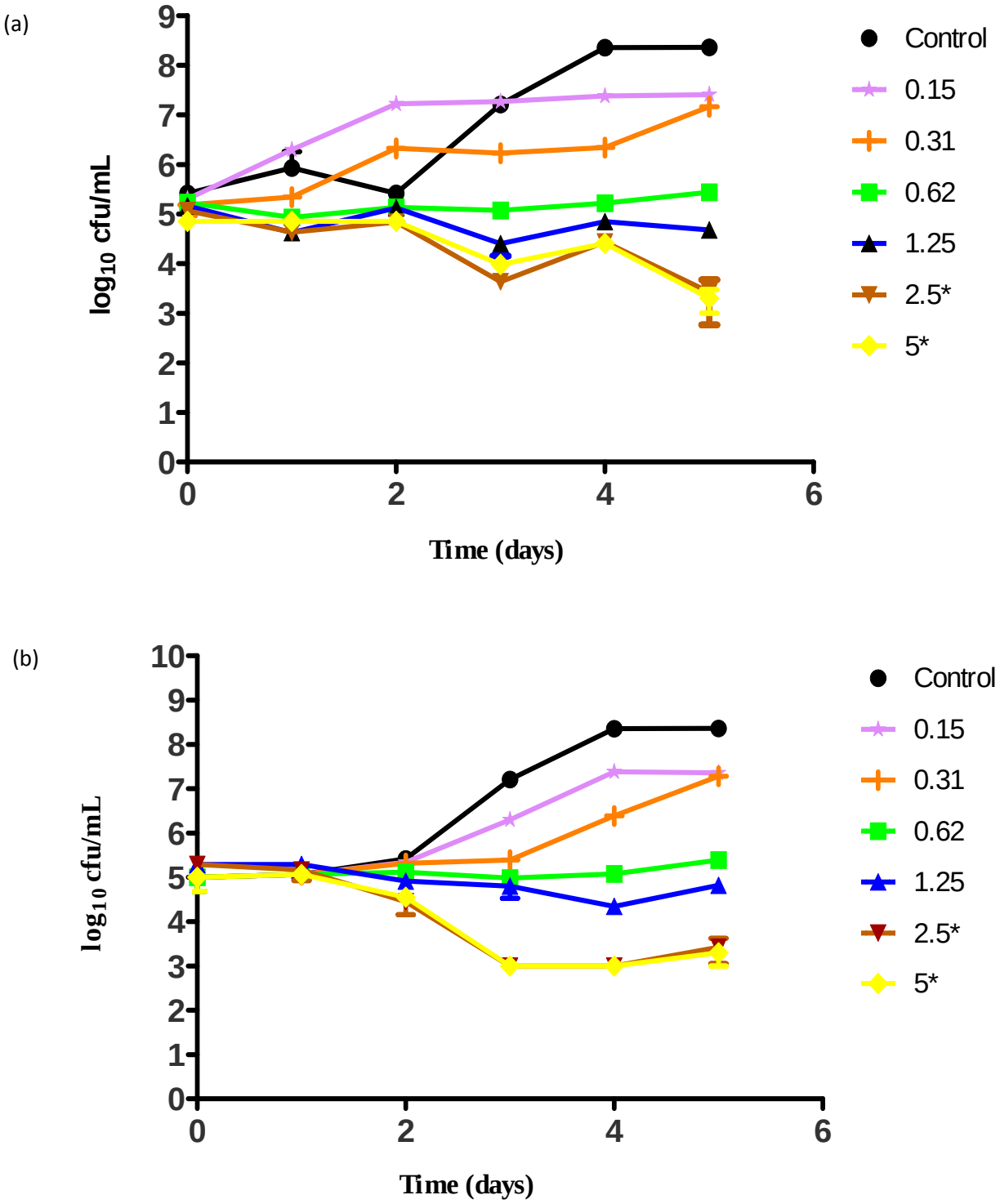


Figure 3.3: The bactericidal effect of clofazimine formulations on biofilm-producing *Mycobacterium smegmatis*. The MBCs and rates of killing of biofilm-producing *M. smegmatis* bacilli exposed to varying concentrations (0.15-5 mg/L) of (a) NC and (b) SDC over five days. The results are of three separate experiments performed in triplicate and are presented as the mean values ± SEM. Statistically significant differences are illustrated by (*), which represents $P<0.05$.

