Anti-tuberculosis drug design based on a possible mimicry between host and pathogen lipids

by

Selaelo Ivy Sebatjane

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(*Phil 4:13 & Joshua 1:9*)

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LIST OF ABBREVIATIONS

ABS	adult bovine serum
ACP	acyl carrier protein
AcpM	ACP mycobacteria
AFB	acid-fast bacilli
AIDS	acquired immunodeficiency syndrome
AmB	amphotericin B
APC	antigen prenting cells
BCG	Bacillus Calmette-Guerin
BuOH	butanol
CCD	Counter current distribution
CID	cholesterol targeting isoniazid derivatives
CO_2	carbon dioxide
$dddH_2O$	double distilled deionized water
DIM	phthiocerol dimycocerosate
DNA	deoxyribonucleic acid
DN-T	double negative T cells
DMSO	dimethyl sulphoxide
DOTS	directly observed therapy short course
ELISA	enzyme linked immunosorbent assay
EM	ethambutol
FAS	fatty acid synthase
FCS	fetal calf serum
GI	growth index
HIV	human immunodeficiency virus
^1H MNR	nuclear magnetic resonance
InhA	enoyl-ACP reductase
INH	isoniazid
KasA	β-ketoacyl-ACP synthase
KatG	catalase-peroxidase

LPS	lipopolysaccharides
L-AmB	liposomes-incorporated AmB
CD-AmB	cholesteryl sulphate colloidal dispersion AmB
MA	mycolic acids
MDR	multi-drug resistance
MIC	minimum inhibitory concentration
MNL	mononuclear leucocytes
NAD	nicotinamide adenine dinucleotide
N_2	nitrogen
NH_4	ammonia
NH_4OH	ammonium hydroxide
OH	hydroxyl group
O-SAP	*O*-stearylamylopectin
PAS	para-aminosalicylic acid
PC	phosphatidyl choline
POA	pyrazinoic acid
PZA	pyrazinamide
RES	reticuloendothelial system
RMP	rifampin
RNA	ribonucleic acid
SM	streptomycin
TB	tuberculosis
THF	tetrahydrofurane
WHO	World Health Organization

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CHAPTER 1

GENERAL INTRODUCTION

1.1 TB – The Global Problem

Today Tuberculosis (TB) is a greater global problem than it was at the beginning of the 20th century and has reemerged as one of the leading causes of death. According to the estimates by the World Health Organization (WHO), there were 8.4 million new TB infection cases in 1999 up from 8 million in 1997. This disease claims between 2 and 3 million lives a year worldwide. If present trends continue, 10.2 million new cases are expected by 2005 (Lall and Meyer, 2001). Even though TB is a worldwide problem, exceptionally high incidences occur in sub-Saharan Africa and south-east Asia and this is shown in figure 1.1 which, indicates the geographic distribution of this disease (http://www.sbri.org/Mission/disease/Tuberculosis.asp).

Figure 1.1. Geographic distribution of TB incidences worldwide (http://www.sbri.org/Mission/disease/Tuberculosis.asp).

A great influence in the rising of the global TB epidemic is HIV/TB co-infection and emergence of multi-drug resistant TB (MDR-TB). In 1994, about 10 million people

globally were estimated to be co-infected with HIV and *Mycobacterium tuberculosis*, of which 68% occurred in sub-Saharan Africa (O'Brein and Nunn, 2001; WHO, 2000).

1.2. TB - The Disease

Tuberculosis (TB) is a bacterial disease caused by *Mycobacterium tuberculosis* and usually affects the lungs (pulmonary tuberculosis). Other parts of the body can also be affected, for example lymph nodes, kidneys, bones, joints, (extra-pulmonary TB). The bacillus that causes TB is an airborne bacterium that is easily transmitted by inhalation. The bacilli are carried and dispersed into the air during coughing, sneezing and talking and can remain airborne and infectious for an extended period of time, typically several hours. Inhalation of small droplets containing not more than 2-3 bacilli propelled by an infectious person is sufficient for primary infection. Because the tubercle bacilli are spread by airborne route, many individuals may be put at risk by one person. People who are known to be infected with TB are placed in air-filtered isolation in an in-patient setting to break the chain of transmission. This type of isolation precaution presents a specific challenge in the home environment (Porche, 1999)

The most common clinical presentation of TB consists of a persistent cough that slowly progresses over weeks or months and that may be associated with blood stained sputum. Occasionally, there is a recurring dull, aching pain or tightness in the chest. Generalized symptoms of TB include fever, night sweat, fatigue and weight loss. Several methods are used to diagnose the disease. The most widely used methods include; direct microscopic examination of a smear sputum for acid-fast bacilli (AFB), chest x-ray commonly used to diagnose active TB, tuberculin or Mantoux skin test, nucleic acids amplification techniques (PCR) and serological test using ELISA (Al Zahrani, 2000; Todar, 2002)

1.3. *Mycobacterium tuberculosis*

Mycobacterium tuberculosis, one of several species of the genus *Mycobacterium*, is the etiologic agent of TB in humans. Other pathogens that belong to this genus are *Mycobacterium bovis,* associated with TB in cattle, *Mycobacterium avium* that causes TB-like diseases especially prevalent in AIDS patients, and *Mycobacterium leprae*, the causative agent of leprosy.

Mycobacterium tuberculosis is a fairly large non-motile rod-shaped bacterium of fairly small size (in the order of 0.5-1 micron). It is an obligate aerobe, which explains the fact that in classic case of TB, the bacterium is found in the well-aerated upper lobes of the lungs. The bacterium is a facultative intracellular pathogen, usually found in macrophages, and has a slow generation time of 24 hours or more, a physiological characteristic that can contribute to its virulence. In the original description of *Mycobacterium tuberculosis,* Koch noted that the bacteria formed braided microscopic bundles or distinctive serpentine cords; a morphology that was later called "cording". Robert Koch then associated cord factor with virulent stains of the bacterium. Further studies demonstrated an association between the virulence of an individual mycobacterial strain and the strength of its cording, indicating that the components of the cell envelope are responsible for both cording and virulence, but these components had not yet been identified (Bedino, 1999; Todar, 2002).

Mycobacterium tuberculosis is often referred to as "gram neutral" bacillus due to its resistance to decolourization by acid-alcohol solutions. Hence, laboratory identification of *Mycobacterium tuberculosis* is often based on its acid-fast staining characteristics. It was for this reason that *Mycobacterium tuberculosis* could not be identified prior to Robert Koch's discovery of a staining technique that allowed him to visualize the bacteria. The acid fast staining properties has been found to be based in the unique waxy cell wall, which is predominantly comprised of long-chain fatty acids called mycolic acids. These mycolic acids are unique and found in the entire genus of *Mycobacterium*, but do occur in other forms in species like *Corynebacteria* and *Nocardia* (Grange, 1988).

1.3.1. Cell Wall Structure

The cell envelope structure of *Mycobacterium tuberculosis* is unique among prokaryotes and provides a major determinant of virulence for the bacterium. The lipid bilayer cell membrane of Gram-positive bacteria is covered by a porous peptidoglycan layer, which does not exclude most antimicrobial agents. A gram-negative bacterium contains an outer membrane with an inner and outer leaflet outside the peptidoglycan layer. The outer leaflet contains low fluidity lipopolysaccharides (LPS) and the inner leaflet is of higher fluidity (Brennan and Nikaido, 1995). The LPS-containing outer membrane of Gram-negative bacteria serves as an efficient permeability barrier (Nikaido, 1994). These differences in cell wall structure and arrangement are shown in figure 1.2.B & C.

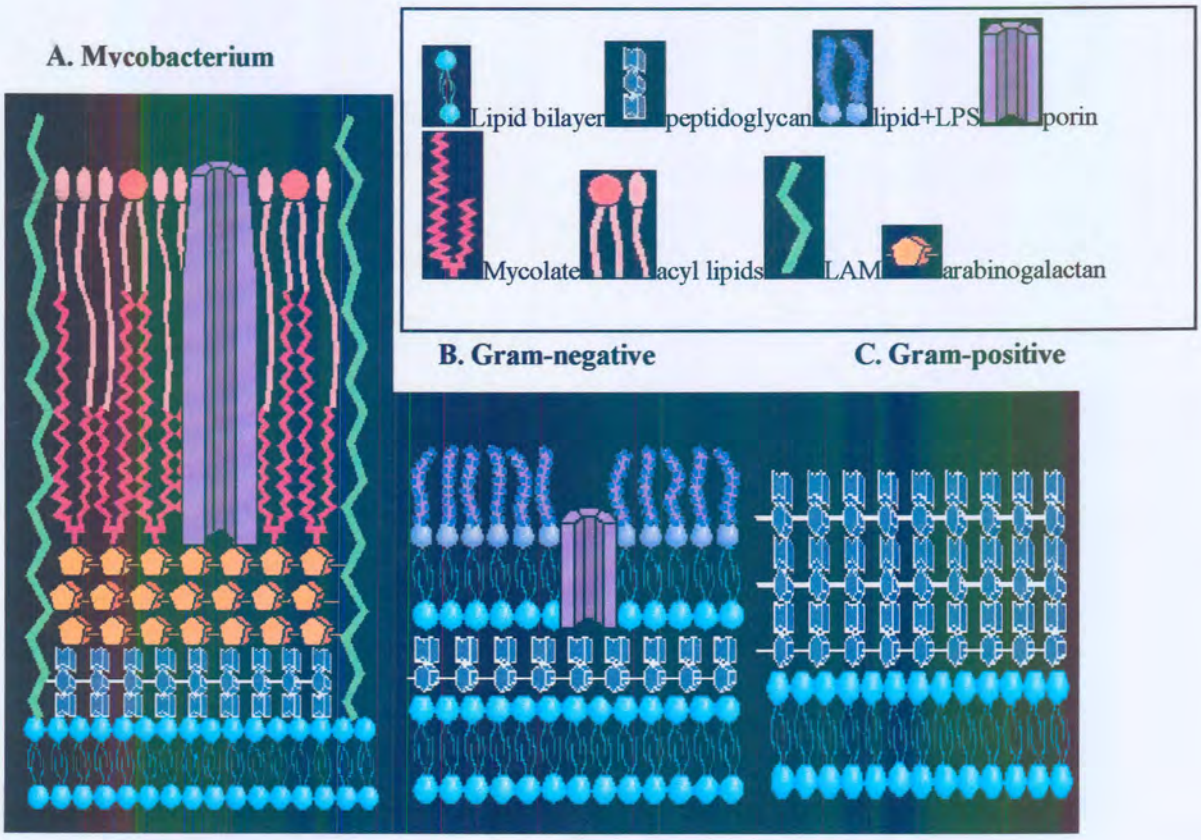


Figure 1.2. Cell wall of mycobacterium in comparison to that of gram-negative and gram-positive bacteria (http://web.uct.ac.za/depts/mmi/lsteyn/cellwall.htlm).

The cell envelope of *Mycobacterium tuberculosis* consists of a plasma membrane, a cell wall and an outer capsule. The cell wall complex contains peptidoglycan, and very high content of complex lipids accounting for over 60% of the cell wall mass. The lipids consist of a wide range of compounds, some being similar to those found in other organisms and others being unique to the genus of *Mycobacterium* (Grange, 1988; Todar, 2002).

Mycolic acids are unique alpha-branched lipids uniquely found in cell walls of *Mycobacterium*, *Nocardia* and *Corynebacterium* (Dubnau *et al.*, 2000; Yuan *et al.*, 1998). In the thick wax-like mycolate-rich region of the cell wall hundreds of mycolic acid residues are covalently linked to a common head group of arabinogalactan forming a permeability barrier of extremely low fluidity. An arabinogalactan is covalently linked to the underlying peptidoglycan structure as illustrated in figure 1.2.A (Nikaido, 1994). Hence, the mycolates are tightly packed in a compact and well-ordered inner-leaflet monolayer, which serves as an exceptionally efficient barrier that accounts for the strong hydrophobicity of the cell wall and the intrinsic resistance (Aínsa *et al.*, 2001; Draper, 1998). The outer leaflet is of higher fluidity and made up of other lipids (Dubnau *et al.*, 2000; and Yuan *et al.*, 1998).

1.3.2. Mycolic acid structure and their biological activities

Mycolic acid was isolated from a waxy extract of human tubercle bacillus (*Mycobacterium tuberculosis*) and its structure was elucidated in 1950 by Asselineau J. It was characterized as a β-hydroxy-α-alkyl branched fatty acid that could contain diverse functional groups such as methoxy, keto, epoxy esters and cyclopropane. Mycolic acids residues contain 70-90 carbons with the main carbon chain in each mycolic acid containing 50-60 carbons, whereas the α-branched side chain has a fixed length of 24 carbon atoms (Liu *et al.* 1995, 1996). Considering the fact that conventional fatty acids in animals contain between 16 and 20 carbons, the complexity of these lipids can be appreciated. The carbon chain lengths of mycolic acid are more characteristic of waxes

than of fats or oils. Hence, the mycolic acids were regarded as primarily responsible for producing a continuous outer waxy coat of the organism (Dubnau *et al.*, 2000; Ehlers, 1993).

Mycolic acids with no oxygenated functional groups in the α- or β- chain are called α-mycolic acids. They are synthesized in abundance by all mycobacterial species and may contain unsaturated bonds and cyclopropane rings that may either be in a cis or trans configuration. Other species of mycolic acids are oxygenated. These comprise the methoxy, keto, wax esters, ω-1 methoxy and epoxy mycolic acids that may all contain unsaturated bonds and a mixture of cis and trans cyclopropanes and are comparatively more restricted in distribution. The keto- and methoxy- mycolic acids are the major classes of oxygenated mycolates in *Mycobacterium tuberculosis*. Ketomycolates are the most widely distributed of the oxygenated mycolic acids and occur in various combinations with other oxygenated types. Methoxymycolates are more limited and occur mostly in slow-growing species, always in combination with ketomycolates (Barry III *et al.*, 1998; Yuan *et al.*, 1998a).

All members of the *Mycobacterium tuberculosis* complex (*Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis* and *Mycobacterium microti*) are pathogenic and synthesize the same combination of mycolic acids, i.e. α-mycolic acid ketomycolic and methoxymycolic acid. The attenuated vaccine strain, *M. bovis* BCG Pasteur, however, only produces α-mycolic and ketomycolic acid. *Mycobacterium tuberculosis* and *Mycobacterium leprae* have been shown to decorate their mycolic acid with unique cyclopropyl groups. Mutations that affect cyclopropanation of mycolic acid have been shown to render *Mycobacterium tuberculosis* unable both to cord and to infect in mice (Dubnau *et al.*, 2000; Yuan *et al.*, 1998).

Using spin label studies, Liu *et al.* (1995) verified the proposed asymmetric bilayer model of the mycobacterial cell wall where the mycolic acids containing inner leaflet has extremely low fluidity, while the moderately fluid outer leaflet is composed of lipids containing shorter chain fatty acids. The low fluidity inner leaflet is thus expected to

result from lipids containing longer hydrocarbon chains and fewer double bonds that tend to become tightly packed against each other. This low fluidity of the inner leaflet may thus account for the low permeability of the cell wall (Liu *et al.*, 1995; 1996). Mycolic acid is more than merely a wax. Several studies have shown that this molecule is antigenic. Mycolic acid has been shown to elicit specific antibodies (Pan *et al.*, 1999), and to induce CD4, 8-double negative (DN) T cells (Beckman *et al.*, 1994).

1.4. Anti-TB Therapy

1.4.1. History of TB treatment

Anti-TB therapy began way before the discovery of *Mycobacterium tuberculosis* as a causative agent of TB. Before the availability of anti-microbial drugs, the prescribed treatment was much rest in open air in specialized sanatoria. Patients were sent to cold, usually mountain climates amongst fir trees where they rested in the fresh, frosty air (Aínsa, *et al.*, 2001) and were provided with good nutrition. Improving social and sanitary conditions and ensuring adequate nutrition were all that could be done to strengthen the body's defense against the bacilli. Sanatoria, which were found at that time throughout Europe and US, provided a dual function: they isolated the sick and the source of infection from the general population, while the enforced rest, together with proper diet and the well-regulated hospital life assisted the healing processes.

The discovery of *Mycobacterium tuberculosis* as the causative agent of tuberculosis initiated the fight against humanity's deadliest enemy. One important development was provided by Calmette and Guerin, who used specific culture media to lower the virulence of the bovine TB bacterium, creating the basis for the BCG vaccine which is still in widespread use today (Warren, 2001).

Although the chemotherapy of infectious diseases with sulfonamides and penicillin had been underway for several years, these compounds were ineffective against *Mycobacterium tuberculosis*. Alternatives to penicillin were sought amongst antibiotic

producing moulds. A soil microbiologist, Selma Waksman, discovered the marked inhibitory effect of certain fungi, especially actinomycetes, on bacterial growth. Amongst the isolated antibiotics was an effective anti-TB antibiotic, actinomycin, which proved to be too toxic for use in humans and animals (Warren, 2001). Success came in 1943, when he isolated streptomycin from *Steptomyces griseus*, which showed maximal inhibition of *Mycobacterium tuberculosis* with very low toxicity. Streptomycin (SM) was administered for the first time to a critically ill TB patient who made a rapid discovery and had bacteria cleared from his sputum. This was a breakthrough, but resistant mutants began to appear in about 80% of the patients. This was overcome by combination therapy (Duncan, 2003; Grosset, 1995; Warren, 2001).

Combination therapy started with the use of para-aminosalicylic acis (PAS) and isoniazid (INH) when they became available in 1946 and 1952, respectively (Duncan, 2003; Sacchettini and Blanchard, 1995; Whitney and Wainberg, 2002). The idea was to treat patients with a combination of available drugs, because each drug was active against mutants resistant to other drugs; thus preventing the emergence of resistant tubercle bacilli. Since the goal was to prevent drug resistance and to sterilize the organs and tissue, 18 – 24 months of treatment was required when using a combination of INH + SM + PAS. This prolonged treatment time was changed when rifampicin (RMP) became available. RMP was effective not only against actively multiplying organisms but also against the resting or persisting organisms responsible for relapse after treatment was stopped. RMP in combination with INH and SM effected the cure of almost 100% of patients in treatment period of 9 months (Grosset, 1995; Van Loenhout-Rooyackers and Veen, 1998). Pyrazinamide (PZA) was discovered in 1954 but caused side effects in the dosages used, hence it was only introduced as a frontline drug in short course chemotherapy given in lower doses in the 1970's. It was shown to be bactericidal to the organism located in an acidic environment, especially inside the macrophages (Duncan, 2003; Grosset, 1995; Petrini and Hoffner, 1999).

Today TB is still treated with a cocktail of drugs; and the most commonly used are RMP, INH, PZA and SM or ethambutol (EM) with INH and RMP being the most potent drugs

(Rattan *et al.*, 1998). Other drugs of choice include quinolones (in multi-drug resistant cases), rifabutin (in RMP resistant) and rifapentine (a new analogue for RMP). The course of treatment is usually 6 – 9 months. When adherence to the regimen is assured this four-drug regimen is highly effective (Bedino, 1999; Grosset, 1995;Todar, 2002).

1.4.2. Isoniazid

Originally synthesized in 1912, Isoniazid (isonicotinic acid hydrazide, INH) was later found to be the most potent of a series of mycobactericidal compounds in the carboxylic acid hydrazide family (Heym and Cole, 1997; Slayden and Barry III, 2000; Wei *et al.*, 2003). As a result it has become a frontline antituberculosis therapy and is still the most widely used drug in tuberculosis regimens. INH demonstrates an amazing specificity and activity against the MTB complex (*M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*), with very low MICs ranging from 0.02 to 0.06 µg/ml for susceptible strians (Cocco *et al.*2000; Rattan *et al.*; 1998; Sacchettini and Blanchard, 1995; Whitney and Wainberg, 2002) and little or diminished activity against even closely related mycobacterial species including the opportunistic pathogens of *M. avium* complex and no activity against other unrelated bacteria (Barry III, 1997; Chen and Bishai 1998; Klopman *et al.*, 1996; Slayden and Barry III, 2000; Whitney and Wainberg, 2002).

INH is bactericidal (Sacchettini and Blanchard, 1995) for bacilli dividing intracellularly in the macrophages and extracellularly in the necrotic tissue. It also quickly reduces the size of populations of multiplying tubercle bacilli that abound in the periphery of the tuberculous cavity. Over the past decade the efficiency of this frontline regimen has been declining with the emergence of wide spread INH resistance (Barry III *et al.*, 2000; Phetsuksiri *et al*, 1999; Slayden and Barry III, 2000; Van Loenhout-Rooyackers and Veen, 1998).

INH is a prodrug that requires cellular activation by the endogenous mycobacterial enzyme catalase-peroxidase (katG) in a poorly understood chemical mechanism, before

exerting its toxic effect on the bacillus (Barry III *et al.*, 2000; Sacchettini and Blanchard, 1995; Wei *et al.*, 2003). The requirement for katG activation was discovered shortly after the introduction of INH in monotherapy. Resistance to the drug became clearly evident, with 50% of this resistance accompanied by deletions and mutations in the *katG* gene, encoding the catalase-peroxidase activity. Because of its selective toxicity for the tubercle bacilli, its mechanism of action needed to be known. Several hypotheses have been proposed to account for its mode of action and include (1) inhibition of mycolic acid synthesis; (2) inhibition of a catalase~peroxidase system causing INH biotransformation; (3) depletion of intracellular nicotinamide adenine dinucleotide (NAD), and (4) reaction of INH with tyrosine residues in the mycobacterial proteins. It is assumed that these modes of action do not necessarily exclude one another and that the antimycobacterial activity of INH may be due to their cumulative effect. However, the first two hypotheses are favoured by more recent studies (Chen and Bishai, 1998; Klopman *et al.*, 1996).

The toxicity of INH is potentiated by its interaction with the mycobacterial catalase-peroxidase (Rouse *et al.*, 1995); this results in production of reactive species (Vilchèze *et al.*, 2000; Whitney and Wainberg, 2002). This active form of INH is an inhibitor of enoyl-ACP reductase, which is responsible for the elongation of long chain fatty acids to mycolic acid. This is one of the ways in which INH exert its mycobactericidal activity (Sacchettini and Blanchard, 1995; Vilchèze *et al.*, 2000). The potential targets for INH thus far include KasA, a β-keto acyl synthase of Mtb, and InhA, the enoyl-ACP reductase of M. *smegmatis* which both appear to function in mycolic acid biosynthesis. Thus the exact sites and mechanism of action of INH for mycolic acid synthesis are varied and may be species specific (Barry III *et al.*, 2000; Phetsuksiri *et al.*, 1999; Slayden and Barry III, 2000).

1.4.3. Other Drugs

Other drugs used for the treatment of TB include rifampin and pyrazinamide, which are mainly used together with INH as the mainstay of anti-TB therapy. Others that can be

introduced during the course of treatment include ethambutol and streptomycin (Barry III *et al.,* 2000; Slayden and Barry III, 2000).