3.4 Effects of the clofazimine formulations on biofilm-encased *Mycobacterium smegmatis* bacilli

To determine the lethal activities of clofazimine against non-replicating *M. smegmatis* bacilli, the MBCs and time-kill kinetics were performed on organism encased in preformed *M. smegmatis* biofilm. The results are shown in Figure 3.4 and 3.5. No lethal activity of either clofazimine formulation against the non-replicating, biofilm-encased *M. smegmatis* bacilli was evident, indicating that these *M. smegmatis* sessile bacilli are either tolerant or inaccessible to clofazimine. Furthermore, it was also noted that both clofazimine formulations became colorless in the culture media during the five-day incubation period, compatible with oxygen depletion in the biofilm environment (Figure 3.4b).

(a)

(b)

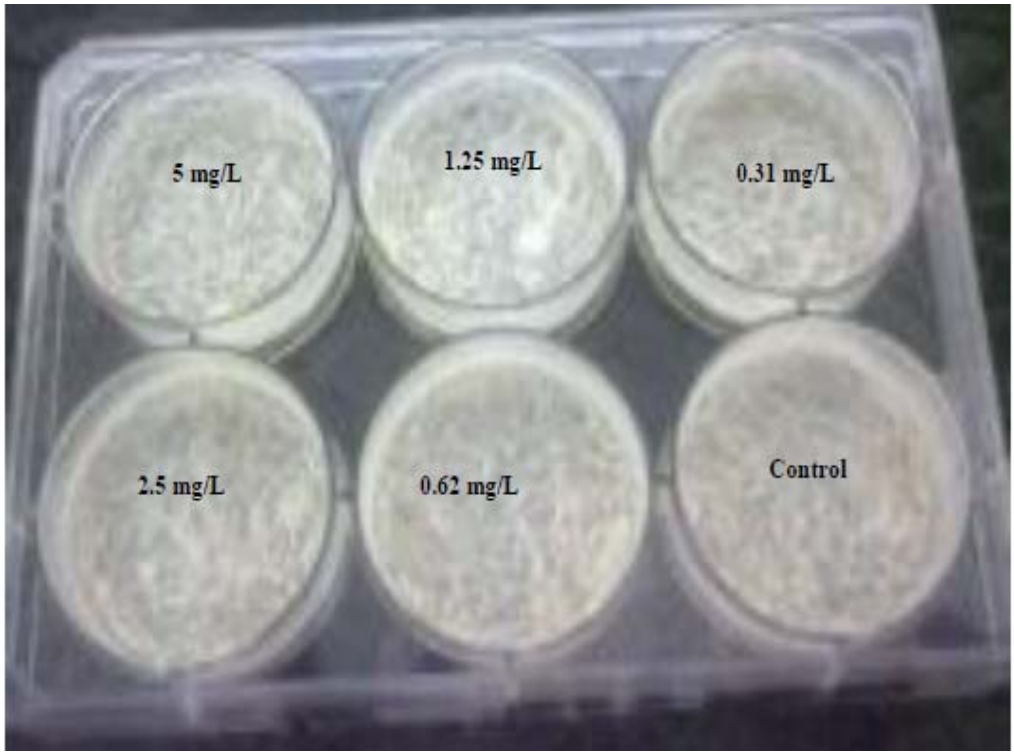
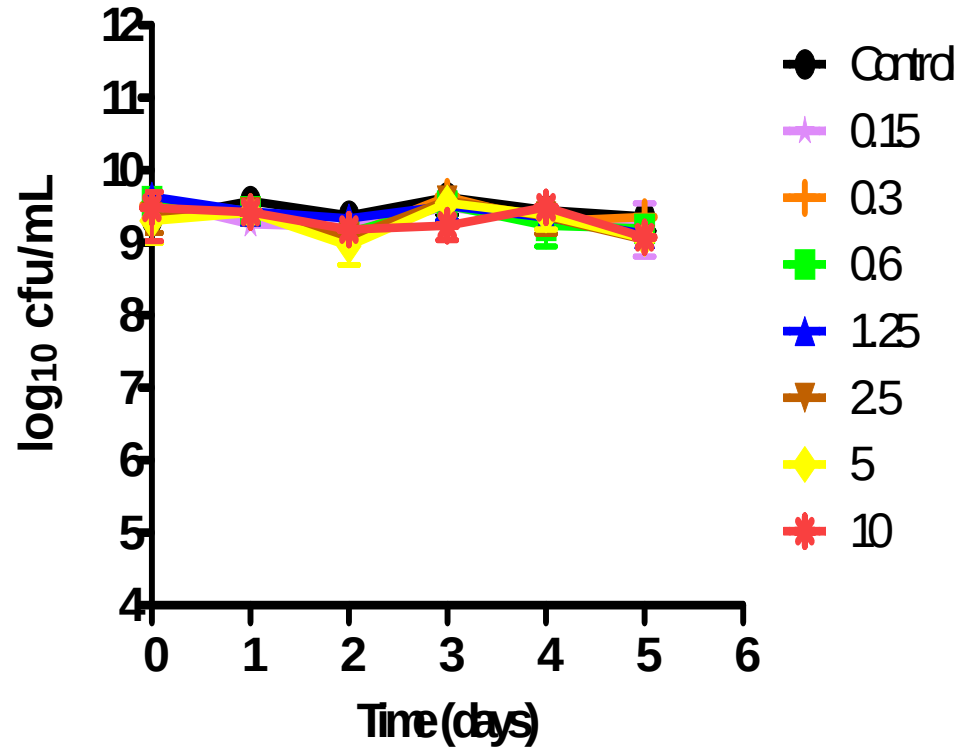


Figure 3.4: Treatment of *M. smegmatis* encased bacilli in preformed biofilm with clofazimine formulations on (a) day 0 and (b) day 5. Biofilm still remained intact at day 5 inside the wells, attaching itself to the walls of the plates even after it was injected with clofazimine. The original reddish-orange color of clofazimine turned colorless, which indicated that there was a reduction of clofazimine as the days progressed.

(a)



(b)

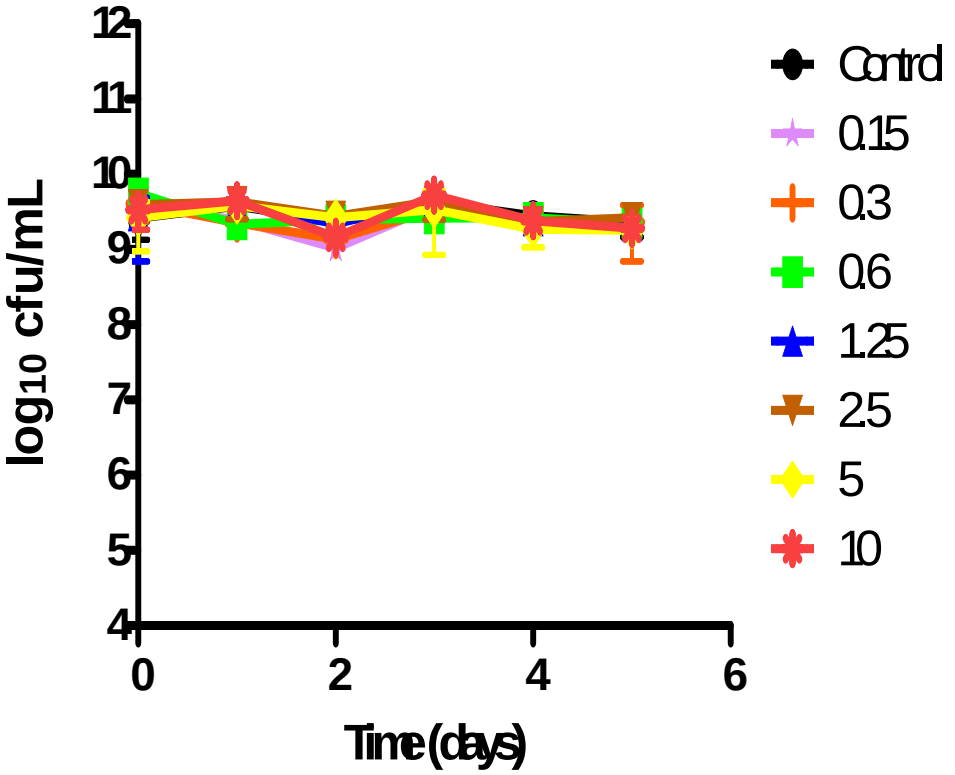


Figure 3.5: The effects of varying concentrations (0.15-10 mg/L) of (a) NC and (b) SDC on biofilm-encased sessile *M. smegmatis* populations. The results are of three separate experiments performed in triplicate and are presented as the mean values ± SEM.

3.5 Effects of clofazimine on *Mycobacterium smegmatis* biofilm formation

In order to determine the effects of clofazimine on the formation of biofilm by *M. smegmatis*, the bacteria were monitored over a five day incubation period. The results are as shown in Figure 3.6. Both formulations, exhibited a comparable dose-dependent inhibition of biofilm formation. The inhibitory activity was evident from 0.31 mg/L with no detectable biofilm from 1.25 to 5 mg/L.