PZA was discovered in the early 50's and became a frontline drug in the 70's (Petrini and Hoffner, 1999). It is a bactericidal agent which only works in an acidic environment and shows excellent activity against intracellular organisms. As a result, PZA is especially important in the initial phase of treatment (Van Loenhout-Rooyackers and Veen, 1998). Like INH, PZA is thought to be a prodrug, which requires activation to the active pyrazinoic acid (POA) by the endogenous enzyme pyrazinamidase encoded by the gene *pncA* (Barry III, 1997).

Rifampin is a lipophilic (Heym and Cole, 1997) bactericidal agent, which is active against intracellular and extracellular bacilli, including persistent dormant bacilli during their short spurts of metabolic activity. It is the key component of current antimycobacterial therapy. This drug therefore is of great importance for sterilization of the organs and thus for the prevention of relapse. Sterilization requires a drug with the power to kill the slowest metabolizing bacillus from the lesions of infection. The introduction of this compound in TB treatment was instrumental in shortening therapy duration from 18 months to 9 months; hence acquired resistance to this drug significantly lengthens treatment duration. It acts by inhibiting the DNA-dependent RNA polymerase, causing protein synthesis of bacilli to halt (Barry III, 1997; Van Loenhout-Rooyackers and Veen, 1998).

Streptomycin belongs to a family of aminoglycosides and was the first antibiotis used for treatment of TB. This drug acts on the 30S subunit of the ribosmes (Heym and Cole, 1997). Other members are amikacin and kanamycin, drugs that block the synthesis of ribosomal RNA. These drugs are bactericidal in aerobic conditions. Streptomycin's bactericidal activity is limited to extra-cellular, actively multiplying mycobacteria e.g., in the cavities caused by infection.

Ethambutol influences the RNA-synthesis of the bacillus and inhibits mycolic acid to become part of the cell wall. It is both bactericidal and bacteriostatic depending on the dose being used but has no effect on the viability and metabolism of non-growing cells (Heym and Cole, 1997). EMB is also advocated in disseminated *M. avium* complex infections, particularly in HIV-infected persons (Rattan *et al.*, 1998). Other drugs include the fluoridated chinolones like ciproflaxacin and ofloxacin, which are bactericidal and inhibit the DNA gyrase of the bacilli. In case of multi-drug resistance (at least resistance to INH and rifampicin), chinolones are drugs of first choice. Others used are clofazimin, prothionamid, rifabutin, cycloserin, thioacetazone and *para*-aminosalicylic acid (Heym and Cole, 1997; Rattan *et al.* 1998; Van Loenhout-Rooyackers and Veen, 1998).

1.4.4. Directly Observed Therapy Short Course (DOTS)

In the 60's and 70's, effective use of antibiotics lead to a significant decrease in TB cases in industrialized countries. Due to inadequate control practices, the cases of TB began to rise. Hence it was slowly realized that provision of effective TB control required an adequately functioning health infrastructure that could support TB diagnosis and treatment services. A strategy called DOTS (Directly Observed Therapy Short Course) was developed by the WHO, aiming mainly at detection and cure of TB. DOTS combines five essential components: (1) Microscopic examination of sputum for acid-fast bacilli (AFB), (2) use of highly effective treatment regimens with direct observation of treatment, (3) reliable diagnostic and drug supplies, (4) monitoring systems and reporting of treatment outcomes, and (5) political commitment from responsible governments. This strategy has been raked as one of the "most cost-effective global interventions" available today (O'Brien and Nunn, 2000; WHO 2000).

In 1991, WHO expressed two global aims for TB control: Curing 85% of newly diagnosed patients and diagnosis of 70% of new patients with infectious TB. This was achieved in several countries where about 95% of cure rates were produced by DOTS. However, DOTS implementation in most of the 22 countries that contain 80% of the

world's burden of TB has been slow to take on. The reasons for this are diverse, but of paramount importance is the lack of commitment by governments and donor agencies in affected countries (O'Brien and Nunn, 2000).

Some factors hindering the goals of DOTS include inadequate tools for diagnosis and ineffective treatment regimens. The AFB microscopy used in DOTS detects only those patients with advanced pulmonary TB and is time consuming. The treatment regimens used are such that a patient uses a combination of drugs, and is required to take them all at once. It was recommended that treatment be directly observed by a health care provider, especially during the first 2 months of treatment and whenever rifampin is being used. Hence the course of treatment is more likely to be completed outside hospital, where there is less supervision. This can easily lead to non-compliance of patients to medications, resulting in increased mortality and drug resistant strains (O'Brien and Nunn, 2000).

1.4.5. Multi Drug Resistant-TB (MDR-TB)

The problem of drug resistance in the treatment of TB is almost as old as the first anti-TB drug. The first cases of drug resistance were reported after the use of streptomycin monotherapy. Within 3 months of treatment, about 80% of bacterial isolates from patients were found to be streptomycin resistant (Petrini and Hoffner, 1999).

The use of a single, sub-optimal concentration of drugs and too short treatment periods allow the TB bacilli to be exposed to sub-lethal concentrations of the anti-mycobacterial compounds, which allows outgrowth of the most resistant bacteria population. Although combination therapy can avoid drug resistance, the failure of patients to complete therapy as well as poorly functioning TB control programmes have lead to emergence and distribution of *Mycobacterium tuberculosis* strains resistant to more that one anti-TB drug (MDR-TB). In such cases, a combination of "second-line" drugs, which are not only more expensive but also more toxic and less effective, are used. As such, MDR-TB is

considered a man-made phenomenon and its spread is a threat to the existing TB control programmes throughout the world. MDR-TB strains resistant to up to 9 different agents have been reported. Hence, mortality is high in MDR-TB, often because of concomitant factors such as HIV (Barry III, 1997; O'Brien and Nunn 2000; Petrini and Hoffner, 1999; Slayden and Barry III, 2000).

1.4.6. TB and HIV/AIDS treatment

HIV exerts a profound influence on the course of *Mycobacterium tuberculosis* infection. TB is one of the opportunistic diseases that usually accompany HIV infection as a result of co-infection, causing early deaths (Orme, 2001).

For HIV-infected patients with pulmonary TB the same treatment schedules apply as for patients without HIV infection, but there are several pharmacokinetic issues that can complicate the treatment, including the possibility of malabsorption of anti-TB drugs with HIV-related eteropathy, the complex drug-drug interactions between anti-retroviral drugs and other anti-TB drugs and an increased risk for adverse side-effects. Hence, during the treatment blood levels of drugs must be controlled and, in cases where treatment proves to be of decreased efficiency, increased duration of treatment is recommended (Van Loenhout-Rooyackers and Veen, 1998). Moreover several studies have shown that rifampin lowers the serum concentration of the therapeutic anti-HIV protease inhibitors, thereby contributing to the resistance to these drugs. Anti-retroviral drugs in turn increase the level of free rifampin, thereby inducing the risk of toxicity. Hence, in the case where protease inhibitors are prescribed, rifampin is usually replaced with rifabutin (Burman and Jones, 2001; Hannan *et al.*, 2000; Van Loenhout-Rooyackers and Veen, 1998).

1.4.7. Challenges in the development of future chemotherapy

Despite the availability of current treatment regimens there is still a great need for the development of new anti-mycobacterial compounds (Coleman *et al.*, 1999; David *et al.*, 2001). Finding new drugs represent a challenge (Orme, 2001) since the aim is to eliminate problems associated with treating TB. Hence new compounds must have maximal anti-bacterial activity coupled with low-toxicity (Coleman, 1999; 2000) and hopefully act by mechanisms different from those of existing drugs (Baohong, 1998). Other requirements for the new compounds include the development of faster-acting drugs to shorten the duration of treatment regimens (Orme, 2001; Van Loenhout-Rooyackers and Veen, 1998) in order to encourage patient compliance and slow down the development of drug resistance (Barry III, 1997).

In addition to this, the new compounds must be effective against the growing number of drug-resistant strains. For this purpose, they should target tubercle bacilli that are at different levels of metabolic activities, specifically latent bacilli, to prevent development of active disease through re-activation (Barry III, 1997; Orme, 2001; Van Loenhout-Rooyackers and Veen, 1998). Thus treatment must be both bactericidal and sterilizing (Van Loenhout-Rooyackers and Veen, 1998).

1.5. Host Pathogen interaction

M. tuberculosis is an intracellular pathogen that can infect and survive within host cells. A key to the pathogenic potential of mycobacteria lies in their capacity to resist destruction by macrophages as well as by being able to survive within macrophages for a long period of time without being lethal to the infected host. Mycobacteria enter the macrophages via a variety of receptors including complement-, mannose-, Fc- and scavenger receptors, ending up in the so-called mycobacterial phagosomes wherein they are protected from degradation by lysosomes. Several studies showed that this route is only applicable to live mycobacteria, because killed mycobacteria are rapidly exposed to the killing agents residing in lysosomes (Bermudez and Sangari, 2001; Pieters and Gatfield, 2002; Pieters, 2001).

1.5.1. Role of cholesterol in mycobacterial entry and survival

Early studies showed that cholesterol and cholesterol ester levels in macrophages infected with mycobacteria are much higher when compared to non-infected cells both *in vitro* and *in vivo*. Kondo and Kanai, (1976a) also showed that coating mycobacteria with cholesteryl oleate, with or without phthiocerol dimycocerosate (DIM), had an infection-promoting effect resulting in longer persistence of avirulent bacilli, and less sensitivity to the protective mechanism generated by BCG immunized mice against virulent bacilli (Kondo and Kanai, 1976a, 1976b). This suggested a role of cholesterol in the pathogenicity of *Mycobacteria*. Gatfield and Pieters (2000) performed a study that showed that the entry of *M. tuberculosis* into the macrophages depends on the presence of cholesterol. Using cholesterol specific dyes, it was observed that cholesterol accumulates at the site of mycobacterial uptake. Cholesterol depleted macrophages were unable to internalize mycobacteria. This inhibition was specific to mycobacteria, since other parasitic microorganisms such as *Salmonella typhimurium*; *Yersinia pseudotuberculosis*, *Escherichia coli* and *Lactobacillus casei* were able to enter cholesterol-depleted macrophages. The reasons for this are still obscure, but one possible explanation could be that the extremely glycolipid-rich mycobacterial cell wall contains components that interact directly with cholesterol. Supporting this possibility is the finding that mycobacteria bind to cholesterol with high affinity, indicating the presence of cholesterol-binding sites at the mycobacterial cell surface (Pieters, 2001; Pieters and Gatfield, 2002). In addition to this, the cholesterol association may determine the subsequent intracellular events, since cholesterol mediates the phagesomal association of the tryptophan aspartate-containing coat protein (TACO), which prevents the maturation of the phagosome to a phagolysosome (Aínsa et al., 2001; Ferrari *et al.*, 1999).

1.5.2. Role of oxygenated mycolic acids in the virulence of M. tuberculosis

The components of the mycobacterial cell wall have been proposed to play a role in the virulence and pathogenesis of this bacterium. Dubnau *et al.* (2000) provided evidence that the oxygenated mycolic acids of *M. tuberculosis* are indeed required for the virulence

of this pathogen. Using a mutant strain of *M. tuberculosis* that didn't synthesize oxygenated mycolic acids, these authors showed that the mutant was attenuated in mice, implying that the oxygenated mycolic acids are important for the pathogenicity of the bacilli.

1.5.3. Anti-mycolic acid antibodies recognize oxygenated mycolic acid

It has been shown by Beckman *et al.* (1994) that in human peripheral blood cells, mycolic acid is presented by antigen-presenting cells (APC) through a mechanism that doesn't involve MHC-class I or II molecules. Instead, mycolic acid is presented on CD1 on APC, which then stimulate CD4- and CD8- double negative T cells (Barry III *et al.*, 1998; Beckman *et al.* 1994). It could well be that antibodies to mycolic acid come about with DN-T cell help to B cells. In a study done by Pan *et al.* (1999) the antigenicity of mycolic acid was demonstrated. Their results indicated that TB patient antibody specificity against cord factor resided in the mycolic acid structure, specifically oxygenated mycolic acid. Among the three subclasses of mycolic acid that make up the *Mycobacterium tuberculosis* cord factor, TB patients' sera reacted most prominently against methoxy mycolic acid (Pan *et al.*, 1999).

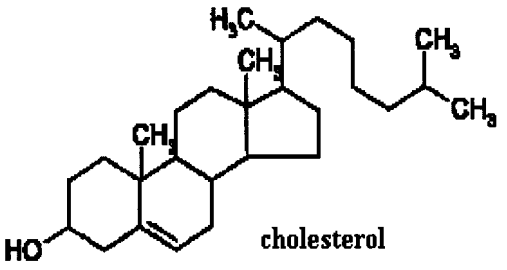
1.5.4. Possible structural mimicry between cholesterol and mycolic acid

A structural mimicry between cholesterol and mycolic acid was proposed based on the outcome of the study by Siko (2002). In this study antibodies to mycolic acids were characterized using a resonant mirror biosensor. The binding properties of serum antibodies from a TB positive a patient serum was analyzed on a biosensor cuvette surface coated with liposomes containing either cholesterol or mycolic acids. The results showed that antibodies bound equally well to both surfaces, implying that there might be a cross-reactivity of binding of antibodies or serum components to cholesterol and mycolic acid. ELISA of patient serum on wells coated with either cholesterol or mycolic acids was used to confirm that the cross-reactive binding agent was immunoglobulin of

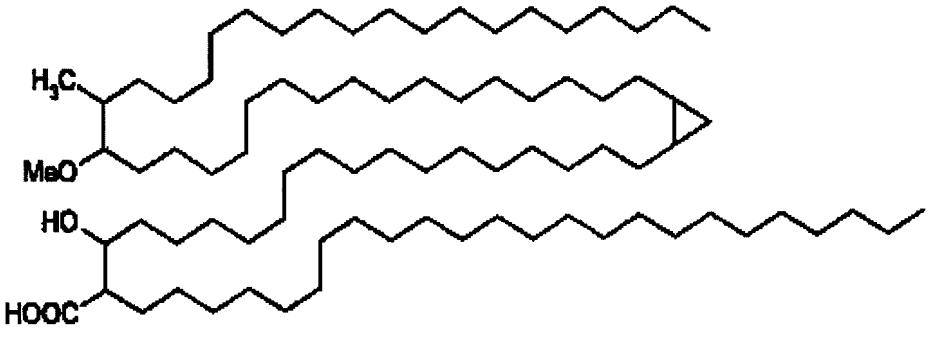
nature. These results might be interpreted as an indication that cholesterol and mycolic acid are recognized by the same antibodies, thereby pointing to a possible mimicry between cholesterol and mycolic acid.

Chemical structures of the two oxygenated mycolic acid species, the keto- and methoxy-mycolic acid) were drawn to illustrate the feasibility of a folding parttern that may mimic the structural features of cholesterol. In its folded structure with all the oxygenated groups clustered on one side of the molecule and a hairpin bend induced at the cyclopropane moiety of the long hydrocarbon chain of mycolic acid, a mimicry to the cholesterol structure appeared likely (Fig. 1.3) with the methoxy group of mycolic acid corresponding to the hydroxyl position of cholesterol. This model corroborates the results of Pan *et al.* (1999), who showed that TB patients have antibodies that recognized specifically the methoxy mycolic acid.

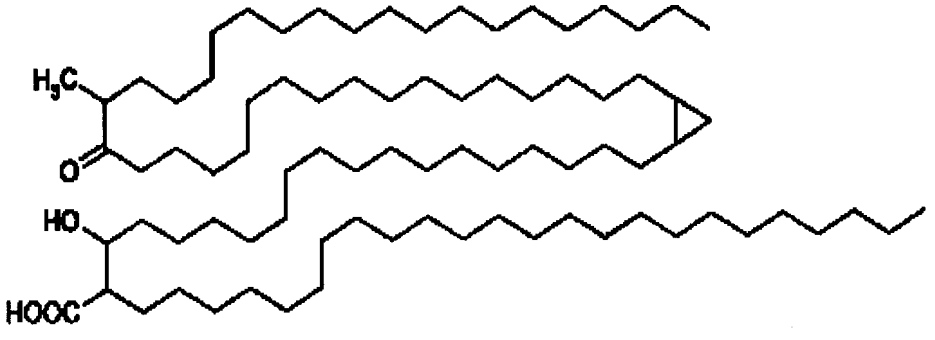
A result of the molecular mimicry between the hydrophobic mycolic acid and cholesterol will be a binding affinity of one to the other. This was indeed demonstrated by Siko (2002) in real time binding studies on the biosensor.



cholesterol



methoxy mycolic acids



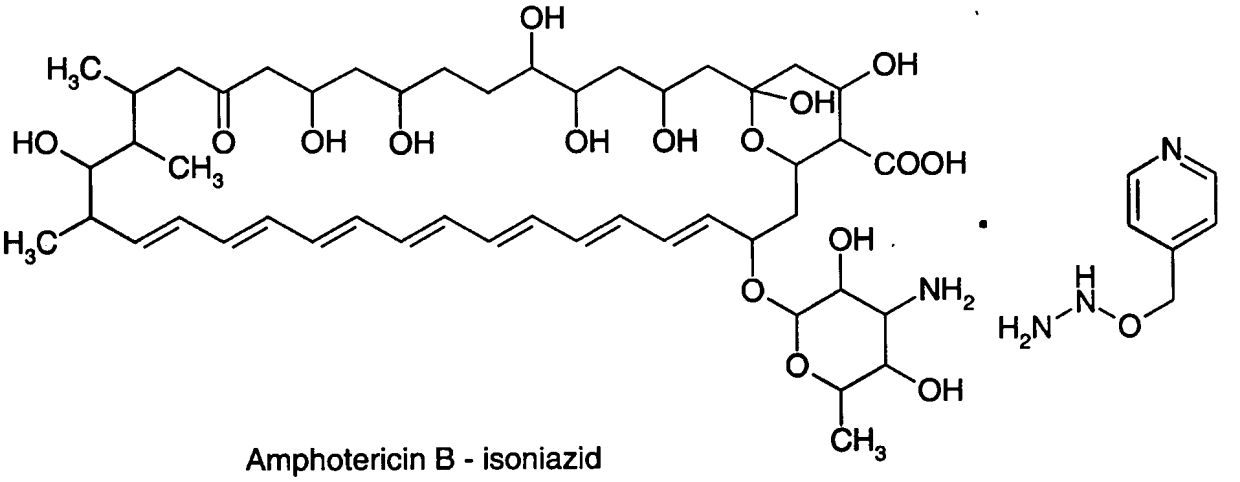
keto mycolic acids

Figure 1.3. **Structures representing the proposed mimicry between the oxygenated mycolic acid and cholesterol** (Siko, 2002).

1.6. The concept of Cholesterol Targeting Mycobactericidal Drug design

The proposed structural mimicry between cholesterol and mycolic acid may be used in the design and development of anti-TB compounds. Cholesterol Targeting Isoniazid Derivatives (CID) were proposed that combine a cholesterol binding molecule, amphotericin B (AmB) to an anti-mycobacterial drug, INH as non-covalent conjugate or as a Schiff base (Fig. 1.4). For this study the non-covalent conjugate (Fig. 1.4.A) was synthesized and tested.

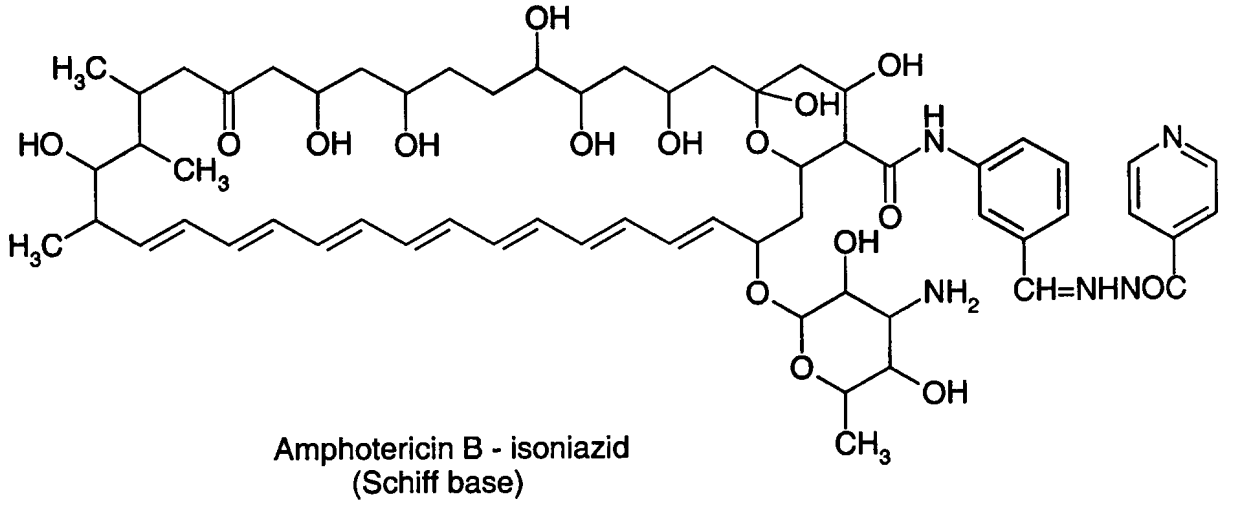
A



Amphotericin B - isoniazid

(Non-covalent)

B



Amphotericin B - isoniazid
(Schiff base)

Figure 1.4. **Two proposed structures of cholesterol targeting isoniazid derivatives (CID).** Amphotericin B and INH were combined as (**A**) a non-covalent conjugate or (**B**) a Schiff base.

1.7. AIMS of the study

The previously proposed MA-cholesterol mimicry may be exploited by specifically targeting the mycolic acid on the bacillus cell wall with cholesterol-binding drugs. If the cholesterol-binding drug can be linked to a mycobactericidal drug such as INH, then it may improve the efficiency of the latter. Alternatively it may be that the cholesterol-binding drug does not interact directly with the mycolic acids but instead it requires the MA-cholesterol surface complex. The proposed molecular mimicry between cholesterol and mycolic acids predicts that mycobacteria will accumulate cholesterol from their physiological surroundings in the body. Hence introduction of cholesterol *in vitro* may provide a model to investigate such a possibility and also address the question of whether the presence of cholesterol in the growth media will have an effect on the efficiency of INH since this is the toxophore to be targeted to the MA-cholesterol surface complex by AmB.

This study aimed to determine if there is any advantage in a cholesterol-targeting drug that has AmB as haptophore to bind to mycolic acids or cholesterol and INH as toxophore to kill *Mycobacterium tuberculosis*, either in the absence or presence of cholesterol.