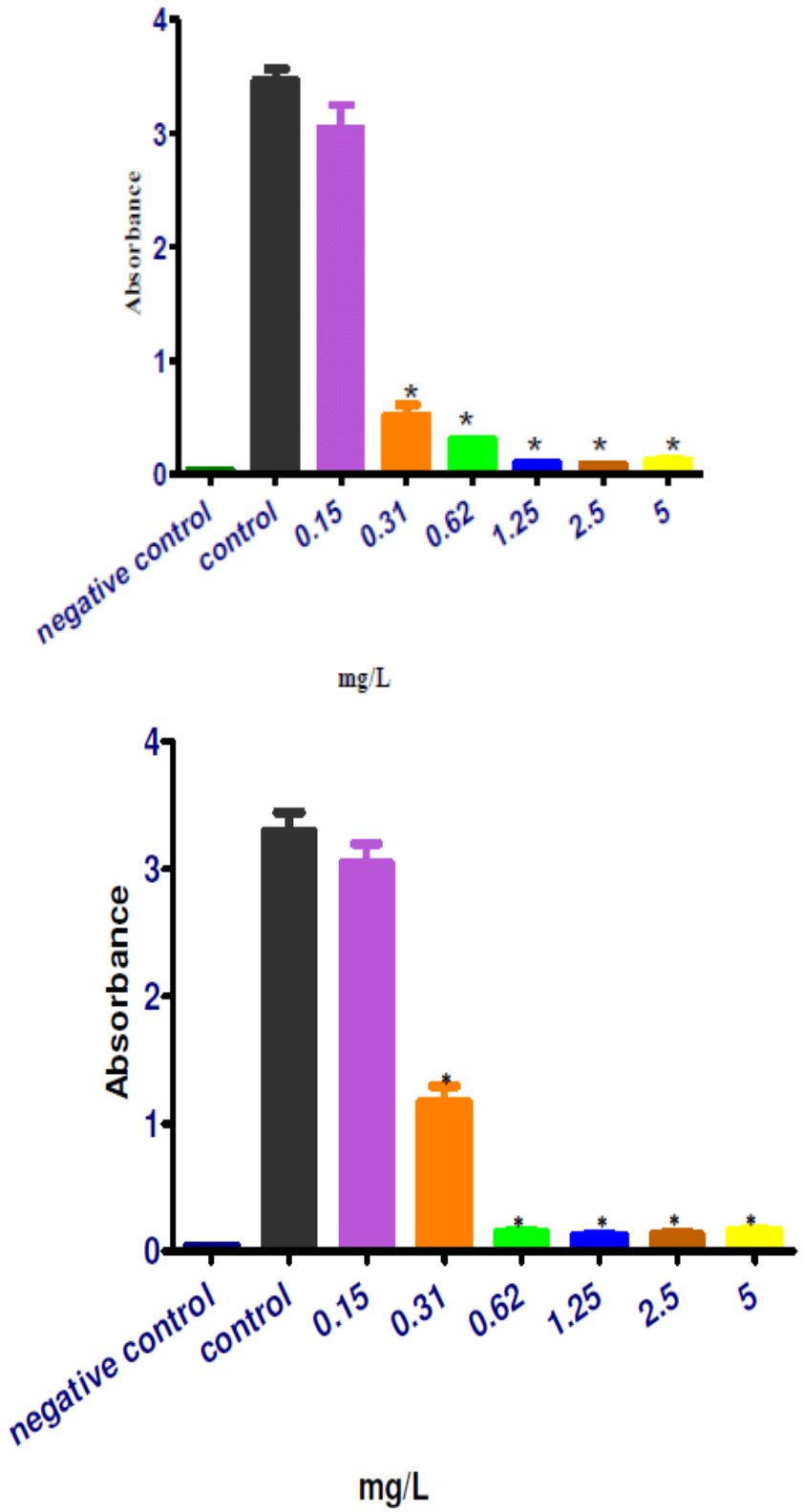


Figure 3.6: The effects of clofazimine on biofilm formation by *M. smegmatis*. The results are of three separate experiments performed in duplicate for each concentration of each preparation of the antibiotic, and are presented as the mean values ± SEM. Statistically significant differences are illustrated by (*), which represents $P<0.05$. The negative control is the absolute value for the growth medium without cells while the positive control is the absolute value for the drug-free control system as described in the Materials and Methods section.

3.6 Effects of clofazimine on the lipid composition of *Mycobacterium smegmatis* biofilm

To determine the effects of clofazimine on lipid composition of *M. smegmatis* biofilm, the FM constituents of the planktonic and biofilm-producing and biofilm-encased cultures were analysed. The quantities of the FM of *M. smegmatis* in each sample, were determined in comparison with those of *M. tuberculosis* standard, run on a separate TLC plate.

As NC and SDC at various concentrations had shown similar effects on FM production by planktonic, biofilm-producing and biofilm-encased cultures, only NC results are illustrated and are shown in Figure 3.7. The FM spots in these TLC plates have been encircled. By visually examining the plates, the quantities of FM in planktonic cultures were smaller than those of the biofilm cultures, by at least a 100-fold, as the spotted biofilm cultures have been diluted 100x. Although clofazimine seems to have inhibited the production of FM by planktonic cells, with the absence of visible spot clearly being shown at 2.5 mg/L, the drug has clearly inhibited the production of FM by biofilm-producing bacilli significantly, which was clearly visible at 0.3 mg/L (Figure 3.7c). This effect on FM production by biofilm-producing cells coincided with the effects of clofazimine on biofilm quantity of the biofilm-producing cultures, illustrated in Figure 3.6. However, clofazimine did not affect the quantity of FM from biofilm-encased *M. smegmatis* bacilli (Figure 3.7d).