CHAPTER 2

PURIFICATION OF ISONIAZID-AMPHOTERICIN-B NON-COVALENT CONJUGATE AND ITS ACTIVITY AGAINST *MYCOBACTERIUM TUBERCULOSIS*

2.1. Introduction

There is an urgent need for more effective anti-TB agents. It has been recommended that further research on existing drugs, especially hydrophilic ones like INH, should be directed towards enhancement of drug permeability rather than towards modifications of the structure of the active functionality. This could be achieved by using hydrophobic or amphiphatic prodrugs or carriers with higher permeability that would release the drug at its target site (Klopman *et al.*, 1996). The hypothetic cholesterol mimicry of mycolic acids in the cell wall of *M. tuberculosis*, lead to the suggestion that amphotericin-B (AmB) could be coupled to INH to investigate whether this would improve the efficiency of the latter, by specifically targeting it to the mycobacterial cell wall or to the cholesterol-rich cell membrane of the infected macrophage.

Amphotericin B is a polyene macrolide antibiotic (Gaboriau *et al.*, 1997; Konopka *et al.*, 1999) that has been extensively used in the treatment of systemic fungal infections including those that often affect immune-compromised patients (Baginski *et al.*, 2001; Barwicz and Tancrède, 1997; Charbonneau *et al.*, 2001). Despite its anti-fungal activity, AmB doesn't have any anti-bacterial activity (Jackson et al., 2000). Its toxicity is mainly due to its high affinity for sterols, with greater affinity for ergosterol-containing membranes of fungi as compared to the cholesterol rich mammalian cell membranes. Even though the structural differences between the two sterols are minute, AmB has been shown to interact more strongly with ergosterol than with cholesterol (Baginski *et al.*, 2001; Charbonneau *et al.*, 2001; Hartsel and Bolard, 1996). The interaction of AmB with sterols occurs through hydrophobic interactions and Van der Waals forces, which are

stronger and more favourable with ergosterol (Charbonneau *et al.*, 2001; Brajburg *et al.*, 1990).

AmB interaction with sterols results in production of aqueous pores/ion-permeable channels (Matsuoka and Murata, 2002) consisting of eight AmB molecules linked hydrophobically to the membrane sterols. This configuration gives rise to a pore or an ion channel in which the polyene OH-residues face inwards, leading to altered permeability and leakage of vital cytoplasmic components that eventually leads to cell death (Baginski *et al.*, 2001; Ghannoum and Rice, 1999). Using UV-V spectroscopy and differential scanning calorimetry, Charbonneau *et al.*, (2001) showed that AmB interacts with several types of sterol molecules including the principal oxidation product of cholesterol, 7-ketocholesterol and, with even stronger affinity than for ergosterol, a cholesterol precursor, 7-dehydrocholesterol (Charbonneau *et al.*, 2001).

Isoniazid is an inexpensive, relatively safe drug that continues to be well suited for the treatment of TB. Its mechanism of mycobactericidal action as well as the mechanism whereby it confers resistance to its bactericidal effects on mycobacteria is complex and not completely understood. However, several studies suggest that INH inhibits the biosynthesis of mycolic acids, thereby making the bacterium susceptible to reactive oxygen species and other hostile factors unleashed on it by the immune system of the host (Cocco *et al.*, 1999). INH shows specific activity against *Mycobacterium tuberculosis* (Barry III, 1997; Chen and Bishai 1998; Cocco *et al.*, 1999; Klopman *et al.*, 1996; and Slayden and Barry III, 2000).

Several attempts have been made to include the antimycobacterial pharmacophore moiety of INH in several molecules to improve the activity against *Mycobacterium*, reduce toxicity and overcome resistance to the drug (Cocco *et al.*, 1999). A number of INH-variants have been studied and shown to be of lesser toxicity to the host while retaining their mycobactericidal activity. Bavin *et al.* (1955) showed that the *o*-hydroxy-benzal isonicotinylhydrazone (salizid) derivative indicated in Fig. 2.1.A showed low toxicity in mice and high activity against *Mycobacterium tuberculosis* H37Rv. This was a follow-

up to the work done by Hart *et al.* (1954) who used the same compound under the name N-isonicotinyl-N′-(salicylidene) hydrazine (salizid), which also showed the ability to inhibit *Mycobacterium tuberculosis* H37Rv, with only slight inhibitory effect on other organisms. Another early example of an INH derivative was its metal ion complex, used to improve its efficiency. Voyatzakis *et al.* (1968) studied the activity of Cu-isoniazid complex (Fig 2.1.B), which was shown to increase the activity of INH *in vitro* (Voyatzakis et al. 1968). Another example of INH derivatives are compounds that are obtained by reacting INH with a *para*-sustituted benzaldehyde. One example of such compounds is pasiniazid (Fig 2.3.C), which is an INH-aminosalicylic acid conjugate (Derivatives of Isonicotinic acid hydrazide, Patent No. 737,271; The London patent office). Using combinatorial chemistry techniques, Cocco *et al.*, (1999) synthesized a series of isonicotinoylhydrazones and tested these against *Mycobacteria*, Gram-positive and Gram-negative bacteria. Several compounds showed good activity against *M. tuberculosis* H37Rv, some of which were even moderately active against a clinical isolate of *M. tuberculosis* resistant to INH. Aminohydrazone derivatives, structurally correlated to INH, were synthesized by combinatorial chemistry techniques and evaluated for *in vitro* toxicity and activity against *Mycobacterium fortuitum* in *in vitro* studies by Coleman *et al.*, (1999, 2000). Some of their compounds showed high activity against the bacterium with much reduced toxicity to human erythrocytes and human mononuclear leucocytes (MNL).

Conjugating INH to carriers has also been explored as a way to improve the efficiency of this compound. This was mainly done to enhance the penetration of INH through the hydrophobic cell wall of *M. tuberculosis*. In a study by Rastogi and Goh (1990) INH was coupled to palmitic acid and this resulted in an increased anti-mycobacterial activity of the parent compound against *M. avium* complex (Brennan and Nikaido, 1995; Jarlier and Nikaido, 1994; Klopman *et al.*, 1996; Rastogi and Goh 1990). Acyl groups have also been widely used as prodrugs or carriers for INH, mainly because of their ability to release the active parent compound at the target site (Klopman *et al.*, 1996).

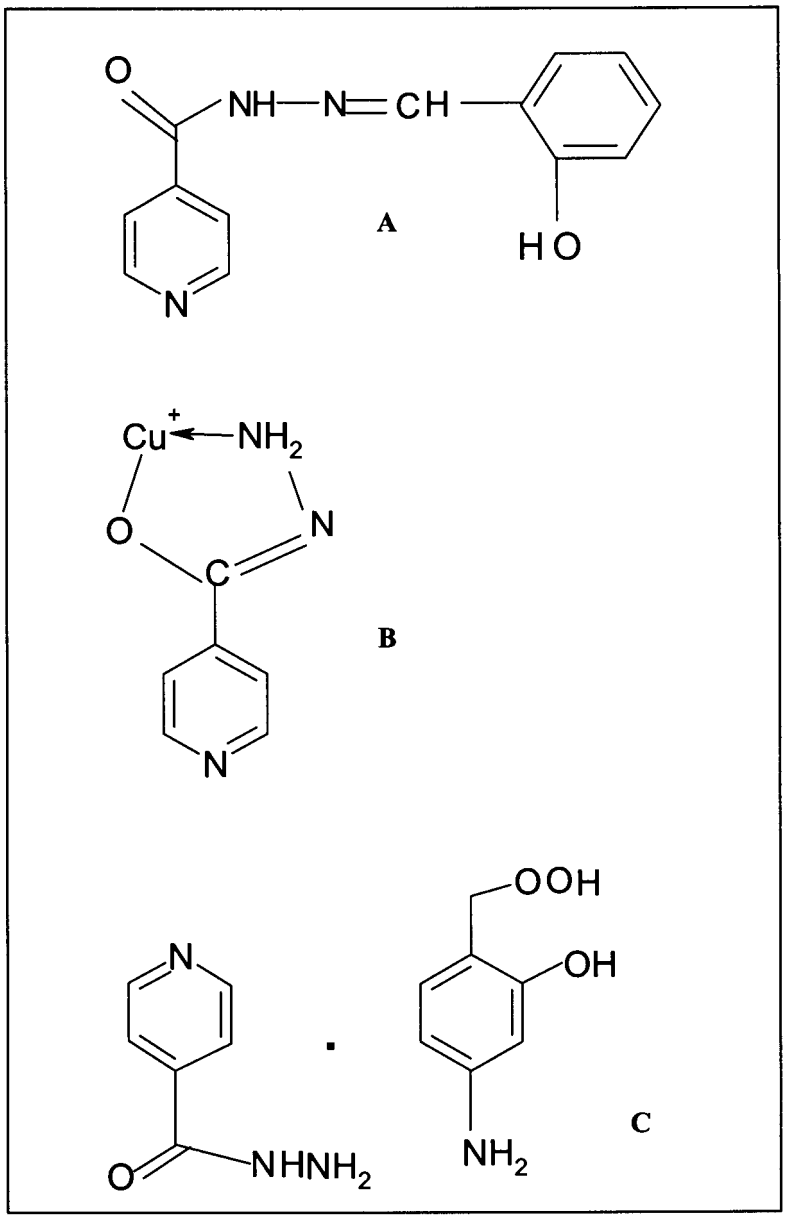


Figure 2.1. Chemical structures of some of the INH derivatives investigated earlier. A represent salizids investigated by Hart *et al.* (1954) and Bavin *et al.* (1955), while B represents the Cu-isoniazid complex investigated by Voyatzakis *et al.* (1968) and C represent the structure of pasiniazid (Derivatives of Isonicotinic acid hydrazide, Patent No. 737,271; The London patent office).

INH is the most efficient drug to reduce the number of actively replicating mycobacteria, even though it has little effect on dormant mycobacteria (Rattan *et al.*; 1998; Van Loenhout-Rooyackers and Veen, 1998). It is therefore ideally suited to provide proof of principle for the concept of targeting drugs to cholesterol like molecules on the surface of mycobacteria, as replication and proliferation of mycobacteria can easily be monitored *in vitro* by BACTEC. While INH is therefore probably not the pharmacophore of choice to solve the problem of dormant mycobacteria (Rattan *et al.*; 1998; Van Loenhout-Rooyackers and Veen, 1998) and extended regimens of chemotherapy, it remains the ideal toxophore to test whether its efficiency is affected by targeting it to cholesterol-like molecules by combining it with a cholesterol binding drug.

Hydrophobic ion pairing (HIP) is a technique that increases the hydrophobicity of molecules containing ionizable groups by stoichiometrically replacing polar counterions with more hydrophobic ones. This process has been used to solubilize ionic molecules in non-polar solvents, enhance the transport of proteins and DNA, and increase the bioavailability of ionic drugs. INH is an uncharged, hydrophilic molecule, thus it is not amenable to ion pairing, but this can be corrected by converting it into an ionizable prodrug. Zhou *et al.* (2002) synthesized a charged prodrug of INH, sodium INH methanesulfonate, which was ion paired with hydrophobic cations, such as alkyltrimethylammonium or tetraaalkylammonium (Zhou *et al.*, 2002). Since INH is not ionisable, it cannot be ion-paired. Hence in this study, CID was synthesised in a manner analogous to that employed for the preparation of pasiniazid, which is an isoniazid-aminosalicylic acid conjugate (Derivatives of Isonicotinic acid hydrazide, Patent No. 737,271; The London patent office). In this approach the acid component (sugar in AmB case), which is zwitterionic (or ionised) allows an ion-dipole interaction with the pyridino nitrogen of INH, which has an affinity for the protonated amino group of the amino acid component. The carboxylate approaches the electrophilic carbon of the hydrazide to complete the interaction (Report from Dr C. Parkinson).

Due to its cholesterol binding ability, AmB was used as a haptophore linked to INH as a toxophore in the design of cholesterol targeting isoniazid derivatives (CID). In the

configuration of an INH-AmB non-covalent conjugate, it is expected to accumulate onto cholesterol containing molecular complexes, or chemical entities with cholesterol structural mimicry like the mycolic acids that occur on the surface of *Mycobacterium tuberculosis*, from where it will supposedly leak its toxophore to the cell wall anchored enzyme target of INH.

2.2. Hypothesis and Aims of the study

Based on the possible mimicry between cholesterol and oxygenated mycolic acids, the cholesterol binding AmB of the INH-AmB non-covalent conjugate is expected to bind to the mycolic acids and target the INH-AmB complex to the mycolic acids within the cell wall. Another property of AmB is that it is amphiphatic, hence can make the complex more hydrophobic as compared to INH. This is expected to concentrate the complex to the hydrophobic mycolic acids, hence improve the efficiency of INH. The aims of this study are:

1. Purification of INH-AmB non-covalent conjugate using counter current distribution
2. Investigation of whether the efficiency of INH is improved by coupling it to AmB

2.3. Materials

2.3.1. List of reagents

Acetone, 99.5% purity identified by IR spectrum (Merck, Halfwayhouse, RSA)

Ammonium Solution -25% (NH_4OH), chemically pure 25% (NH_3) (Saarchem, UniLAB, Krugersdorp, RSA)

Amphotericin B from Streptomyces, 80% purity identified by HPLC (Sigma-Aldrich, Steinheim, Germany)

n-Butanol, 99.9% purity (Saarchem, Midrand, RSA)

Dimethyl sulphoxide for cell culture, (Sigma Chemical Co., St Louis, USA)

Dimethyl sulphoxide, 99.5% purity (LABCHEM, Edenvale, RSA)

Ethanol (Merck, Halfwayhouse, RSA)

Isoniazid (Sigma-Aldrich, Steinheim, Germany)

NaCl (Merck, Darmstadt, Germany)

Silica gel TLC plates (0.20 mm) with fluorescent indicator UV_{254}, (Macherey–Nagel, GmbH & Co., Dűren, Germany)

Tetrahydrofurane (THF), 99.9% purity (Riedel-de Haën Ag, Seelze, Germany)

Triethyl amine (Adros organics, New Jersey, USA)

2.3.2. List of apparatus

BACTEC performance test kit (Becton Dickinson and Co., Maryland, USA)

BACTEC 460 radiometric apparatus (Becton Dickinson, Johnston Laboratories, USA)

Countercurrent distribution apparatus (H O POST, Instrument Co. Inc., NY)

2.3.3. List of disposables

Alcohol swabs (TYCO Health care (PTY) LTD, Midrand, RSA)

Amber vials (Separations Pty Ltd, Randburg, RSA)

Disposable pipette tips (Bibby Sterilin, Serowell; Bibby Sterilin Ltd, Stone, UK)

Disposable 50ml and 10ml sterile centrifuge tubes (Bibby Sterilin Ltd, Stone, UK)

Membrane filters – 0.22 µm filter, hydrophobic (Sartorius AG, Goettingen, Germany)

Insulin 1 ml syringe with integrated 29G needles (B. braun Petzold GmbH, Switzerland)

7H12 Middlebrook TB medium culture vials (Becton Dickinson International, Belgium)

2.4. Methods

2.4.1. Synthesis of the INH-AmB non-covalent conjugate

An INH-AmB non-covalent conjugate was synthesized by Dr Chris Parkinson, CSIR, Modderfotein, South Africa and provided as a crude synthetic mixture. AmB (66 µmol) was dissolved in dH_2O (12.2 ml) and mixed with triethyl amine added in two aliquots of 9.2 µl each at RT for 30 minutes to form a heterogeneous solution. To this, an equal volume of ammonia solution was added at RT (23 ^{O}C). After 20 minutes of stirring, the solution became homogeneous at pH 12 – 13. The aqueous INH solution (63 µmol in 1 ml) was added in 4 portions over 1hour. The mixture was then stirred overnight at RT. Acetic acid was added drop-wise to generate a pH ~ 5 and the solvents were removed on a rotary evaporator (Buchi) at a temperature below 40 ^{O}C.

To create a comparable AmB blank, AmB (33 µmol) and 6.0 ml dH_2O were mixed for 5 minutes, and then 9.2 µl triethyl amine added. The solution was stirred for 10 minutes at RT and ammonia solution (6 ml) was added, resulting in homogeneity. The solution was stirred overnight as above. The pH of the solution was adjusted to pH 5 and the solvents were removed as above. After synthesis the product was assessed with NMR.

2.4.2. Development of Tri-component bi-phasic solvent system.

Different ratios of double distilled deionized water ($dddH_2O$) and butanol (BuOH) were mixed in an Erlenmeyer flask to form immiscible solutions. Each solution was then weighed and titrated with tetrahydrofurane (THF) until the two phases became miscible. This was also weighed and the amount of THF used to reach this point was calculated for each flask. The amount of THF used was obtained as follows:

$$M_{H2O + BuOH + THF} - M_{H2O + BuOH} = M_{THF}$$

$$V_{THF} = M_{THF} / \rho_{THF}$$

Where, M=Mass, V=Volume and ρ=density. The percentage of each compound in the solvent system was calculated and used to construct a phase diagram.

2.4.3. Counter Current Distribution (CCD) of the INH-AmB non-covalent conjugate and AmB blank

The solvent system used in countercurrent separation was made up of: 15 % v/v THF, 57 % v/v $dddH_2O$ and 9 % v/v butanol. These components were mixed in a separatory funnel and the phases were allowed to separate. The phases were collected separately. The composition of the upper phase and the lower phase were determined from the phase diagram.

The lower phase was made up of: 8% v/v THF, 83% v/v $dddH_2O$ and 9% v/v butanol.

The upper phase was made up of: 22% v/v THF, 30% v/v $dddH_2O$ and 48 % v/v butanol.

Both the INH-AmB non-covalent conjugate and the AmB blank were separated with the countercurrent distribution in one run under the same experimental conditions. A countercurrent distribution train comprising of 21 tubes numbered 0 – 20 was used each for the INH-AmB non-covalent conjugate and AmB blank. A sample of 30 mg INH-AmB non-covalent conjugate and about 15mg of AmB were dissolved separately in 10 ml of the lower phase. This was loaded into the first tubes numbered "0" of each train. Quantitative transfer was ensured by rinsing of the above samples with 10 ml of the upper phase, which was also loaded into the first tube of each train. Into the remaining tubes 10 ml of the lower phase was introduced. The contents of the first tubes were mixed and allowed to separate, after which the first CCD transfer was made. To the first tubes, 10 ml of the clean upper phase was introduced after each transfer and the contents were mixed and allowed to separate, followed by another transfer. This cycle was repeated until all the 20 transfers were complete. Emulsions were observed during the separation, which were slow to break up. This resulted in prolonged equilibration time between transfers.

Thin layer chromatography was used to analyse and characterize the contents of the tubes. From each tube, 5 µl of the upper phase and lower phase was spotted onto a TLC silica gel coated plate. The plate was allowed to dry using a hair dryer and developed with n-butanol:ethanol:ecetone:25% NH_4OH (2:5:1:3). The plate was dried as above and observed under UV light at 366 and 254 nm.

2.4.4. Removal of Solvents

After TLC, both the AmB blank and INH-AmB non-covalent conjugate CCD tube train distributions were divided into four concurrent fractions collected and concentrated by removal of solvents. The solvents to be evaporated were made up of THF, $dddH_2O$ and butanol that were difficult to remove on a standard rotary evaporator (Buchi) below 40 ^{O}C. The solvents were therefore evaporated by ebulition. Briefly, the samples were evaporated under positive N_2 pressure at a temperature maintained between 35 ^{O}C and 40 ^{O}C under high vacuum. Solvents were captured in a liquid nitrogen trap. The concentrated samples were resuspended in $dddH_2O$ and aliquoted into pre-weighed, dry amber vials. These samples were then frozen prior to freeze-drying. The freeze-dried samples were weighed to determine the mass in each fraction and were stored at 4 ^{O}C until use.

2.4.5. Thin Layer Chromatography (TLC) of CCD fractions

Fractions 1 and 4 were spotted on TLC plates at 3 mg/ml (5 µl); and 2 mg/ml for fractions 2 and 3 (5 µl) and compared with the commercial INH and AmB, which were spotted at the approximate molar equivalent of 0.75 mg/ml and 3 mg/ml respectively. All the compounds were dissolved in a 1:1 ratio of dimethyl sulphoxide (DMSO) and $dddH_2O$ and spotted onto a silica gel-coated TLC plate which was then developed with n-butanol:ethanol:acetone:25% NH_4OH (2:5:1:3). The plate was then dried as in section 2.4.3. and visualized under a wavelength of 254 nm and 366 nm.

2.4.6. In vitro testing of compounds on M. tuberculosis cell culture

Test compounds used in this study included INH-AmB non-covalent conjugate, INH, AmB, and the DMSO solvent as such. The DMSO and AmB were included to confirm their non-toxicity to the bacteria at the concentrations used, since DMSO was used as a solvent and AmB forms a major part of the CID. INH was tested at its MIC, 10-fold and 100-fold dilutions thereof. All the other test compounds were tested at the concentration equivalent to the minimum inhibitory concentration (MIC) of INH and also at 10- and

100-fold dilutions thereof. Stock solutions were prepared by dissolving the test compounds in a 1:1 ratio of double distilled deionized ($dddH_2O$) and DMSO prior to use. The stock solutions were then diluted with sterile saline, and filter-sterilized through a 0.22 μm filter. The filter-sterilized solutions were used to prepare the 10- and 100-fold dilutions in saline.

The susceptibility testing was done using the BACTEC 460 radiometric apparatus. In this method, bacterial growth is determined by measuring the amount of ^{14}C-labelled CO_2 released during mycobacterial metabolism, which is presented in terms of a radiometric Growth Index (GI). The cultured bacteria were harvested after reaching a GI of 300 – 500, triturated through a 29G needle and transferred into a sterile tube. The tube was allowed to stand to settle any clumps that might be present. The upper homogeneous suspension was transferred into a second sterile tube. From this suspension, 0.1 ml aliquots were inoculated into the experimental vials, which contained 0.1 ml of each test. Each test compound was tested in triplicate in each experiment performed. Drug-free control vials consisted of undiluted and 1:10 diluted bacterial inoculum in saline. Negative control vials containing saline without any bacteria or drugs were included to check if any unwanted bacterial contamination occurred during the experiment. All the vials were flushed with gas that contained 4-5% CO_2 for aerobic culturing as to maintain optimum culture conditions and then maintained at 37°C. The vials were read in this manner daily until the GI of the undiluted bacterial inoculum reached 999.

2.5. Results

2.5.1. Development of a tricomponent bi-phasic solvent system to purify the INH-AmB non-covalent conjugate

In order to develop the relevant phase diagram (Fig. 2.2) different ratios of H_2O and butanol were titrated with THF until the two liquid phases became miscible. The amount of H_2O and butanol were prepared according to Table 2.1, which also shows the percentage of each compound in the solvent system that was determined. The curve in Fig. 2.2 represents the solvent composition at which the titration mixtures became miscible, while the area under the curve represents the mixtures of solvents that separated into two phases. Line LU was plotted on the phase diagram, where point L represents the composition of the lower phase and point U represents the composition of the upper phase. The point X, which is the mid-point of line LU, represents the point at which the upper phase and the lower phase have equal volumes. The phase composition at this point was 15% THF, 57% H_2O and 28% n-butanol and this was used for purification of CID by counter current distribution.

Table 2.1: Percentage of each solvent used to develop the solvent system for CCD purification of the INH-AmB non-covalent conjugate.