Due to time-constraints, the effects of clofazimine on the absolute quantities of FM in these experimental settings could not be determined. However, the work will be extended in future to include *M. tuberculosis*.

Apart from FM there were two additional spots representing different lipids from FM in the planktonic cultures, which were non-responsive to clofazimine treatment.

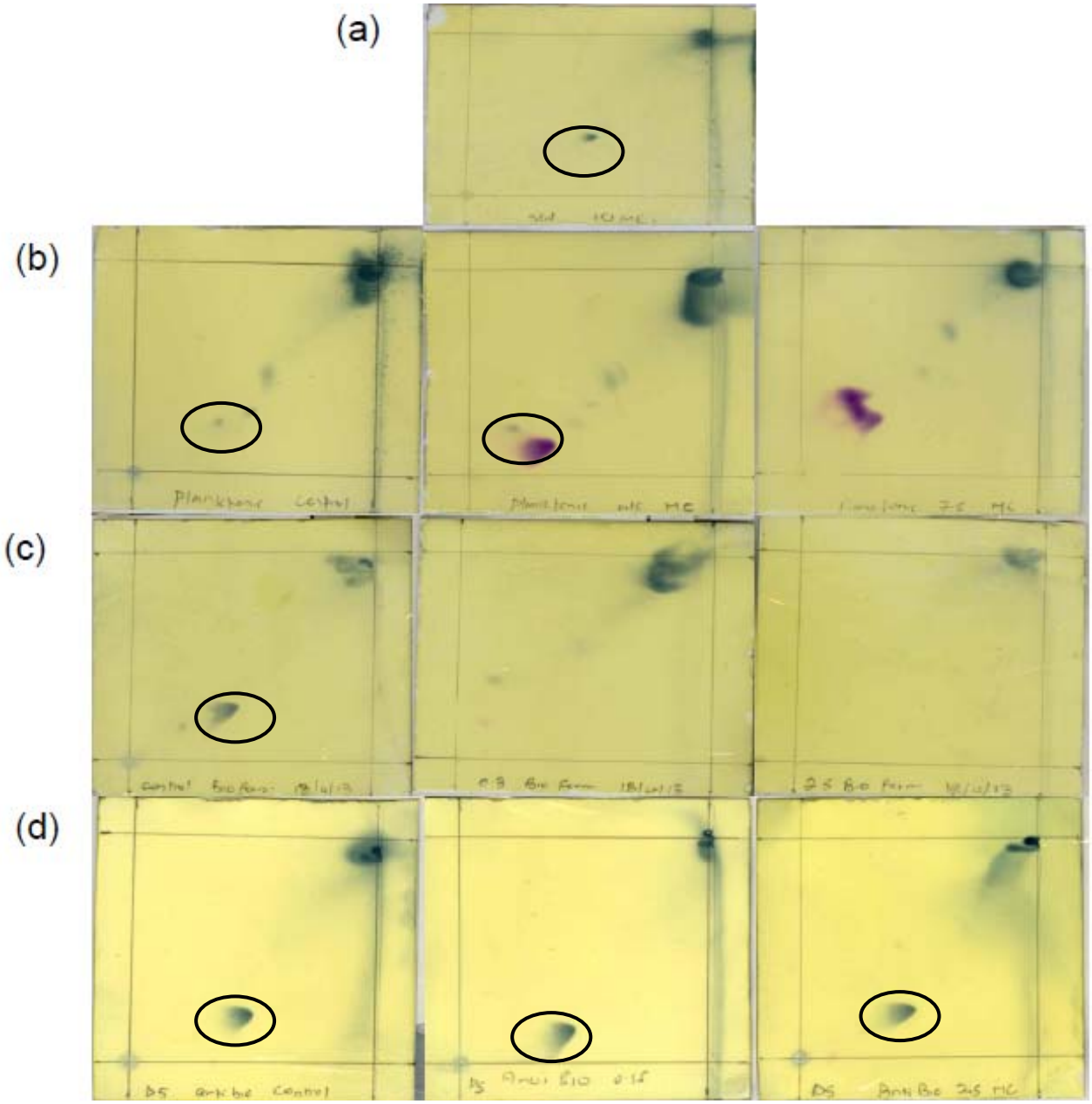


Figure 3.7: The analysis of the effects of clofazimine on FM production by planktonic and biofilm cultures *M. smegmatis*. The panels are: (a) 10 µL of *M. tuberculosis* FM standards, (b) from left to right, control and clofazimine (0.15 and 2.5 mg/L)-treated planktonic cultures (c) from left to right, the control, 0.3, and 2.5 mg/L of biofilm-producing bacilli (d) from left to right, the control, 0.15 and 2.5 mg/L of biofilm-encased bacilli. The spotted samples of the control and 0.15 mg/L of biofilm-producing cultures and all of those of the biofilm-encased organisms have been diluted 100-fold.

CHAPTER 4: DISCUSSION

The chemotherapy of human TB requires 6-9 months treatment with anti-TB drugs (Hopewell et al., 2006). One of the reasons for such a long period of treatment is the prevention of re-emergence of the disease, which is caused by the reactivation of surviving drug-tolerant, dormant and persistent bacilli (Ojha et al., 2008). The current anti-TB therapeutics work effectively during the first 14 days of treatment, by killing the actively-growing bacteria (Jindani et al., 2003; Sirgel et al., 2005), while the drug-tolerant bacilli remain and survive in the granuloma.

The dormant and persistent organisms develop as a result of unfavourable growth conditions, such as nutrient limitation and low oxygen tension, occurring in the granuloma, triggering their metabolic downshift. Their tolerance/resistance to antibiotics may possibly be due to either their altered metabolic state or inaccessibility of the bacteria to the potent antimicrobial agent by the protective granuloma membrane. These dormant *M. tuberculosis* bacilli are mostly associated with latent disease, which is responsible for the prevalence of TB in a third of the world's population. The dormant organisms may not cause any clinical disease as they are non-replicating, but may replicate when favourable conditions, such as immunosuppression, occurs in the host, resulting in the re-occurrence of the disease (Gengerbacher and Kaufmann, 2012).