Flask	ddd H_2O		Butanol		THF		Total
No.	(ml)	(%)	(ml)	(%)	(ml)	(%)	(ml)
3	18.0	81.1	2.0	9.0	2.2	9.9	22.2
4	17.0	72.5	3.0	12.8	3.5	14.7	23.5
5	15.0	61.0	5.0	20.3	4.6	18.7	24.6
6	13.5	53.1	6.5	25.6	5.4	21.3	25.4
7	10.0	38.0	10.0	38.0	6.3	24.0	26.3
8	7.0	27.5	13.0	51.1	5.5	21.4	25.5
9	5.0	21.6	15.0	64.9	3.1	13.4	23.1
10	4.0	18.4	16.0	73.7	1.7	7.9	21.7

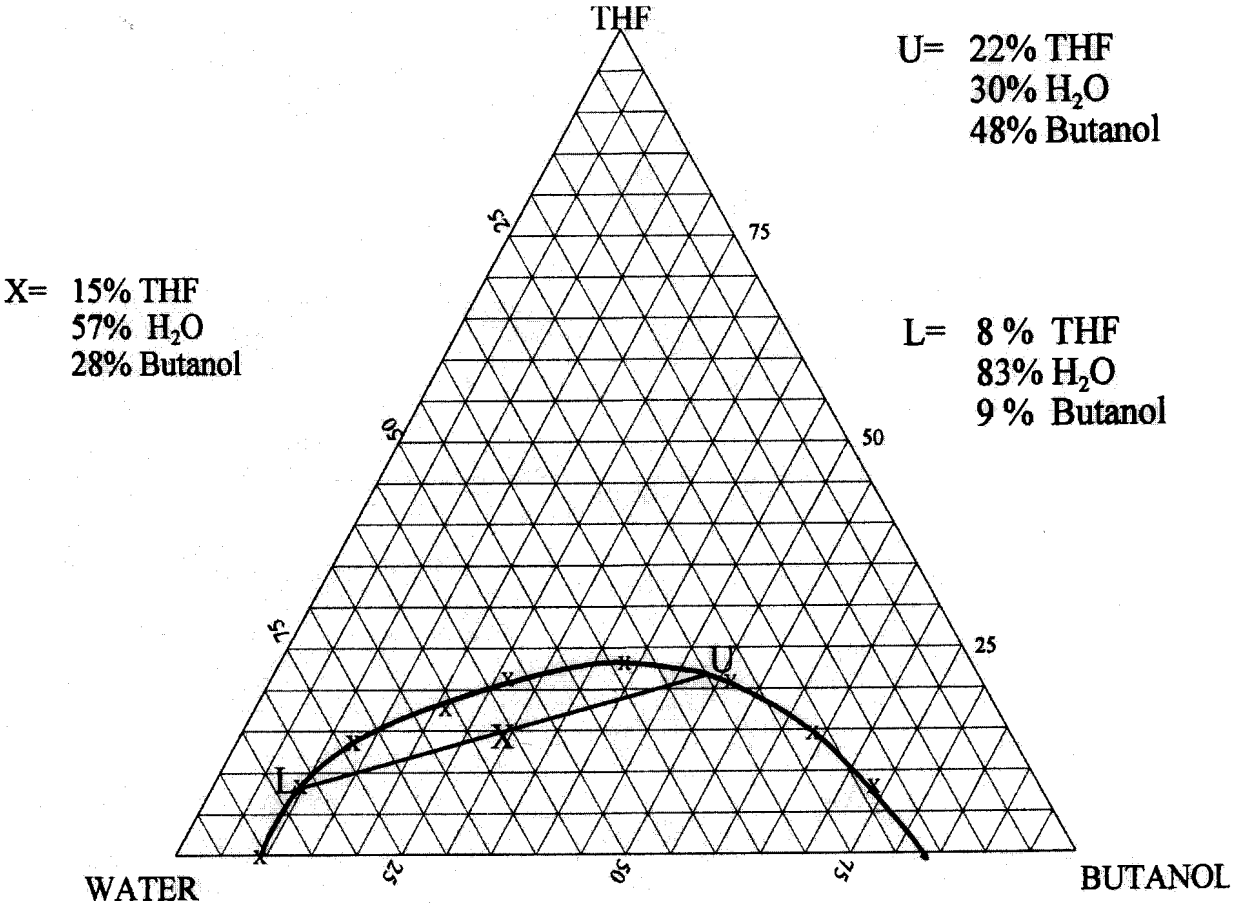


Figure 2.2. Phase diagram obtained from titration of H_2O and n-butanol with THF.

2.5.2. Countercurrent distribution purification of INH-AmB non-covalent conjugate and TLC analysis of CCD fractions

About 30 mg of INH-AmB non-covalent conjugate was dissolved in equal volumes of the lower and the upper phases and loaded into the first tube of the CCD. The AmB blank (about 15mg), which was exposed to the same conditions as INH-AmB non-covalent conjugate, but did not contain INH, was also dissolved in the same manner and loaded in the first tube of the second tube train. The compounds were allowed to separate over 21 tubes of the counter current after 20 cycles and analysed with TLC.

The TLC analysis showed that the INH-AmB non-covalent conjugate separated into 4 distinct fractions while the AmB separated into 3 distinct fractions. The contents of each fraction were grouped according to their appearance under the UV light as summarized in Table 2.2. Since the two compounds were separated under the same conditions, prior to concentration of the contents of each fraction, the AmB was separated into 4 fractions just like the INH-AmB non-covalent conjugate for comparison. The four fractions of each compound were then concentrated using the ebulition system. The concentrated fractions were resuspended in about 3ml – 6ml $dddH_2O$ and aliquoted into pre-weighed dry vials, freeze-dried and weighed to determine the mass of the compound in each fraction. The masses and percentage of the contents of each fraction are also summarized in Table2.2.

Table 2.2. Summary of INH-AmB non-covalent conjugate and AmB fractions after CCD separation and percentage yield of each fraction.

		CID FRACTIONS			AmB FRACTIONS		
Fraction Number	**Tube Number**	**Yield**		**Fraction identity**	**Yield**		**Fraction identity**
		(mg)	**(%)**		**(mg)**	**(%)**	
1	0 - 3	9.71	32.7	Salts (C1)	3.70	34.3	Salts (A1)
2	4 - 8	6.39	21.5	INH-AmB non-covalent conjugate 2 (C2)	1.73	15.8	None (A2)
3	9 - 14	4.11	13.8	INH-AmB non-covalent conjugate 3 (C3)	2.46	22.4	None (A3)
4	15 - 20	9.48	31.9	AmB (C4)	2.94	26.8	AmB (A4)
Total Yield (mg)		**29.69**			**10.83**		

2.5.3. Thin Layer Chromatography analysis of INH-AmB non-covalent conjugate and AmB concentrated fractions.

In order to determine any differences between the contents of each fraction, a TLC was performed. Fraction 1 of INH-AmB non-covalent conjugate was analysed at the same mass concentration as that of fraction 1 of AmB and the same was done for the other fractions. The results obtained (Fig. 2.3) indicate that under a wavelength of 366 nm the commercial AmB showed a dark brown spot at Rf 0.75 which was also observed with fractions 4 of INH-AmB non-covalent conjugate (C4) and AmB (A4), implying that these fractions contained just AmB. Fractions 2 and 3 of AmB (A2 and A3) didn't show any spots except slightly bright long white smears from the origin. Fractions 2 and 3 of INH-AmB non-covalent conjugate (C2 and C3) showed the same spot as AmB at Rf 0.75 but it appeared as a bright cream – whitish spot and there was another spot at Rf 0.9 that was bright white. The only difference between C2 and C3 was the intensity of their spots, which was higher on C3. Fractions 1 of INH-AmB non-covalent conjugate (C1) and AmB (A1) showed long thin bright whitish smears from the origin.

Under a wavelength of 254nm, the bright white spot at Rf 0.9 observed on C2 and C3 appeared as a darker spot and was found only on the INH-AmB non-covalent conjugate fractions. It was difficult to derive the identities of the spot in C2 and C3, hence the experiment was repeated, with commercial INH run as an additional control.

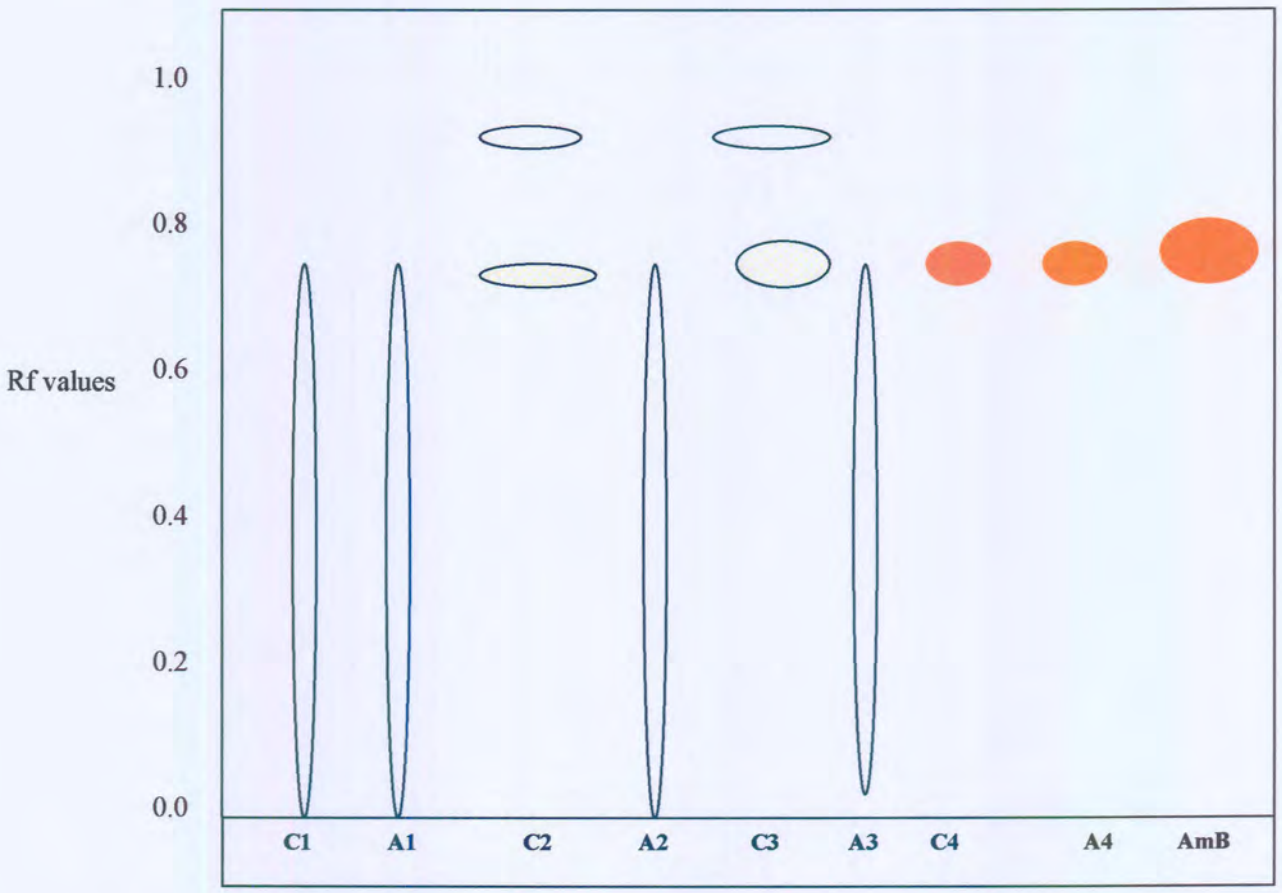


Figure 2.3. TLC analysis of separated INH-AmB non-covalent conjugate and AmB blank fractions. The plate was developed with n-butanol:ethanol:acetone:25% NH_4OH (2:5:1:3) and observed at a wavelength of 366nm. C1 –C4 represent INH-AmB fractions 1 – 4 and A1 – A4 represent AmB fractions 1 – 4.

Fractions 3(C3) and 2(C2) of INH-AmB non-covalent conjugate were analysed together with commercial INH and AmB in order to investigate the nature of the top spot of C3 and C2. The developed TLC plate was observed under different wavelengths (366nm and 254nm). The results obtained (Fig. 2.4) indicate that INH shows maximal absorption under a wavelength of 254nm and this is highlighted by a dark purple spot at RF 0.9 on the plate. Under the same wavelength, C3 and C2 showed the same spot as that of INH but with a lighter colour and less intensity, while AmB showed very little absorption at this wavelength. Under a wavelength of 366nm, AmB shows maximal absorption and

this is highlighted by a dark brown spot at Rf 0.75 on the plate. At the same place as the AmB spot, the C3 and C2 showed a bright cream - whitish spot while the INH spot was not visible at all. The INH-like spot of C3 was clearly visible at this wavelength, but it was no longer light purple as observed under a wavelength of 254nm. Instead it appeared as a bright white spot.

These observations showed that the compound in fraction 3 of INH-AmB non-covalent conjugate (C3) is the same as AmB except that they both show different absorption properties under different wavelengths. This might imply that C3 is a derivative of AmB. C2 and C3 did not show any difference except relative intensities of the spots.

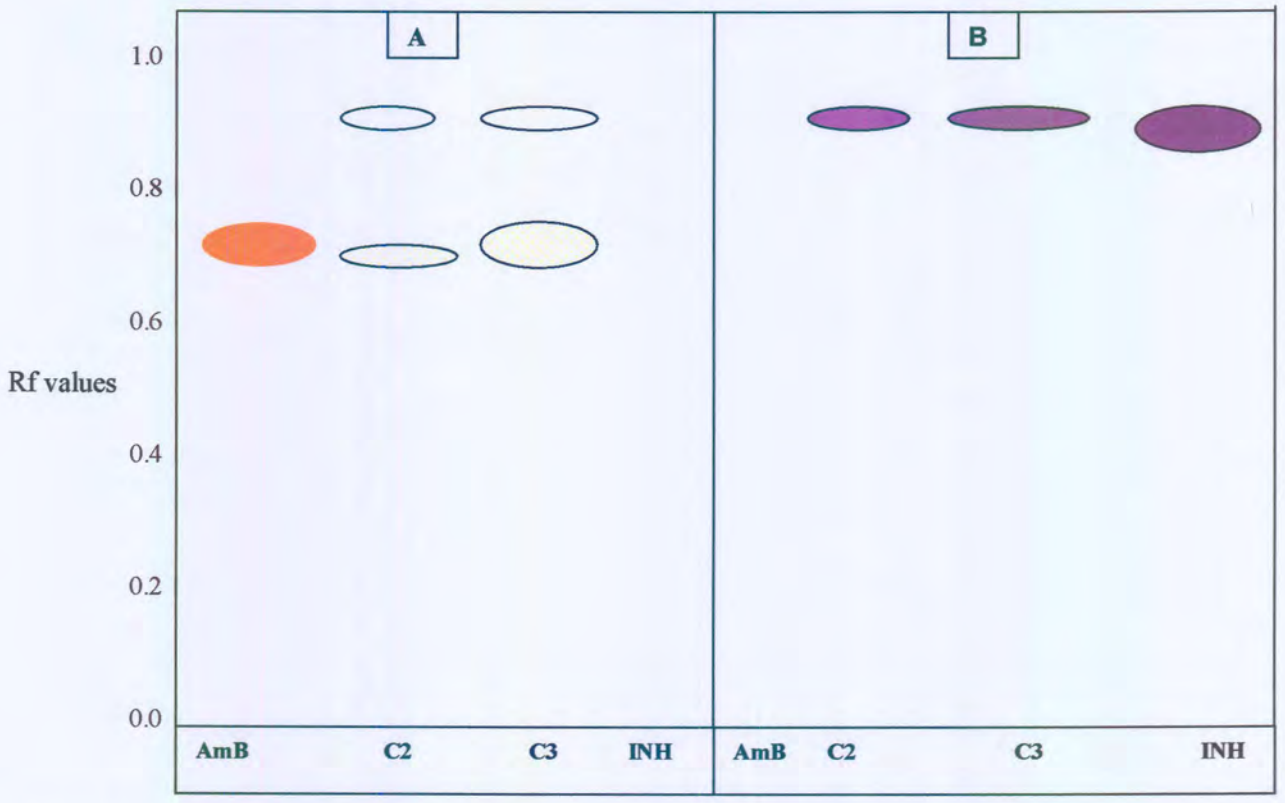
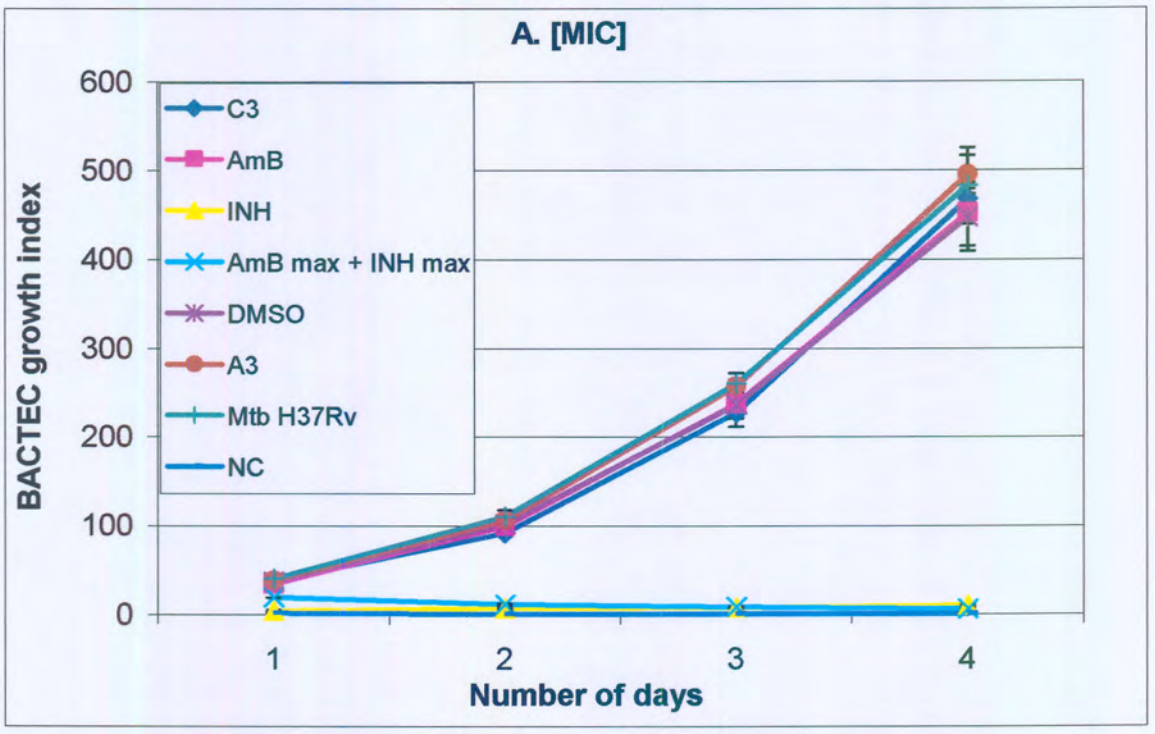


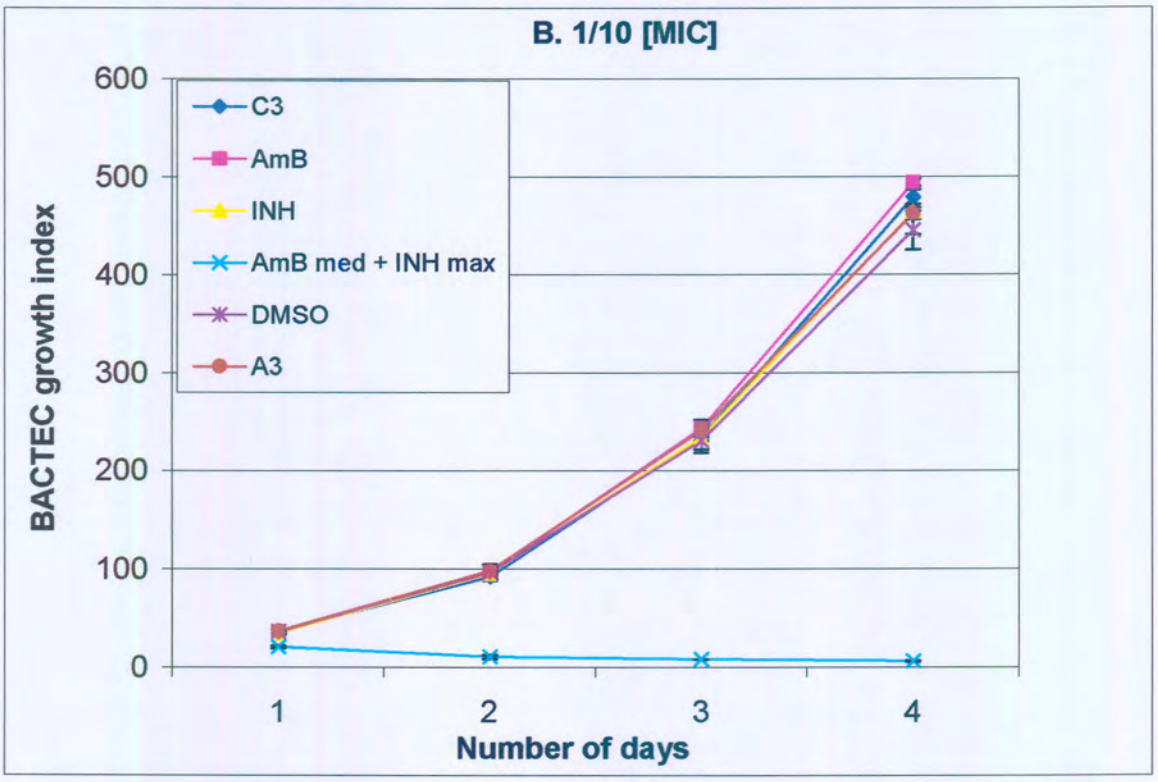
Figure 2.4. **TLC analysis of fractions 2(C2) and 3(C3) of INH-AmB non-covalent conjugate in comparison to the commercial INH and AmB.** The plate was developed with n-butanol:ethanol:acetone:25% NH_4OH (2:5:1:3) and observed at a wavelength of 366nm(A) and 254nm(B).

2.5.4. *In vitro testing of separated INH-AmB non-covalent conjugate fractions on M. tuberculosis*

The activity of the purified INH-AmB non-covalent conjugate fractions against *Mycobacterium tuberculosis* H37Rv was tested in the BACTEC 460 radiometric system. The activity of fraction 3 of INH-AmB non-covalent conjugate (C3) and fraction 3 of AmB (A3) was tested at the same molar concentrations as the MIC of INH. The results obtained, shown in Fig. 2.5, indicate that C3 and A3 at all the tested concentrations did not inhibit the growth of *Mycobacterium tuberculosis*.