To date, no anti-TB agent, that is able to eliminate these bacterial populations, has been included in the chemotherapy of TB. Although PZA is reported to be active against dormant *M. tuberculosis* bacilli, it has not contributed to the elimination of these bacilli from the patients' lesions. Several newly synthesized anti-TB agents with inhibitory activity on the non-replicating dormant bacilli, including the nitroimidazopyran PA-824, are being evaluated for their mechanisms of action (Stover et al., 2000) but their effects on the granuloma have not been determined.

Despite this information, an ideal agent, with the ability to act on the slow/non-replicating persistent/dormant bacilli, predominantly found in the granuloma, is still required. Based on the overlapping anatomical and physiological features of the granuloma and mycobacterial biofilm, in supporting persisters and dormant bacilli in a lipid-rich matrix, the response of these bacterial populations to a lipophilic anti-TB agent, using *M. smegmatis* biofilm as a surrogate environment, was studied, specifically clofazimine as native and spray-dried formulations. Clofazimine is a riminophenazine antibiotic, used previously for the treatment of TB, but was discontinued because of its poor pharmacokinetic and physiochemical properties (Barry et al., 1957). In the current study, it was investigated for its lethal activity on the slow/non-replicating bacilli in *M. smegmatis* biofilm. The potential of clofazimine, together with its nano-particle formulation (Verma et al., 2013), to

target the actively-growing, as well as the slow-growing and non-replicating *M. smegmatis* dorganisms in biofilm environments has then been investigated (Ojha et al., 2008). Both formulations have demonstrated comparable *in vitro* inhibitory and lethal activities, against *M. smegmatis*, illustrating that the spray-dried preparation has retained the antimycobacterial activity of native clofazimine.