The experiment was repeated with C3 tested at a concentration of 10X the MIC of INH. Fraction 2 of INH-AmB non-covalent conjugate (C2) and fraction 2 of AmB (A2) were tested as well. The results shown in Fig. 2.6.A indicate that the C3 at a concentration 10X the MIC, shows growth inhibition. The compound was able to show the same inhibitory effects on *Mycobacterium tuberculosis* at the higher concentration, as INH did at its MIC (Fig. 2.6.B). C2 and A2 did not show any inhibitory effect on the bacterium. Even though the TLC analysis did not show differences between C2 and C3, these results indicate that the two compounds have different biological activities against *Mycobacterium tuberculosis*. Hence from this, it can be predicted that the fraction with the biologically active compound is the 3rd fraction of INH-AmB non-covalent conjugate i.e. C3.


A. [MIC]
BACTEC growth index
600
500
400
300
200
100
0
C3
AmB
INH
AmB max + INH max
DMSO
A3
Mtb H37Rv
NC
1
2
3
4
Number of days


B. 1/10 [MIC]
BACTEC growth index
600
500
400
300
200
100
0
C3
AmB
INH
AmB med + INH max
DMSO
A3
1
2
3
4
Number of days

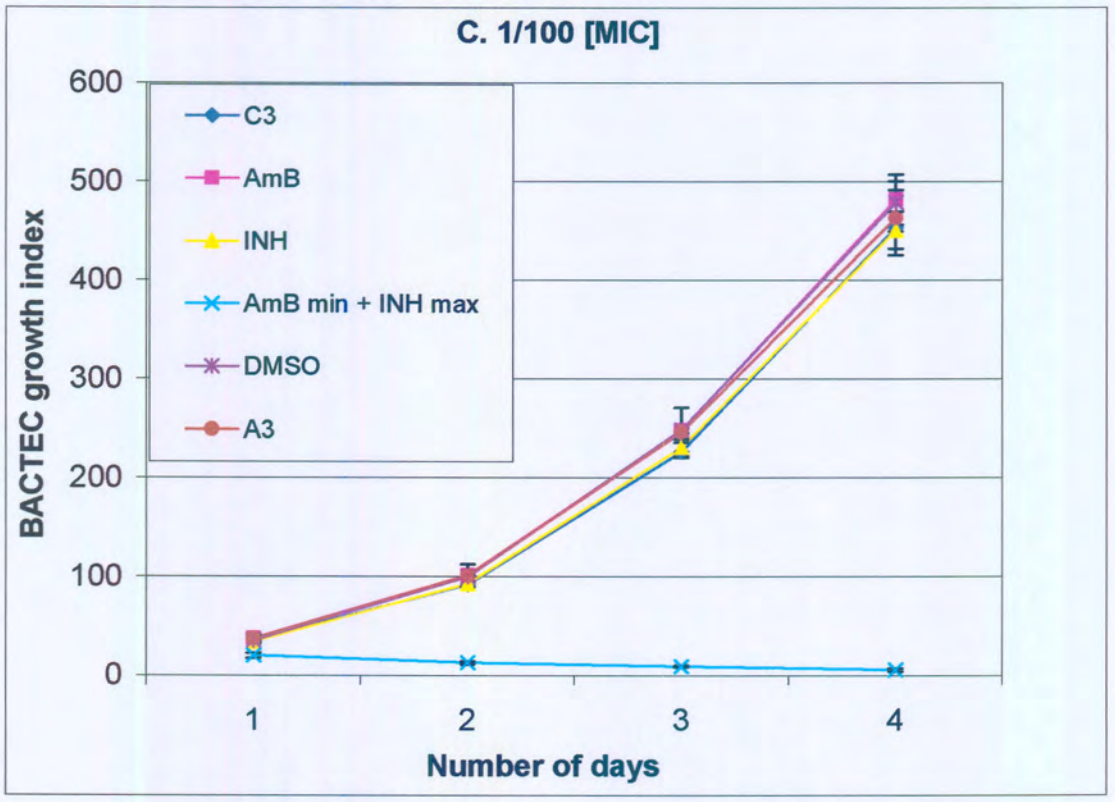


Figure 2.5. BACTEC growth index of *M. tuberculosis* H37Rv upon treatment with various test compounds at or below the MIC of INH. The Negative control (NC) received saline without any bacteria. Mtb H37Rv represents the untreated bacteria. Compounds were tested at (A) the same concentration as the MIC (max) of INH, (B) 1/10 MIC (med), and (C) 1/100 MIC (min). The results represent one experiment done in triplicate.

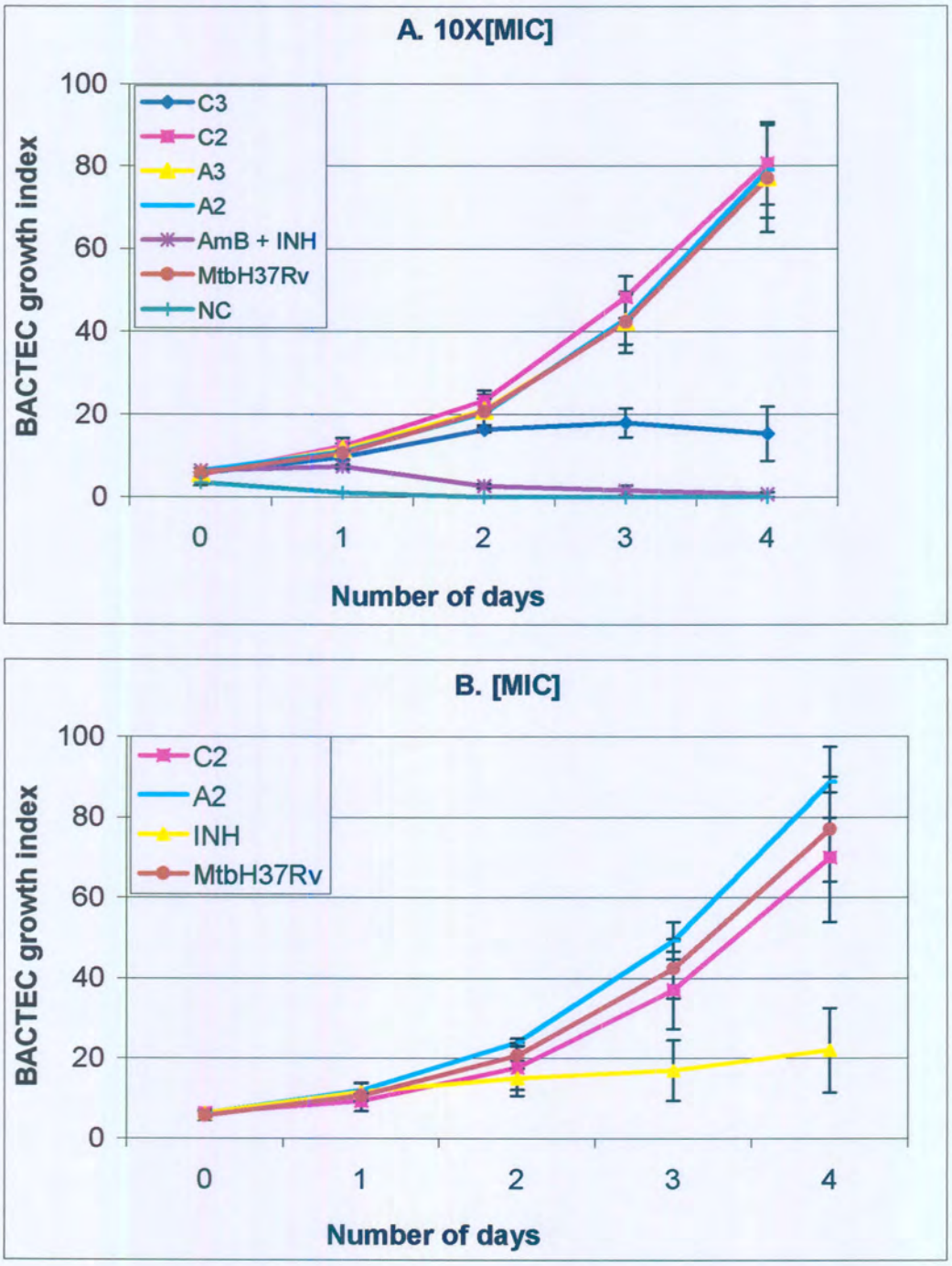


Figure 2.6. BACTEC growth index of *M. tuberculosis* H37Rv upon treatment with various test compounds at or higher than MIC of INH. The Negative control (NC) received saline without any bacteria. Mtb H37Rv represents the untreated bacteria. Compounds were tested at (A) 10X MIC, and (B) MIC. The results represent one experiment done in triplicate.

To confirm these observations, the remaining fraction1 (C1) and fraction 4 (C4) of INH-AmB non-covalent conjugate were also tested to see if they possess any activity against the bacteria. The results showed that C1 did not have any activity against *Mycobacterium tuberculosis*, while C4 showed activity (Fig. 2.7) to the same level as that of C3. In the previous experiment (Fig. 2.6) C3 at a concentration of 10X the MIC of INH showed growth inhibition with the activity of more or less the same as that of INH at its MIC. AmB was also tested at higher concentrations and it showed no inhibition at all. This was expected to be the case with C4 as well, since according to TLC analysis this compound showed the same RF and absorption properties as AmB. From this it can be concluded that both C3 and C4 contained the presumed CID biological activity, but no advantage was gained over pure INH by conjugating INH and AmB in a non-covalent association since the activities exerted by the complex were less than those of INH.

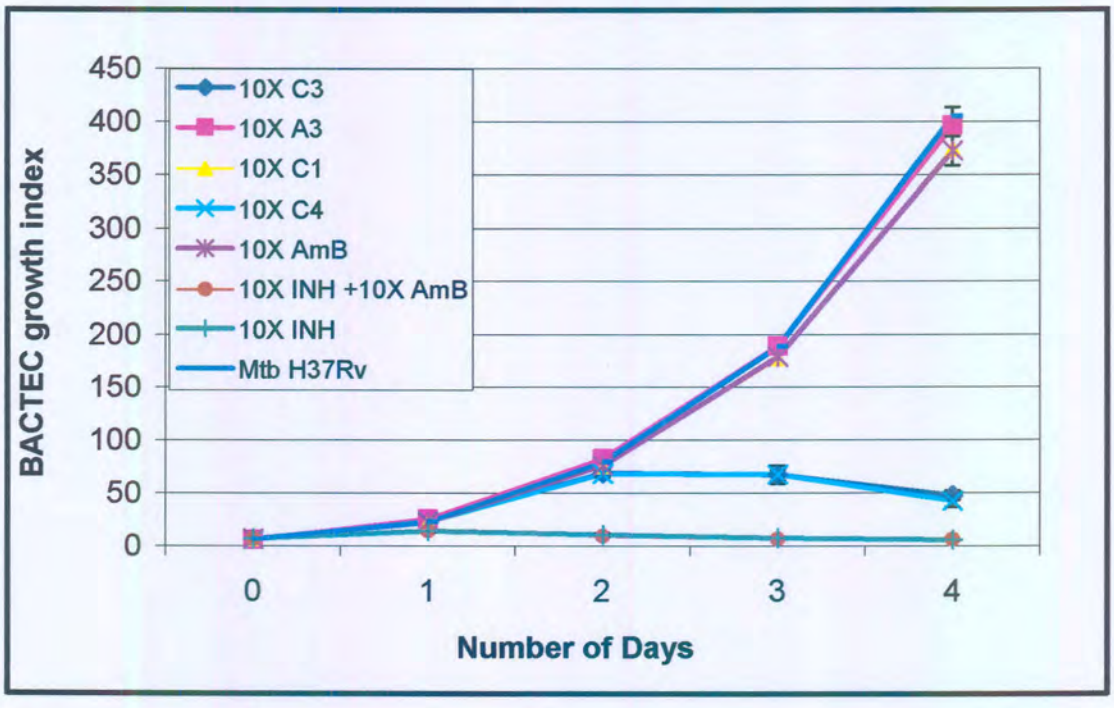


Figure 2.7. **BACTEC growth index of *M. tuberculosis* H37Rv upon treatment with the remaining test compounds at or higher MIC of INH.** Mtb H37Rv represents the untreated bacteria. The results represent one experiment done in triplicate.

2.5. Discussion

Both the cholesterol binding nature of AmB and its use as a second-line treatment of systemic fungal infections that accompany HIV infection (Ehrenfreund- Kleinman *et al.*, 2002; Paquet *et al.*, 2002) were considered when AmB was chosen as the carrier molecule for INH. In cases of AIDS patients co-infected with *M. tuberculosis* and fungal infections, the INH-AmB non-covalent conjugate may fulfil a dual purpose. Konopka *et al.*, 1999 showed that in addition to its anti-fungal activities AmB has anti-HIV-1 activities at non-toxic concentrations. The authors also tested the activity of liposomes-incorporated AmB (L-AmB) and cholesteryl sulphate colloidal dispersion AmB (CD-AmB), since these forms of AmB have been shown to be less toxic than the parent compound (Baginski *et al.*, 2002). Their results showed that the L-AmB has no anti-HIV-1 activity while the CD-AmB was less effective than AmB, but its antiviral activity was significantly enhanced by maintaining the drug throughout the culture period (Konopka *et al.*, 1999).

During the course of the study it was observed that the crude synthetic mixture of INH-AmB non-covalent conjugate was hygroscopic. There were changes in the physical properties of the compound during storage, probably induced by accumulation of water. After two years of storage at 4 ^{o}C, the mass of the INH-AmB non-covalent conjugate had almost increased 10 fold. Although the compound was tested soon after purification, its concentration during testing may have been somewhat less than indicated. In addition to this the synthesis of the compound was found to be poorly reproducible. According to Zhou *et al.* (2002) INH, which is neutral and hydrophilic, cannot undergo ion pairing. Instead, it must be converted into an ionizable compound that will allow ion pairing to take place. Hence an ion-dipole interaction was allowed between the amino acid component of AmB and the pyridino nitrogen of INH. Dr C. Parkinson who synthesized the CID, reported that "the evidence from the nuclear magnetic resonance for this interaction comes through the observation of the protons on the pyridine ring. In the parent INH, there are two pairs of equivalent protons while in the conjugate, broadening and splitting of these protons is observed indicating slight non-equivalence. This is

observed through encapsulation of the molecule in a chiral environment- the INH conjugate".

Isoniazid was non-covalently conjugated with a cholesterol-binding drug, amphotericin-B, in order to investigate if this will enhance the activity of INH by targeting the INH-AmB non-covalent conjugate to the presumed cholesterol-like molecules in the *M. tuberculosis* cell wall. Purification of this compound was done with CCD, which lead to separation of the INH-AmB non-covalent conjugate from salts and free/unreacted AmB. The TLC analysis of the separated compounds showed that the INH-AmB non-covalent conjugate had the same RF value as AmB but with different light absorption properties, indicating that the compound is an AmB derivative that is somehow modified by interaction with isoniazid.

Targeting of the INH-AmB non-covalent conjugate compound to the bacterial cell wall components was expected to concentrate INH to its target, hence requiring lower molar concentrations to kill off the bacteria. This study showed that the INH-AmB non-covalent conjugate had some inhibitory activity against the *M. tuberculosis* H37Rv strain, but required concentrations as high as 10 fold the MIC of INH. The anti-mycobactericidal activity was shown not to be due to AmB as such since AmB did not exhibit any anti-mycobacterial activity at the comparable concentrations. Jackson *et al.* (2000) showed that despite the high anti-fungal activity of AmB, it does not posses any anti-mycobacterial activity. These authors tested the activity of AmB against *M. smegmatis*.

In principle, lipophilic compounds should be able to penetrate any biological membrane by dissolving into the hydrocarbon interior of the lipid bilayer. Lipophilic and amphiphatic agents may therefore be expected to penetrate or cross the lipid-rich mycobacterial cell wall through its lipid domain, yet most mycobacteria exhibit intrinsic resistance to a number of such agents (Jarlier and Nikaido, 1994; and Liu *et al.*, 1996). On the other hand, it has been shown that the more lipophilic derivatives of mycobactericidal agents are more active against mycobacteria (Jarlier and Nikaido,

1994). Rastogi and Goh (1990) showed that the efficiency of INH against *M. avium*, which appears to have very low hydrophilic permeability, was improved by converting it into a hydrophobic compound by addition of a C_{16} fatty acyl chain to INH (Brennen and Nikaido, 1995; Jarlier and Nikaido, 1994; Rastogi and Goh, 1990). Due to its cholesterol binding properties, the INH-AmB non-covalent conjugate was expected to be able to improve the efficiency of INH but this was found not to be the case in this study.

The reduced biological activity of INH-AmB non-covalent conjugate in comparison with INH could not be due to the presence of AmB in the compound since treatment of *M. tuberculosis* with INH at its MIC combined with AmB at various concentrations still resulted in complete growth inhibition. Isoniazid has been shown to have a very high affinity for its target catalase-peroxidase (KatG), which is responsible for its activation through oxidation into electrophilic species or active radicals (Rattan *et al.*; 1998; Sacchettini and Blanchard, 1995; Whitney and Wainberg, 2002; and Wei C *et al.*, 2003). Marcinkeviciene *et al.*, (1995) showed that INH is a potent inhibitor (K_i = 3-5 μM) of the *M. smegmatis* catalase-peroxidase (Marcinkeviciene *et al.*, 1995; Sacchettini and Blanchard, 1995). The requirement for a higher concentration of INH-AmB non-covalent conjugate to kill off the bacteria might be due to lower affinity of this compound to catalase-peroxidase, since the catalase-peroxidase must activate INH before its inhibitory effects can be manifested.

When designing the INH-AmB non-covalent conjugate, the AmB part of this molecule was expected to interact with mycolic acid on the cell surface and release the pharmacophore, which in this case is INH, in the lipid rich bilayer. INH is hydrophilic and can only penetrate the lipid bilayer through the water-filled porin molecule embedded within the cell wall (Jarlier and Nikaido, 1994; Liu *et al.*, 1996) to get to its target which is known to have a cell wall location (Raynaud *et al.*, 1998). The path of INH to its target appears not to be blocked by AmB, since the presence of the latter in solution together with INH at its MIC resulted in no detectable decrease in the biological activity of INH.

Due to the difficulties in the synthesis of the CID, Dr Parkinson concluded that the preparation of the CID "was inconsistent and difficult to control, resulting in quality control difficulties." He suggested that we abandon the approach due to the problems that are inherent in the chemical synthesis of CID. There may still be therapeutic value to be discovered in CID but this will need some alterations in the synthesis of INH-AmB conjugate and the conditions under which the CID is tested. A previous patented approach of covalent linkage of INH to an aromatic aldehyde (Farbenfabriken Bayer Akteien-gesellschaft, 1952, London Patent office) without loss of mycobactericidal activity may provide the basis for the design of a covalent conjugate of which the synthesis and quality can be better controlled. Testing of this compound should then also be done with BACTEC in the presence of cholesterol. It may well be that CID accumulates onto *M. tuberculosis*, not by direct contact with the mycolic acids, but by binding to the MA-cholesterol surface complex. Thus, if MA-cholesterol surface is targeted by the CID for effective delivery of the pharmacophore, it is important to know the effect that the presence of cholesterol in the growth media will have on the efficiency of INH. In preparation for this, the next chapter investigated the effect of cholesterol on the biological activities of INH, which was used as the pharmacophore in the CID.

CHAPTER 3

THE EFFECT OF CHOLESTEROL ON THE EFFICIENCY OF ISONIAZID AGAINST *M. TUBERCULOSIS*

3.1. Introduction

Mycobacteria can grow *in vitro* on an extremely wide range of carbohydrates and hydrocarbons with glycerol considered to be the preferred carbon source *in vitro*. Even though cholesterol is mainly a eukaryotic sterol, it can be metabolized by a wide range of microorganisms as a carbon and energy source (Av-Gay and Sobouti, 2000). The ability of actinomycetes, specifically mycobacteria, to bioconvert cholesterol and other sterols into steroid precursors is well documented and has been used for partial chemical synthesis of various novel steroids in pharmaceutical industries (Av-Gay and Sobouti, 2000; Perez *et al.*, 2003). Attempts were made to culture mycobacteria in growth media containing cholesterol. Kondo and Kanai (1976b) cultured *M. tuberculosis* in the presence of cholesterol liposomes (lecithin-cholesterol liposomes). It was shown that lecithin was used as a carbon source while cholesterol was not. In a study by Av-Gay and Sobouti (2000) the ability of various species of mycobacteria to utilize cholesterol as a carbon source was investigated. From that study it was shown that fast-growing, non-pathogenic mycobacteria degrade cholesterol from liquid media and were able to grow on cholesterol as a sole carbon source, while slow-growing pathogenic mycobacteria including *M. tuberculosis* did not degrade or use cholesterol as a carbon source. In addition to this pathogenic mycobacteria were able to take up, modify and accumulate cholesterol. Thus cholesterol could have been accumulated for a particular purpose, indeed, *M. tuberculosis* was shown to use cholesterol for its entry into the macrophages (Pieters and Gatfield, 2002; Gatfield and Pieters, 2000) and survival within these cells (Aínsa *et al.*, 2001; Ferrari *et al.*, 1999).

3.1.1. Cholesterol and Mycobacteria

Despite early studies by Kondo and Kanai, (1976b) that proposed that pathogenic *Mycobacteria* do not use cholesterol as an *in vivo* carbon source, the complete genome sequence of *M. tuberculosis* published by Cole *et al.*, (1998) revealed that it contains a sterol biosynthetic enzyme homologous to the sterol 14α-demethylase biosynthetic enzyme as well as two putative cholesterol degradation enzymes (Av-Gay and Sobouti, 2000; Cole *et al.*, 1998 and Lamb *et al.*, 1998). In a study by Lamb *et al.*, (1998) the ability of *M. smegmatis* to synthesize cholesterol was investigated using gas chromatography-mass spectrometry analysis. These authors found that cholesterol is synthesized but as a minor constituent of the bacterial cell wall representing 0.001 mg/g dry cell weight (Av-Gay and Sobouti, 2000; Lamb *et al.*, 1998). Researchers are already using the sterol biosynthetic pathway as a target for new anti-TB agents. Jackson *et al.* (2000) investigated the activity of a couple of azole anti-fungal agents against *M. smegmatis* that are known to inhibit the sterol 14-demethylase. Amongst the tested compounds, clotrimazole, econazole, miconazole and tebuconazole were found to be bactericidal with MICs ranging from 2 – 8 μg/ml (Jackson *et al.*, 2000).

3.1.2. Mycolic acids synthesis and its inhibition by INH

Biosynthesis of mycolic acids in *Mycobacteria* requires the interaction of two fatty acid synthase (FAS) systems, the FAS-I and FAS-II system. In the FAS-I system each enzymic activity resides on the single polypeptide chain of a very large multifunctional enzyme (Rozwarski *et al.*, 1999; Schaeffer *et al.*, 2001), while in the FAS-II system consists of dissociable enzyme components, which act upon a substrate bound to an acyl carrier protein (ACP) designated as AcpM in *Mycobacterium tuberculosis* (Kremer *et al.* 2002). This FAS-II system prefers C16 as a starting substrate and can extend up to C56, indicating that the mycobacterial FAS-II system utilizes the products of the FAS-I system as primers to extend fatty acyl chain lengths even further. The FAS-I system is mainly responsible for the *de novo* synthesis of C16 – C26 fatty acyl primers. The longer chain products of the FAS-II system are the precursors of mycolic acids (Rozwarski *et al.*, 1999, Schaeffer *et al.*, 2001). Briefly, upon synthesis of the short chain primers

(substrate) by the FAS-I system, these primers are transferred to the AcpM. The various acyl-AcpM (C_{16}-ACP) molecules are elongated using malonyl-coA by the FAS-II system components, which include β-ketoacyl-ACP synthase (KasA and KasB), enoyl- ACP reductase (InhA) and MabA (Yuan *et al.*, 1998b) resulting in the formation of the meromycolic acids (C54-C56). The meromycolate chain that is bound to the AcpM then undergoes some modification reactions, which include desaturation, methylation and oxygenation (Assellineau *et al.*, 2002; Barry III *et al.*, 1998 and Wheeler and Anderson, 1996). Yuan *et al.* (1998) showed that the cell free methylation of the meromycolate is inhibited by antibodies to AcpM implying that the mycolate modification occurs parallel with the synthesis of the AcpM-bound meromycolate chain thus the growing meromycolate chain remains attached to AcpM during modification and synthesis (Yuan *et al.* 1998b).

Some of the enzymes in the FAS-II system have been identified as targets of INH. *M. tuberculosis* InhA (Wheeler and Anderson, 1996) and KasA (Mdluli *et al.*, 1998b) have been pointed out as targets of the activated form of INH (Assellineau *et al.*, 2002; Parikh *et al.*, 2000; Parrish *et al.*, 2001; Slayden *et al.*, 2000b; Wei *et al.*, 2003). InhA catalyses NADH dependent reduction of *trans* double bond between positions C2 and C3 of the fatty acyl substrate of preferably 16 carbon atoms or greater, since this enzyme has specificity for C16 fatty acyl substrates (Marrakchi et al., 2000; Rozwarski *et al.*, 1999). Mycobacteria utilize the products of InhA catalysis to create the mycolic acids (Rozwarski *et al.*, 1998). Even though the connection between INH action and InhA inhibition is somewhat complicated, the general perception is that the activated form of INH is responsible for inhibition of this enzyme. Structural studies by Rozwarski *et al.* (1999), showed that upon activation of INH by KatG, the activated form of INH becomes covalently attached to the nicotinamide ring of the NADH bound within the active site of InhA, creating an NADH adduct that acts as a very tightly bound inhibitor. InhA binds this inhibitor with an affinity much higher than for NADH, resulting in complete inactivation of InhA *in vitro* and inhibition of mycolic acids synthesis (Chouchane *et al.*, 2000; Parikh *et al.*, 2000; Rozwarski *et al.*, 1999; Wei *et al.*, 2003). In contrast, the S94A mutation which is within the NADH binding region in INH-resistant InhA, has decreased

affinity for NADH (Parikh et al., 2000; Rozwarski *et al.*, 1998, 1999, Sacchettini and Blanchard, 1996; Wei *et al.*, 2003) and such enzymes are protected from activation or bind the INH-NAD adduct less effectively (Larsen *et al.*, 2002; Rozwarski *et al.*, 1999). INH-dependent inhibition of wild type-type InhA requires the presence of NADH, while inhibition of the S94A mutant occurs only when the concentration of NADH is increased, which implies that there is a correlation between the ability of the enzyme to bind NADH and to become inhibited by activated INH (Rozwarski *et al.*, 1998).

Because the complete mycolic acid appears to bear resemblance to cholesterol structure, there may well be an effect of the presence of cholesterol on the INH activity, as competitive inhibitors for the reactive centre of the enzyme that is responsible for mycolic acids synthesis.

3.2. Hypothesis and Aims of the study

Based on the possible structural mimicry between cholesterol and mycolic acid (discussed in Chapter 1), it was hypothesized that cholesterol, which may have the ability to accumulate onto the mycobacterial surface, might provide a selective target for CID that may enhance its mycobactericidal effect and efficiency of uptake into infected macrophages. Hence this part of the study aimed to:

1. Determine if the presence of cholesterol in the growth media has an effect on the normal growth of *M. tuberculosis*
2. Determine whether the presence of cholesterol affects the mycobactericidal activity of INH.

3.3. Materials

3.3.1. List of reagents

Adult bovine serum, fetal calf serum (Highveld Biologicals, Johannesburg, RSA)

Cholesterol (Sigma Chemical Co., St Louis, USA)

Chloroform, 99% purity (Saarchem, UniLAB, Krugersdorp, RSA)

Dimethyl sulphoxide for cell culture, (Sigma Chemical Co., St Louis, USA)

Isoniazid (Sigma-Aldrich, St Louis, USA)

Phosphatidyl choline (Sigma Chemical Co., St Louis, USA)

NaCl (Merck, Darmstadt, Germany)

3.3.2. List of apparatus

BACTEC performance test kit (Becton Dickinson and Co., Maryland, USA)

BACTEC 460 radiometric apparatus (Becton Dickinson, Johnston Laboratories, USA)

Branson sonifier, model B30 (Branson Sonic Power Co., USA)

3.3.3. List of disposables

Amber vials (Separations Pty Ltd, Randburg, RSA)

Alcohol swabs (TYCO Health care (PTY) LTD, Midrand, RSA)

Disposable pipette tips (Bibby Sterilin, Serowell; Bibby Sterilin Ltd, Stone, UK)

Membrane filters – 0.22 μm filter, hydrophobic, and– 0.2 μm membrane filters, CA-membrane + GF prefilter (Sartorius AG, Goettingen, Germany)

Sterile, disposable 50 ml and 10 ml centrifuge tubes (Bibby Sterilin Ltd, Stone, UK)

Insulin syringe (1 ml) with integrated 29G needles (B. braun Petzold GmgH, Switzerland)

7H12 Middlebrook TB medium culture vials (Becton Dickinson International, Belgium)

3.4. Methods

3.4.1. Culturing of Mycobacterium

The *Mycobacterium tuberculosis* H37Rv (Mtb H37Rv) strain was used in this study. Its susceptibility to test compounds was determined using the BACTEC 460 radiometric system. To culture the bacteria, frozen stock of Mtb H37Rv was thawed at 37^{O}C for about 60 seconds, 10-fold and 100-fold dilutions were made and 0.1 ml of this was injected into BACTEC 12B vials. All the vials were flushed with gas that contained 4-5% CO_2 for aerobic culturing as to maintain optimum culture conditions. The vials were incubated at 37^{O}C and read everyday at the same time until they gave a Growth Index (GI) of 300 – 500.

3.4.2. Drug susceptibility testing and MIC determination

The susceptibility testing was done using the BACTEC 460 radiometric apparatus as in chapter 2 section 2.4.6. Briefly, 0.1 ml of undiluted bacterial suspension was inoculated into the experimental vials, which contained 0.1 ml of INH dilution. INH was tested at various concentrations. Stock solution was prepared by dissolving INH in a 1:1 ratio of double distilled deionized water (dddH_2O) and dimethyl sulphoxide (DMSO) prior to use. The stock solution was then diluted with sterile saline and filter-sterilized through a 0.22 µm filter. The filter-sterilized solution was used to prepare the 10- and 100-fold dilutions in saline. For the susceptibility testing 0.1 ml of the test compound was injected into the BACTEC 12B vials. INH was tested in triplicates in each experiment performed. Drug-free control vials consisted of undiluted and 1:10 diluted bacterial inocula in saline. Negative control vials containing saline without any bacteria or drugs were included to check if any unwanted bacterial contamination occurred during the experiment. All the vials were flushed with gas that contained 4-5% CO_2 for aerobic culturing to maintain optimum culture conditions. The bacteria were then maintained at 37^{O}C. The vials were read daily until the GI of the undiluted bacterial inoculum reached 999.

3.4.3. Preparation of Liposomes

Liposomes used as one of the carriers of cholesterol were prepared as follows: Stocks of 100 mg/ml were prepared for both phosphatidyl choline (PC) and cholesterol (Chol) in cold chloroform. To prepare PC: Chol liposomes, 90 µl of PC stock solution were mixed with 30 µl of cholesterol stock solution. The "empty" liposomes were prepared with PC stock solution without any cholesterol. The liposomes were dried under a steam of N_2 gas at 85 ^{o}C for 20 – 30 minutes to evaporate the chloroform. To the dry liposomes, 2 ml of saline was added and then sonified at room temperature for 5 minutes at 20% output and 2% duty cycle with the Branson sonifier. An additional control was setup for cholesterol not taken up in liposomes and this was prepared as above but using cholesterol stock solution. The sonified liposomes were then autoclaved for 20 min. at 121 ^{o}C to sterilize them. The required dilutions of liposomes were made in sterile saline.

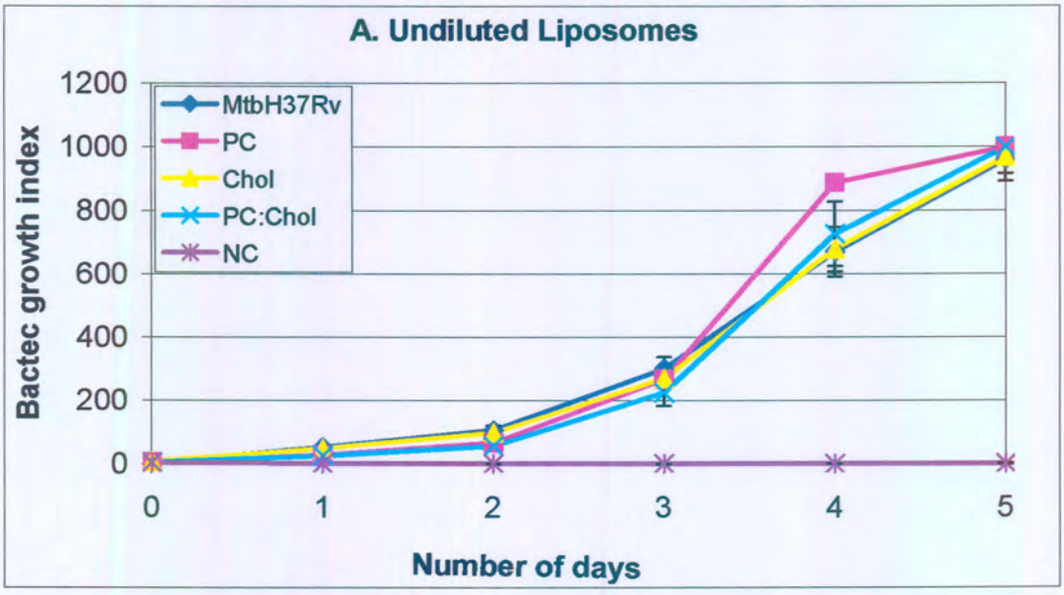
Another source of cholesterol used was adult bovine serum (ABS) and fetal calf serum (FCS). The sera were filtered through 0.2 µm filters. Serum dilutions of 1% and 10% were prepared with sterile saline. Liposomes, ABS and FCS were then used for susceptibility studies performed in the presence of cholesterol. Quantities of 0.1 ml of liposome dilutions were injected in the BACTEC vials.

3.5. Results

3.5.1. Susceptibility testing of isoniazid (INH) in the presence of cholesterol

To test if cholesterol has any effect on the activity of isoniazid against *M. tuberculosis*, the bacteria were cultured in a cholesterol-enriched media. Cholesterol was introduced to the media in a form of phosphatidylcholine:cholesterol (PC:Chol) liposomes and cholesterol without any PC both containing cholesterol at a concentration of 3.9 mM. A preliminary experiment was done in order to see if the presence of PC:Chol liposomes did not have any effect on the growth of *M. tuberculosis*. The results shown in Fig. 3.1 indicate that the cholesterol containing liposomes did not affect the bacterial growth curve while empty liposomes (PC) caused a slight increase in growth of the bacteria.

Having thus proven that PC:Chol liposomes could be used for the susceptibility testing, the activity of INH at its MIC, 1/10 and 1/100 [MIC] was then tested on *M. tuberculosis* in the presence of undiluted cholesterol containing liposomes with cholesterol at a concentration of 3.9 mM. From this experiment it was clear that in the presence of cholesterol liposomes there is no growth inhibition even upon INH treatment at its MIC as indicated in Fig. 3.2.A, and the same was observed with 1/10 and 1/100 [MIC] of INH as shown in Fig. 3.2.B & C. Lack of inhibition by INH at its MIC suggested that the presence of cholesterol inhibits the biological activity of INH.



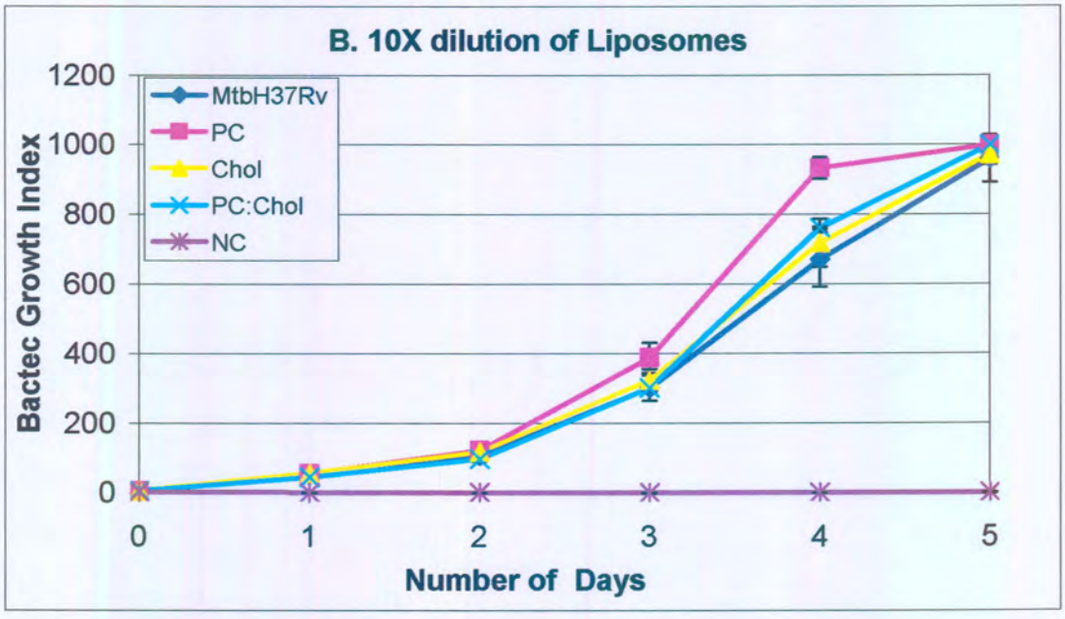
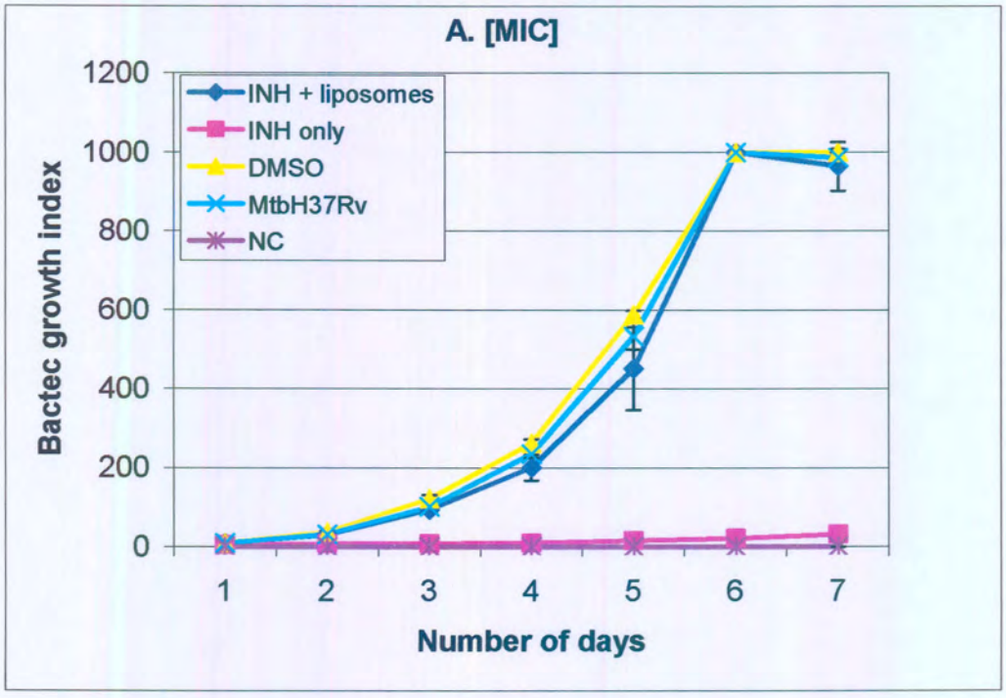
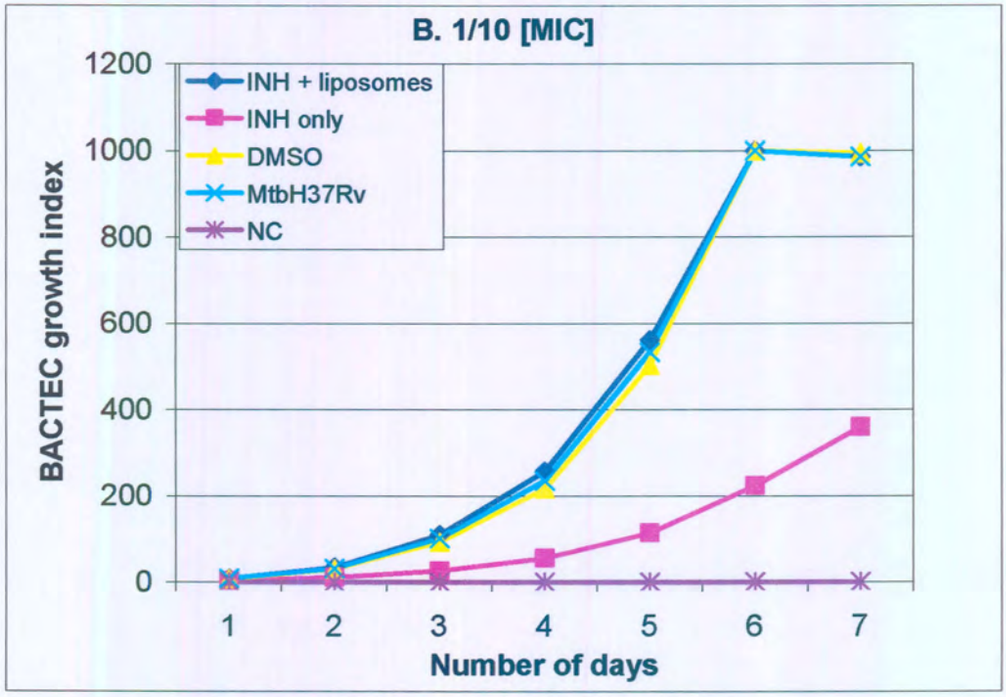


Figure 3.1. Growth curve of *M. tuberculosis* H37Rv cultured in liposome-containing media. Liposomes were introduced into the media as undiluted (A) and 10X diluted (B) empty liposomes [PC], cholesterol containing [PC:Chol] liposomes and cholesterol without PC [Chol]. NC represents saline without bacteria and MtbH37Rv represent untreated bacteria. The results represent the mean of one experiment done in triplicate.


A. [MIC]
Bactec growth index
1200
1000
800
600
400
200
0
INH + liposomes
INH only
DMSO
MtbH37Rv
NC
1
2
3
4
5
6
7
Number of days


B. 1/10 [MIC]
BACTEC growth index
1200
1000
800
600
400
200
0
INH + liposomes
INH only
DMSO
MtbH37Rv
NC
1
2
3
4
5
6
7
Number of days

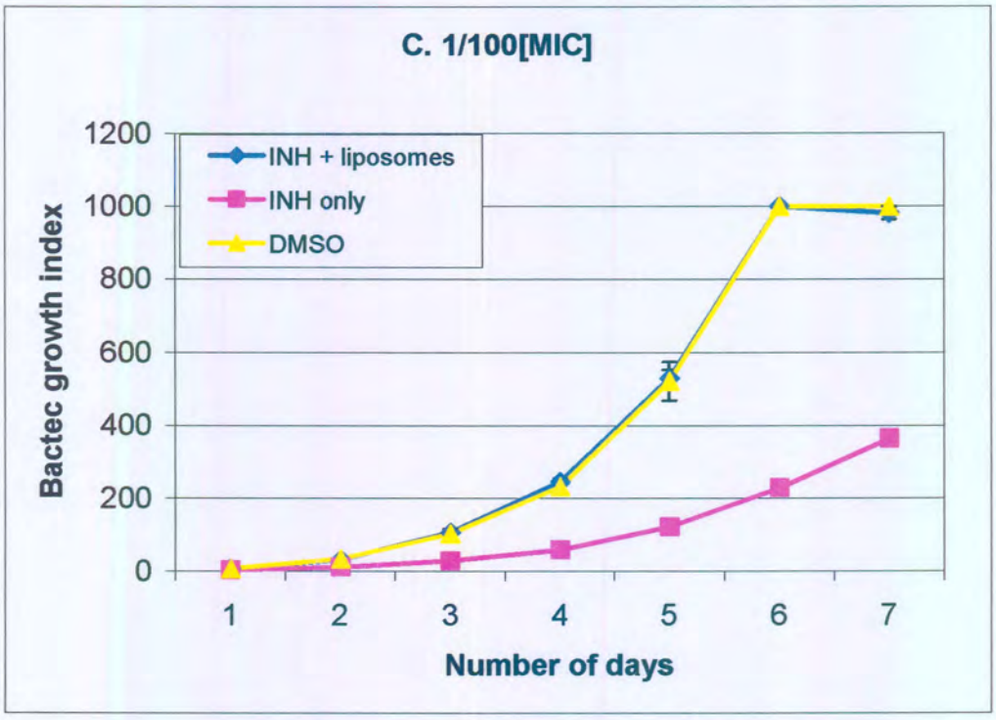


Figure 3.2. Altered activity of INH by the presence of PC:Chol liposomes in the growth media. Susceptibility testing of *M.tuberculosis* H37Rv upon treatment with INH at MIC (A), 1/10 (B) and 1/100 MIC (C) in the presence or absence of undiluted PC:Chol liposomes. NC represents saline without bacteria and MtbH37Rv represent untreated bacteria. The results represent the mean of one experiment done in triplicate.