The two formulations were found to exhibit bacteriostatic rather than bactericidal activities for planktonic *M. smegmatis* bacilli. Failure to establish MBC values in this setting may result from either the insensitivity of rapidly-growing bacilli, or insolubility of the antibiotic at high concentrations (>20 mg/L) in the culture medium.

In the case of slow-growing biofilm-producing bacilli, both formulations, however, exhibited bactericidal activity with an MBC value of 2.5 mg/L. In this experimental setting, they also caused dose-related inhibition of biofilm formation, via the interruption of FM synthesis, which achieved statistical significance at concentrations of ≥0.3 mg/L and was probably secondary to inhibition of bacterial growth. Activity against sessile, biofilm-producing, persistent bacterial pathogens is clearly a beneficial, albeit somewhat uncommon, property of antibiotics (Feldman and Anderson, 2012; Xu et al., 2012), and has recently been described for several cationic amphiphiles, such as antimicrobial peptides (AMP), in pre-clinical development (Beckloff et al., 2007; Findlay et al., 2010). Ideally, these agents should also target viable, biofilm-encased non-replicating organisms.

However, despite the impressive activities against slow-growing *M. smegmatis* bacilli, the clofazimine formulations were ineffective against the non-replicating organisms encased in a mature, pre-formed biofilm matrix, as well as FM synthesis in this environment. The lack of activity in this setting may possibly be as a result of one or more of the following: i) insensitivity of dormant bacilli to the antibiotic; ii) the impenetrable nature of the viscous *M. smegmatis* biofilm mass; and iii) inactivation/immobilisation of clofazimine by the biofilm environment, resulting in reduced activity of the antibiotics (Yano et al., 2011). In addition to the above possibilities, failure to inhibit FM synthesis may be due to the absence of microbial targets for clofazimine in the matured biofilm, which may be present during *M. smegmatis* biofilm formation. This statement may be supported by findings by Ojha and co-workers (2010), who have illustrated that the FM precursors, such as TDM and GroEL1, are abundant during the early stage of biofilm formation, but are exhausted as the biofilm matures at day 4 (Ojha et al., 2010), a day before biofilm was treated with clofazimine formulations in this study. Although these substrates have not been evaluated as targets for clofazimine activity, based on their findings (Ojha et al., 2005; 2010) the potential targets

for clofazimine may be substrates/enzymes involved in *M. smegmatis* biofilm formation, probably via the FM synthesis pathway.

Although failure to target intra-biofilm *M. smegmatis* could be considered as a limitation of clofazimine, it is noteworthy that the experimental design of the current study constituted an extremely stringent assessment which may not be representative of the clinical setting of pulmonary TB. *M. tuberculosis* not only grows at a slower rate than *M. smegmatis*, but is also more susceptible to clofazimine (Bopape et al., 2004; Cholo et al., 2006). In addition, the nano-particle formulation may be more effective than the native drug against *M. tuberculosis*, an effect which has been demonstrated in a murine model of experimental TB, in which it reduced the bacterial load in the lungs and spleen by $3\log_{10}$ in comparison to the NC (Verma et al., 2013). Clearly, the current study must be extended to include *M. tuberculosis*.

CHAPTER 5: CONCLUSION

- Native clofazimine and its nanoparticle preparation, are more active against slow-growing than rapidly-growing *M. smegmatis*, inhibiting biofilm formation, via FM synthesis, but fail to target the non-replicating bacilli encased in the biofilm mass.
- As a result, extension of these studies to *M. tuberculosis* is a priority due to differences in susceptibility profiles to clofazimine between *M. smegmatis* and *M. tuberculosis.*
- The antimycobacterial activity of these clofazimine formulations against sessile bacteria may be of benefit in anti-TB chemotherapy, by preventing the formation of granuloma and killing the slow-growing persisters, but this remain to be established.

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