3.5.2. Determination of the best source of cholesterol

One possible explanation for the abrogation of INH activity in the presence of cholesterol-liposomes could be that the complex formation of free cholesterol on mycolic acids in the cell wall of the mycobacteria affects the cell wall permeability to INH. Lower concentrations of free cholesterol, or cholesterol presented in its normal lipoprotein environment of the plasma may be able to leave the permeability of the cell wall for INH unaffected. To investigate this, adult bovine serum (ABS) and fetal calf serum (FCS) were tested for their ability to serve as cholesterol sources. The mycobacteria were cultured in growth media containing either 1% ABS, 1% FCS or 10%

FCS. The results in Fig. 3.3 show that the presence of 1% ABS in the growth media caused growth inhibition, while the presence of 10% FCS and 1% FCS showed normal bacterial growth, hence this serum was used for further investigations.

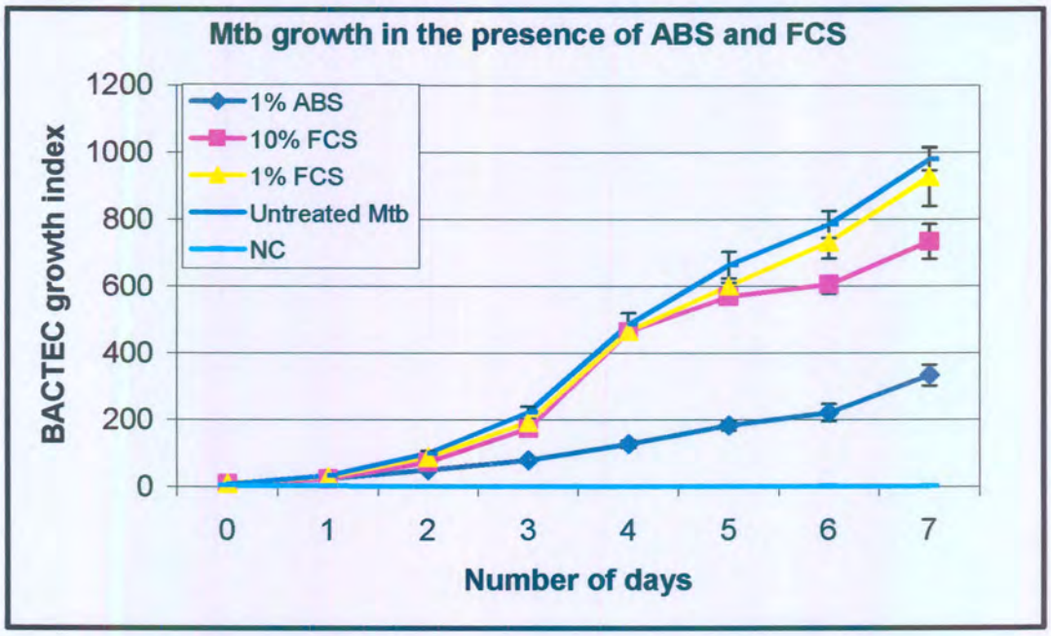


Figure 3.3. Growth curve of *M. tuberculosis* H37Rv cultured in media containing serum. *M tuberculosis* H37Rv was grown in the absence or presence of 1% ABS, 1%FCS and 10% FCS. NC represents saline without bacteria or serum and MtbH37Rv represent bacteria grown without serum or INH. The results represent the mean of one experiment done in triplicate.

3.5.3. Inhibitory effects of cholesterol in its physiological state on INH efficiency

The effect of cholesterol was further investigated in the presence of FCS, which was used as the source of cholesterol. The bacteria were cultured in the presence of 1% FCS and 10% FCS and treated with INH at its MIC. The results obtained (Fig. 3.4) showed an inhibition of INH activity by the presence of the serum. There was an indication that the cholesterol in the FCS has a dose dependent inhibitory effect on the activity of INH, since the degree of inhibition increased as the percentage of the serum increased, with the 10%

FCS showing more inhibition of INH efficiency. Hence it was necessary to determine the concentration of cholesterol in the FCS. The serum was taken to a pathologist for determination of the concentration of total cholesterol in serum and the results showed that the FCS contained 0.8mM cholesterol while the ABS contained 4.2mM cholesterol.

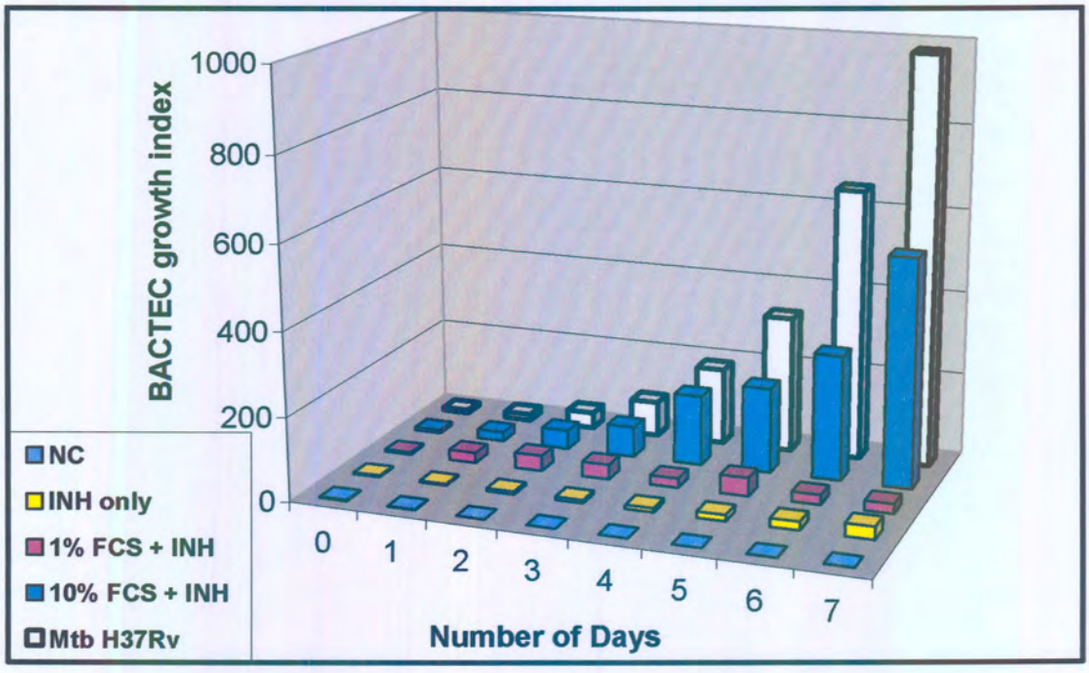
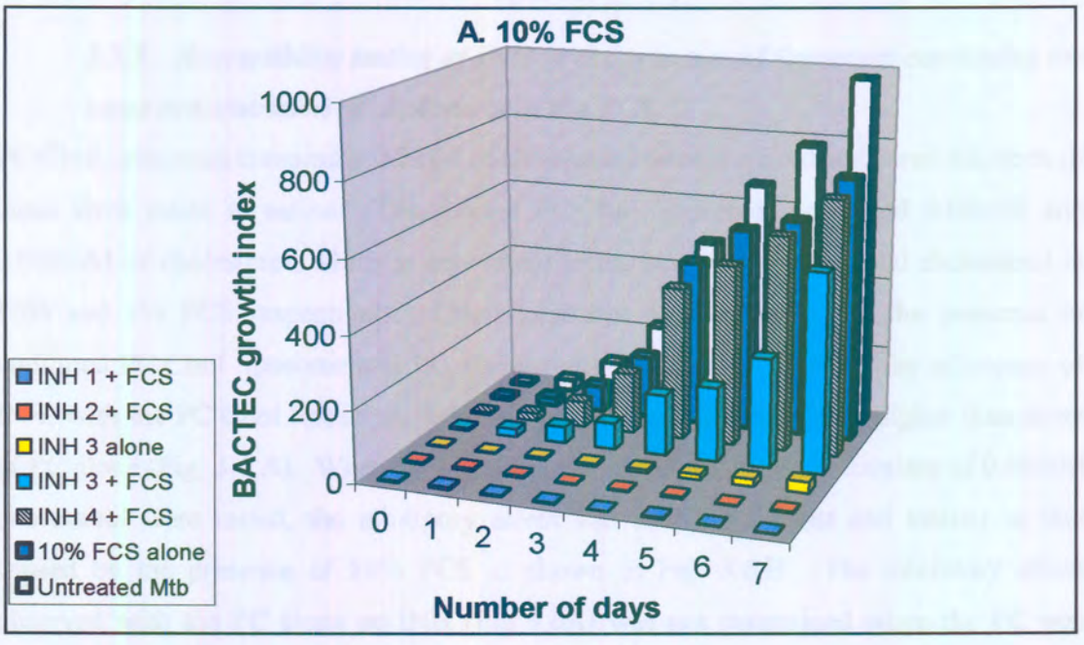


Figure 3.4. Inhibitory effects of serum on the efficiency of INH. The activity of INH against *M. tuberculosis* H37Rv was tested at its MIC in the presence or absence of 1% FCS and 10% FCS. NC represents saline without bacteria or serum and MtbH37Rv represent bacteria grown without serum or INH. The results represent the mean of one experiment done in triplicate.

3.5.4. Determination of the MIC of INH in the presence of 10% FCS and 1% FCS

To determine the concentration of INH that could still inhibit *Mycobacterium tuberculosis* growth in the presence of cholesterol, varying concentrations of INH were tested in the presence of 1% and 10% FCS. As in the previous experiment, the 10% FCS showed significant inhibition of the INH efficiency at its MIC while higher concentrations of INH (3.0 and 0.3 μg/ml) were able to overcome the effect of the serum. In 10% FCS, 0.3 μg/ml INH showed the same inhibitory effects as INH at its MIC without 10% FCS, (Fig. 3.5.A). The 1% FCS did not affect INH efficiency as the 10% FCS did, since the INH at MIC in the presence of this serum concentration showed the same inhibitory effect as IHN at MIC without 1% FCS (Fig. 3.5.B). These results further support the suggestion that cholesterol in the serum has a dose dependent inhibitory effect on the activity of INH. The results obtained (Fig. 3.5.A) indicate that the MIC of INH in 10% FCS lies between 0.3 μg/ml and 0.03 μg/ml.

To confirm that cholesterol in the serum is responsible for the loss of INH efficiency, the concentration of cholesterol in the liposomes was optimized to the same concentration as that in 10% FCS and 1% FCS and this was compared with empty liposomes containing PC only.



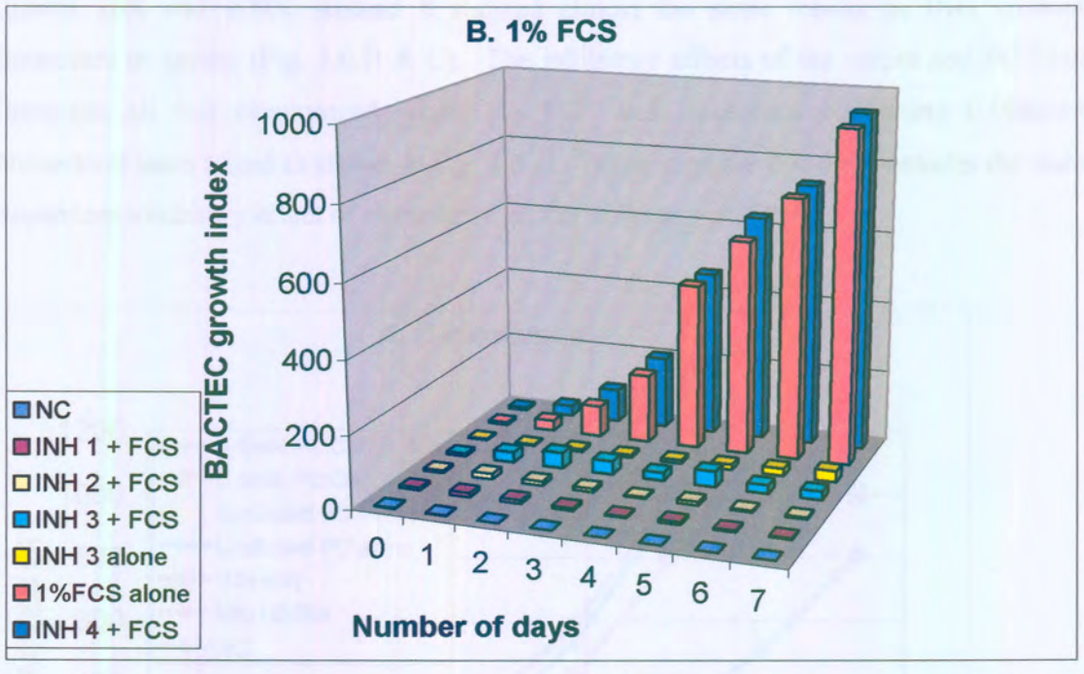
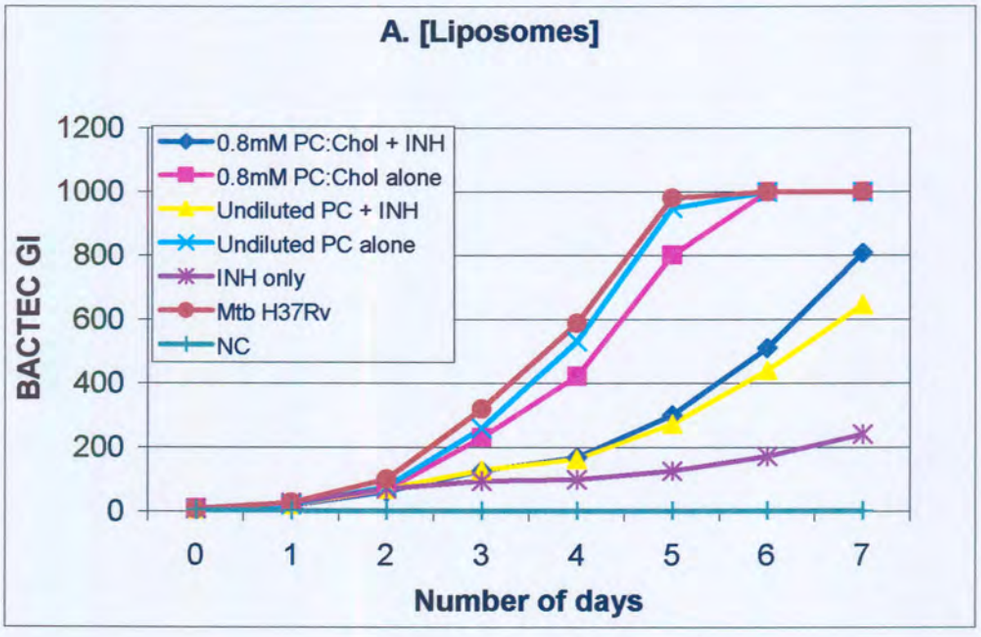


Figure 3.5. Determination of the MIC of INH in the presence of serum. *M. tuberculosis* H37Rv was cultured in a media containing 10% FCS (A) and 1% FCS (B) as sources of cholesterol and treated with INH at varying concentrations, INH 1= 3.0 μg/ml, INH 2= 0.3 μg/ml, INH 3= 0.03 μg/ml and INH 4= 0.003 μg/ml. The results represent the mean of one experiment done in triplicates.

3.5.5. Susceptibility testing of INH in the presence of liposomes containing the same concentration of cholesterol as the FCS.

PC:Chol liposomes containing 0.8mM of cholesterol were prepared and serial dilutions of these were made in saline. The diluted PC:Chol liposomes contained 0.08mM and 0.008mM of cholesterol, which is equivalent to the concentration of total cholesterol in 10% and 1% FCS respectively. Upon treatment of the bacteria in the presence of undiluted PC:Chol liposome and PC, cholesterol was shown to inhibit the efficiency of INH, with the PC:Chol liposomes exhibiting inhibitory effects slightly higher than those of PC alone (Fig. 3.6.A). When PC:Chol liposomes containing an equivalent of 0.08mM cholesterol were tested, the inhibitory effect was more prominent and similar to that caused by the presence of 10% FCS as shown in Fig. 3.6.B. The inhibitory effect observed with the PC alone on INH (Fig 3.6A) was not maintained when the PC was diluted 10X and 100X; instead it showed almost the same results as INH without liposomes or serum (Fig. 3.6.B & C). The inhibitory effects of the serum and PC:Chol liposome all but disappeared when 1% FCS and liposomes containing 0.008mM cholesterol were tested as shown in Fig. 2.6.C. Taken together this demonstrates the dose dependent inhibitory effect of cholesterol on the efficiency of INH.



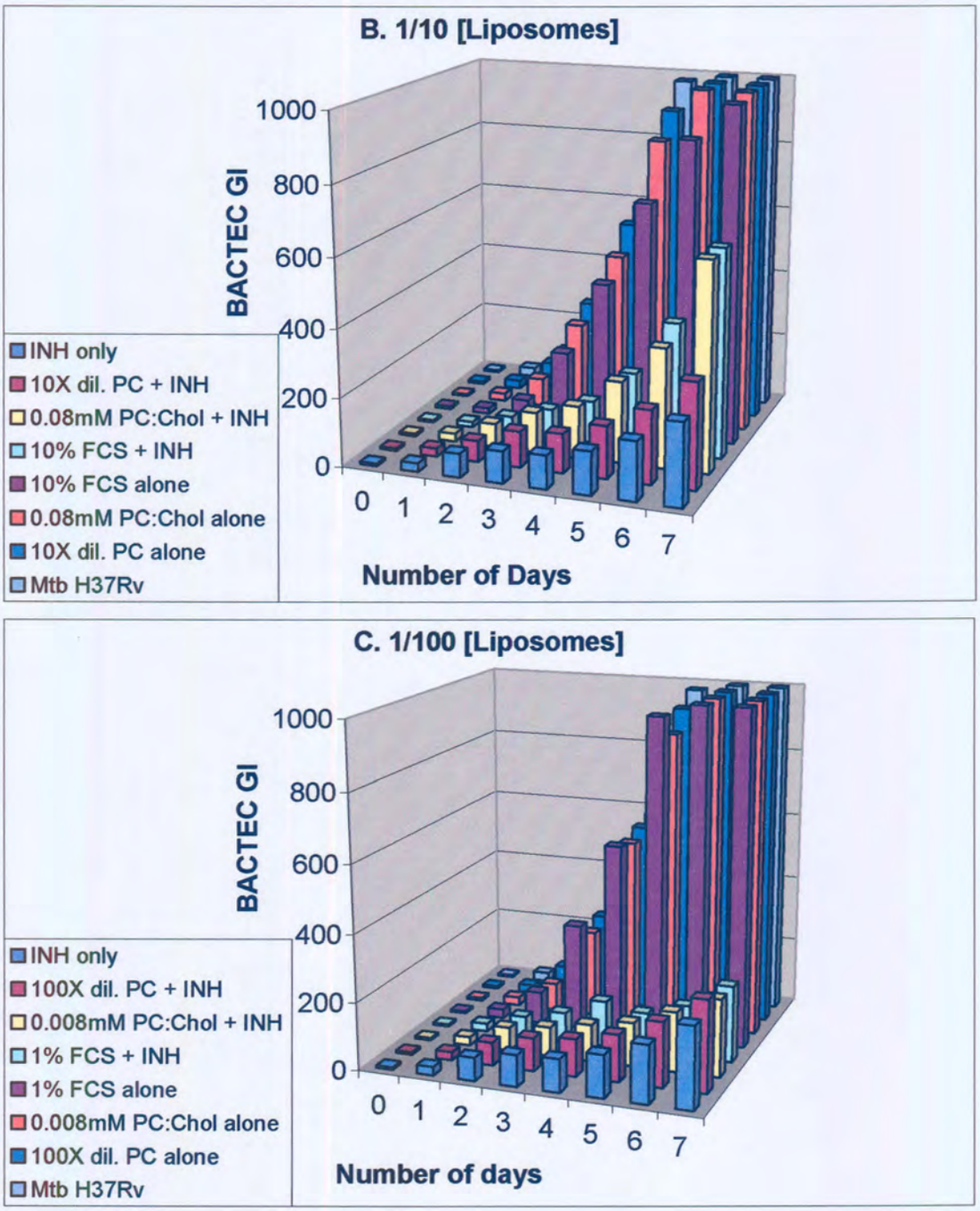


Figure 3.6. Inhibitory effects of liposomes on the efficiency of INH. The activity of INH against *M. tuberculosis* H37Rv was tested at its MIC in the absence or presence of (A) undiluted PC and PC:Chol (0.8 mM chol.) liposomes; (B) 10X diluted PC, PC:Chol (0.08 mM chol.) liposomes and 10% FCS; and (C) 100X diluted PC, PC:Chol (0.008 mM chol.) liposomes and 1%

FCS. Mtb H37Rv represents bacteria without INH, liposomes or serum. The results represent the mean of one experiment done in triplicates.

3.5. Discussion

The proposed mimicry between MA and cholesterol suggested that cholesterol might associate with MA on the bacterial cell surface and form a MA-cholesterol complex to be targeted by the CID. Since the pharmacophore to be delivered by the AmB is INH it was also important to investigate the effect of the presence of cholesterol in the growth media on the efficiency of INH against *M. tuberculosis in vitro*. Thus, the bacteria had to be cultured in growth media containing cholesterol. Cholesterol was introduced into the growth medium carried in liposomes. In this way the amount of free cholesterol introduced into the media could be controlled. In the body however, cholesterol is transported in serum lipoproteins. To establish whether observations made for cholesterol in liposomes will also apply in the physiological environment, FCS and ABS were also used as carriers of cholesterol.

Normal bacterial growth was observed in the presence of both cholesterol containing liposomes and FCS while the presence of the ABS in the growth media inhibited the growth of the bacteria. Hence further investigations were done by comparing cholesterol-containing liposomes to FCS only.

In this study, it was shown that the presence of cholesterol, either in liposomes or in fetal calf serum, negatively affected the mycobactericidal activity of INH *in vitro*. In the presence of 10% FCS, *Mycobacteria* grew normally but upon treatment with INH, the inhibitory effects of INH were decreased approximately ten-fold to reach MIC. Feigin *et al.*, (1997) showed that the presence of cholesterol in lipid bilayers greatly reduces their sensitivity to an anti-fungal agent syringomycin E. Early studies proposed that pathogenic mycobacterium species grown in a media containing lecithin-cholesterol liposomes utilized lecithin as a source of carbon but not cholesterol, which was esterified indicating that the tubercle bacilli interacted with the lecithin-cholesterol liposomes during growth *in vitro* (Kondo and Kanai, 1976b). This lead to the suggestion that cholesterol in the liposomes associated with the growing bacilli on the cell wall surface. The results of Siko, (2002) clearly showed that cholesterol interacts with the mycolic acids of *M. tuberculosis*.

Our results could imply that the interaction of cholesterol with the mycolic acids on the cell wall of the bacterium is in such a way that they both increase the hydrophobicity and further lower the permeability of the cell wall to INH, thus reducing its efficiency against *Mycobacterium tuberculosis*. The hydrophobicity of the cell wall is also known to contribute to the low permeability or slow uptake of hydrophilic compounds (Draper, 1998; and Brennan and Nikaido, 1995). Interestingly, like the mycolic acids in the mycobacterium cell wall, cholesterol, which is the major sterol in the eukaryotic cell membranes (Barenholz, 2002; Miao *et al.*, 2002), has been shown to contribute to low fluidity and permeability of these membranes (Ohvo-Rekilä *et al.*, 2002 and Miao *et al.*, 2002) wherein the fluidity in biomembranes could be diminished particularly by enrichment with cholesterol (Bastiaanse *et al.*, 1997). Since the uptake of hydrophilic molecules had been shown to be limited by the permeability barrier and the hydrophobicity of the wall (Jarlier and Nikaido, 1990; and Mdluli *et al.*, 1998), the uptake of such molecules is likely to be via a water-filled porin molecule embedded within the cell wall. The presence and function of the porin molecule may be influenced by mycolate composition. The ketomycolate has been shown to have an effect on the function of these integral cell wall proteins (Trais *et al.*, 1992; Trais and Benz, 1993; and Yuan *et al.*, 1998). In addition to resistance to INH due to mutations in the *katG* gene (Chen and Bishai, 1998), it has been suggested that resistance to this drug is related to failure of the drug to penetrate the mycobacterial cell wall or be taken up by the organism (Aboul-Fadl and Hassanin, 1999).

Even though the serum contains other components, Av-Gay and Sobouti (2000) showed that pathogenic mycobacterium species (*Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG) accumulated cholesterol but did not metabolize it or use it as a source of carbon, implying that cholesterol is utilized by the bacterium for other activities. The non-metabolized cholesterol might be utilized by the bacterium for uptake by macrophages (Gatfield and Pieters, 2000). Gatfield and Pieters (2000) showed that *Mycobacterium tuberculosis* requires cholesterol in the membranes of target cells to effect uptake of the organism. This was observed only with *Mycobacterium tuberculosis* and not with other organisms (Pieters and Gatfield, 2002; and Gatfield and Pieters, 2000).

Treatment of macrophages with liposomes containing mycolic acids resulted in foam cell formation accompanied by increased levels of cholesterol content (Korf, 2002). Kondo and Kanai (1976b) showed that macrophages infected with *M. tuberculosis* had much higher concentrations of cholesterol and cholesterol esters as compared to non-infected cells (Kondo and Kanai 1976b). Cholesterol appears to be essential in the infection of macrophages by *M. tuberculosis* and the latter's subsequent survival inside the host cell (Gatfield and Pieters, 2000; and Pieters and Gatfield, 2002). Thus through the accumulation of cholesterol onto the cell wall mycolic acids, the bacilli can mimic cholesterol-containing lipoprotein particles, hence enhancing their uptake through the scavenger receptors (Av-Gay and Sobouti 2000).

It was also observed that cholesterol in the liposomes did not affect the growth of *M. tuberculosis*. The uptake of essential nutrients by the bacteria was therefore not affected by cholesterol. Hence, cholesterol might posses yet another way of protecting the bacilli from the actions of INH. One such possibility might be the ability of cholesterol to recognize or bind to the INH target enzymes, which are responsible for the synthesis of mycolic acids. One such example is InhA, which is the target of the activated form of INH (isonicotinic acyl). This enzyme catalyzes an NADP dependent reduction of AcpM – bound 2-*trans*-enoyl fatty acids chains with 16 carbon atoms or more. This enzymic activity corresponds to the last step of each two-carbon-elongation of fatty acid biosynthesis pathway (Marrakchi et al., 2000). The next chapter provides an in-depth discussion of the possible mechanism of direct inhibition of INH activity by competitive binding to the active site of this enzyme.

CHAPTER 4

CONCLUDING DISCUSSION

The discovery of antibiotics and their application since the 1940's against *M. tuberculosis* led to a substantial reduction in the incidence of tuberculosis with the widespread view that the war against the disease was won. Three decades later, however TB incidences increased worldwide again (Davis *et al.*, 1998), accompanied by multi-drug resistance and later AIDS (Whitney and Wainberg, 2002). This called for development of novel anti-TB agents and improvement of the efficiency of existing agents. The reality is that not a single anti-mycobacterial agent has been accepted for widespread use since rifampin in the1960's (Barry III, 1997). Developing drugs against *M. tuberculosis* is quite a challenge, due to the complicated physiological and biochemical make-up of this organism. Some of the factors that have to be taken into consideration include the importance of the organism's cell wall in protection against chemotherapeutic agents and the metabolic state of the organism, since the bacilli can be both in an actively growing state or latent state in the host body. The complex pathobiology of *M. tuberculosis*, which permits the organism to persist in a dormant stage for months or years on end, renders treatment difficult (O'Brein and Nunn, 2001).

The complexity of the organism calls for different approaches in developing new anti-TB agents. In consideration of the natural resistance provided by the cell wall, the hydrophobicity of active compounds has been increased by using prodrugs that have the ability to release the active compound (Aboul-Fadl and Hassanin, 1999; Giannola *et al.*, 1992; Klopman *et al.*, 1996). In addition to improving the cell wall penetration by the active compound, prodrugs can also be made to target the drug to a specific receptor on the bacterial surface or to the infected macrophages (Roseeuw *et al.*, 1999; Zhou *et al.*, 2002). One such receptor may be cholesterol, as it has been shown that the bacilli use the cholesterol to facilitate entry into the macrophages (Gatfield and Pieters, 2000) and sustain within the cell (Aínsa et al., 2001; Ferrari *et al.*, 1999).

In this study the contribution of cholesterol towards the pathogenesis of this bacilli was exploited in an attempt to develop new anti-mycobacterial agents. The role of cholesterol was investigated in two ways, firstly the use of cholesterol-like molecules on the cell wall of *M. tuberculosis* as targets for new anti-TB agents, and second cholesterol's effect on the efficiency of anti-mycobacterial agent INH.

On the use of cholesterol like molecules on the mycobacterial cell surface as the target for anti-TB agents, an INH-AmB non-covalent conjugate was designed and its activity tested against *M. tuberculosis* H37Rv. In this case AmB was used as a prodrug, which is expected to improve the efficiency of INH while targeting it to the bacterial cell surface. Prodrugs are mainly bioreversable forms of the drug that have the ability to modulate the pharmacokinetic properties and cellular permeability of a drug. In addition to this, a prodrug must be able to liberate or release the parent drug through chemical or enzymic degradation preferably at the site of infection (Aboul-Fadl and Hassanin, 1999; Roseeuw *et al.*, 1999). However, unless the produg can be readily removed *in vivo*, it will not function as a true prodrug (Zhou *et al.*, 2002). Here the ability of AmB to release the INH toxophore upon interaction with its target molecules was demonstrated, albeit at higher, rather than lower MIC of INH. Prodrugs can also be used as delivery systems capable of specifically targeting the drugs into the macrophages in order to increase the bioavailiabity of drugs since adequate concentrations of the drug are required for it to be effective (Roseeuw *et al.*, 1999; Zhou *et al.*, 2002). The bioavailability of a drug is dependent on three basic parameters: solubility, permeability and the metabolic half-life which in turn affects intestinal absorption, the initial hurdle in oral delivery as well as toxicity (Barry III *et al.*, 2000 Gianola *et al.*, 1992). Due to the poor control over the synthesis of the non-covalent INH-AmB conjugate, its efficiency as a delivery system was not tested *in vivo* in the course of this study.

Roseeuw *et al.* (1999) used polymeric prodrugs to improve the efficiency of the antibiotic norfloxacin. Norfloxacin was linked to the tetrapeptide derivative gly-phe-gly-gly as the α-subsistent of the C-terminal glycine residue and subsequently coupled onto mannosylated dextran known to be recognized by macrophages. By investigating the *in*

vitro and *in vivo* activities of the compound against *M. tuberculosis*, it was found that the uptake of the prodrug by cells occurred by endocytosis, thus allowing for cell specific targeting of the drug and intracellular controlled release at the target site. The modified norfloxacin showed improved activity and efficiency against *M. tuberculosis* with more activity against organisms in the liver (Roseeuw *et al.*, 1999). Since AmB was used here as a prodrug to target INH to the cholesterol-like molecules on the mycobacterial cell wall, and since mycolic acid treated macrophages have been shown to accumulate cholesterol (Korf, 2002), the anticipation is the INH-AmB non-covalent conjugate will also be able to penetrate the infected macrophages to pursue the inactivation of intracellular *M. tuberculosis*, better than INH on its own. This, however, can only be tested once a new preparation of covalently linked INH-AmB becomes available.

Another draw-back in the treatment of TB is the toxicity and severe side-effects of the available drugs. Hence, drug-carriers to target drugs to infected cells are needed in order to maximize the antimycobacterial activity of antibiotics while minimizing their toxicity (Oh *et al.*, 1995). Incorporation of drugs into liposomes has also been used as a unique biocompatible vehicle for drug delivery without any side-effects. Since the prospect of finding newer and more effective anti-TB drugs than existing ones is small, existing anti-mycobacterial agents are being tested in liposomal form for the therapeutic treatment of *M. tuberculosis* and *M. avium* complex infections (Deol and Khuller, 1997; Deol *et al.*, 1997 Quenelle *et al.*, 1999). Liposomes allow the use of large doses of the drug with much lower side-effects and toxicity, which has been confirmed to be the case with liposomal delivery of AmB (Ehrenfreund-kleinman *et al.*, 2002). The ability of liposomes to reduce the toxicity of drugs has also been demonstrated for isoniazid and rifampicin in a study by Deol and Khuller (1997). In that study the cytotoxicity of liposome-encapsulated drugs was tested *in vitro* in peritoneal macrophages and *in vivo* in mice and was found to be less than the toxicity of free drugs (Deol and Khuller, 1997).

In addition to being able to lower the toxicity of drugs, liposomes have the ability to steady the release of encapsulated contents, which is important in increasing the bioavailibility of drugs to the infected cells. Liposomes are taken up avidly by

macrophages and are targeted naturally to the reticuloendothelial system (RES), which include macrophages of the liver and spleen (Deol and Khuller 1997; Oh *et al.*, 1995). Hence for *M. tuberculosis* infections, liposomes that target alveolar macrophages are required. Deol and Khuller (1997) prepared such liposomes by tagging normal liposomes with *O*-stearylamylopectin (O-SAP), which resulted in increased affinity of these liposomes towards lung tissue of mice with the lungs accumulating more of these liposomes than the RES (Deol and Khuller 1997). It is envisaged that the effort to target anti-TB drugs to cholesterol will include liposome delivery of combinations of AmB and anti-TB drugs in future extension of the work of this dissertation.

Treating latent TB infection is very important in eradicating the tubercle bacilli since this will significantly lower the size of human population infected with latent bacteria. This will decrease the number of reactivation cases that might occur due to infections like AIDS and reduce the size of potential reservoirs (Betts et al., 2002; Heym and Cole, 1997). Currently, treating latent infection is not effective, since it is hampered by lack of understanding of the metabolism of the bacterium while in the semi-dormant or latent form. A few of the available drugs are active against latent bacteria. Rifampin that has significant early bactericidal effect on metabolically active bacilli, also exhibit excellent late sterilizing action on semi-dormant organisms (Barry III, 1997; Somoskovi *et al.*, 2001; Van Loenhout-Rooyackers and Veen, 1998) undergoing short bursts of metabolic activity. Another drug with excellent sterilizing activity is pyrazinamide. This explains why introduction of these two compounds into the routine TB treatment reduced the treatment duration from 9 months to 6 months (Somoskovi *et al.*, 2001). Hence, as a way to eliminate latent bacteria, the toxophore in the complex we studied, INH-AmB conjugate can be replaced by either of these compounds. Prior to this, the ability of AmB to interact with the mycolic acid species or cholesterol-MA complex should be studied. The replacement of the toxophore can be accompanied by incorporation of the AmB conjugated anti-mycobactericidals into liposomes to see if this improves the targetability of the AmB conjugated antimycobactericidal complexes.

On the role of cholesterol on the efficiency of INH it was shown that cholesterol negatively affected the efficiency of this drug. Upon interaction of cholesterol with the mycolic acids (Siko, 2002) there could be an effect on the physical properties of the bacterial cell wall. This interaction could be in such a way that the permeability of the cell wall is lowered and its hydrophobicity increased which in turn affects the uptake of INH which is a hydrophilic compound, hence this results in a decreased activity of INH. This could be investigated by examining the fluidity of the cell wall of bacteria (Yuan *et al.*, 1998) cultured in the presence of cholesterol. A widely used method to study this is the uptake of chenodeoxycholate that has been widely used as a marker of the cell wall permeability in strains of *Mycobacterium* (Liu *et al.*, 1996; Yuan *et al.*, 1998). Thus, the ability of cholesterol to lower the permeability and the fluidity of *M. tuberculosis* cell wall can be investigated by studying the uptake of chenodeoxycholate by *M. tuberculosis* cultured in the presence of cholesterol. Another approach to investigating the changes in the cell wall properties is the examination of the cell wall ultrastructure by transmission electron microscopy. The cell wall ultrastructure of mycobacteria is characterized by a typical thick electron-transparent zone outside the plasma membrane, which constitutes the hydrophobic domain of the cell wall dominated by the mycolic acids. This layer has a thickness of about 9-10 nm. The transparency to the electrons in the ultrasructure is mainly due to the extremely hydrophobic nature of the mycolic acids, which prevent water soluble, electron dense heavy metals salts normally used as stains to penetrate the cell wall (Brennan and Nikaido, 1995; Wang *et al.*, 2000). Thus the extent to which these stains penetrate the cell wall can be studied in *M. tuberculosis* cultured in cholesterol to indicate any changes in the ordered stacking and permeability of the mycolic acids layer of the cell wall.

Another possibility for the loss of INH activity due to the presence of cholesterol might be that cholesterol, that mimics the structure of the mycolic acids, is recognized by the enzyme(s) that synthesize mycolic acids and that are INH targets. It could be that the INH target enzyme(s) have sterol recognizing motifs or belong to the same family of enzymes as some of the sterol metabolizing enzymes such that cholesterol competes with INH for binding to these enzymes. Two enzymes that have been identified as INH

targets, InhA and KasA both form part of the FAS-II system. InhA of *M. tuberculosis*, which is an enoyl-ACP reductase, has been shown to be a member of the short chain dehydrogenase/reductase (SDR) family of enzymes. In addition to the FAS-II system enoyl-ACP reductases, numerous steroid dehydrogenases have also been shown to be the members of this family (Benach *et al.*, 2002; Oppermann *et al.*, 2003; Parikh *et al.*, 1999; Rozwarski *et al.*, 1999. The members of this family are important in metabolism of steroids and bile acids, beta-oxidation and retinal metabolism. This family consists of many enzymes of different substrate specificities that act on steroids, prostaglandins, aliphatic alcohols and xenobiotics. The sequence identity in pair-wise comparison between different SDR enzymes is very low (often 10-30%), but the 3D structures display a highly similar α/β folding pattern. Most SDR enzymes have a core structure with 250-350 residues in length, frequently with N- or C-terminal transmembrane domains or signal peptides or form part of multifunctional complexes (Filling *et al.*, 2002, Oppermann *et al.*, 2003). Enzymes of this family catalyse NAD(P)(H)-dependent steroid oxidation/reduction reactions, and bind NAD(P)(H) in a motif known as the Rossmann-fold, which is the co-factor binding site in the majority of dehydrogenases (Brown *et al.*, 2003; Filling *et al.*, 2002, Kallberg *et al.*, 2002). SDR enzymes consist of a conserved sequence region at the active site with a triad of catalytically important residues made up of Ser, Tyr, and Lys of which Tyr is the most conserved in the whole family (Benach *et al.*, 2002; Filling *et al.*, 2002, Rozwarski *et al.*, 1999). The tyrosine and lysine residues of the catalytic triad are strictly conserved and therefore mark the location of a consensus sequence (Tyr-X-X-X-Lys), where the separation between the tyrosine and lysine can vary from three to seven residues. Among the various crystal structures of members of the SDR family, the location of the lysine residue is nearly identical, whereas the position of the tyrosine residue has a tendency to vary somewhat among different SDR family members (Rozwarski *et al.*, 1999).

The 7α-hydroxysteriod dehydrogenase (7αHSDH) of *E. coli*, which is a steroid dehydrogenase, catalyzes the NAD^+-dependent dehydrogenation of the hydroxyl group at position seven on the steroid skeleton of bile acids. Amino acid sequence alignment of *M. tuberculosis* InhA with *E. coli* 7αHSDH shows 22% identity. Rozwarski *et al.*,

(1999) studied the crystal structure of InhA in complex with NAD^+ and a C16 substrate and found that the C16 fatty acyl substrate binds to InhA in nearly the same location that the bile acid substrate binds to *E. coli* 7αHSDH. The 7αHSDH of *E. coli* possesses an SDR family catalytic triad of Ser^{146}-Tyr^{159}-Lys^{163} while InhA possesses Phe^{149}-Tyr^{158}-Lys^{165} as its SDR family catalytic triad. The tyrosine and serine residues of *E. coli* 7αHSDH directly interact with the bile acid substrate OH-group, whereas the lysine residue interacts with the nicotinamide ribose of the substrate NAD^+. It was shown that the side chain of Tyr^{158} of InhA interacts with the C16 fatty acyl substrate thioester carbonyl oxygen while the side chain of Lys^{165} interacts with the 3'-hydroxy oxygen of the nicotinamide ribose of NAD^+ and this was found to be consistent with the interactions in other SDR family members (Rozwarski *et al.*, 1999). This was further verified by Parrikh *et al.*, (1999) who showed that K165A and K165M mutants of InhA were unable to bind NADH, indicating that K165 has a primary role in binding NADH. Thus this enzyme that prefers molecules with 16 carbon atoms or more as substrates and belongs to the same family as other steroid dehydrogenases that are capable of metabolizing sterols might be able to interact with cholesterol in such a way that the activated form of INH cannot bind to the enzyme, leading to prevention of INH activity against the bacilli presenting yet another way in which the bacilli can use the host lipids to protect itself.

Rozwarski *et al.* (1998), studying the crystal structure of an InhA binary complex (InhA and isonicotinic-acyl-NADH), showed that the formation of the isonicotinic-acyl-NADH adduct, which is responsible for the inhibition of InhA occurs within the active site of this enzyme. The crystal structure of INH-inhibited InhA revealed that the location and the orientation of the isonicotinic-acyl group are complementary to the InhA amino acids side chains that surround a specific binding pocket for the isonicotinic-acyl group. When comparing the crystal structure of InhA bound to NADH to InhA bound to isonicotinic-acyl-NADH it was revealed that the only significant difference in the latter is the location of the side chain of Phe^{149}, that had rotated ~90^O to form an aromatic ring-stacking interaction with the pyridine ring of the isonicotinic-acyl group and this structural arrangement would increase the affinity of the isonicotinic-acyl group over NADH alone (Rozwarski *et al.*, 1998; 1999). Taking into consideration the suggestion that cholesterol

interaction with InhA might prevent INH from exerting its biological activities, lead to some questions: (i) does binding of cholesterol to the InhA prevent the isonicotinic-acyl from binding to the NADH hence inhibiting the formation of the isonicotinic-acyl-NADH adduct resulting in normal synthesis of mycolic acids? (ii) does the binding of cholesterol to InhA occur before that of NADH, thereby protecting most of the enzyme from the isonicotinic-acyl (activated form of INH)? (iii) does binding of cholesterol out-compete the isonicotinic-acyl-NADH such that InhA release isonicotinic-acyl-NADH allowing normal substrate catalysis to proceed?

Kinetic isotope analysis of InhA has demonstrated that the binding sequence of NADH and long-chain acyl substrates is not strictly ordered, but there is preference for NADH binding first (Quemard *et al.*, 1995) which would leave most of the enzyme in an NADH-bound form, available for attack by the activated INH. If binding of cholesterol occurred before that of NADH, the enzyme will be protected from attack by activated INH. In contrast, the decreased affinity of the S94A mutant for NADH would promote acyl-ACP substrate binding before NADH, thereby protecting most of the enzyme from activated INH. The inability of the wid-type InhA to release significant amounts of isonicotinic-acyl-NADH effectively creates permanent inhibition of the enzyme and prevents mycolic acids biosynthesis, but if the isonicotinic-acyl-NADH is formed on the mutant enzyme, the lowered affinity for NADH will promote release of isonicotinic-acyl-NADH, allowing normal substrate catalysis to proceed (Rozwarski *et al.*, 1998). It is also clear that the affinity of InhA for NADH plays a very important role in the mechanism of action of INH. Thus the effect of cholesterol might also lie in the ability of the enzyme to interact with it in preference to NADH. Understanding the role of cholesterol in preventing the biological activities of INH against *M. tuberculosis* may help in developing new anti-TB drugs.

Combined, the results of this study indicate how the cholesterol-mycolic acid structural mimicry can be applied in the development of anti-TB agents and improvement of the existing drugs. The CID design based on this molecular mimicry possess an advantage in that the drug to be delivered by the cholesterol-binding molecule might directly target the

infected macrophages that are known to accumulate cholesterol, while on the bacterium it can target the cholesterol-MA complex on the bacterial cell surface. This molecular mimicry also pointed out a possible interesting relationship between cholesterol and some of the enzyme(s) used for the synthesis of the mycolic acids that are normally targets for some prominent anti-TB agents. The possible binding of cholesterol to InhA that ultimately results in decreased efficiency of the anti-TB drug INH implies that INH competes for binding to this enzyme. The proposed ability of INH target to recognize sterols opens the way for exploiting sterols as possible inhibitors for this enzyme, which is involved in the synthesis of mycolic acids. Important, is the fact that the same enzyme targeted by INH can be targeted by sterols as well. This improved understanding of the structural properties of the binding site of the target enzyme of INH opens the way to a fresh approach to the design of mycobactericidal drugs that may be used to overcome INH resistance.

SUMMARY

The need for new anti-TB drugs is increasingly rising because of the resistance of *M. tuberculosis* to existing drugs. The mycobacterial cell wall serves as an impermeable protective barrier for the bacilli from toxins and chemotherapeutic agents, mainly due to the mycolic acids waxy outer layer. The mycolic acids play an important role in the architecture and physical properties of the mycobacterial cell wall. This study was based on the observed mimicry and association between the host cholesterol and the mycolic acids. This may present yet another way in which the TB bacilli survives by manipulating its host and using some of its components for its survival. The research focused on whether the cholesterol-like molecules on the mycobacterial cell surface can be targeted for effective delivery of anti-mycobacterial agents. In order to exploit the ability of *M. tuberculosis* to accumulate cholesterol or interact with it, a cholesterol-binding molecule was used for targeting an anti-TB drug to the mycobacterial cell wall or to the cell membrane of infected macrophages. It was observed that the drug does possess anti-mycobacterial activities even though higher concentrations of the compound were required. This supports the idea that the ability of cholesterol to interact with the mycobacterial mycolic acids can be exploited for designing of anti-TB agents. It was also demonstrated in this study that cholesterol has a negative effect on the activity of INH. Thus cholesterol, which is required for entry and survival of *M. tuberculosis* in the host cells, has yet another protective effect on this pathogen. The possible ability of cholesterol to target the same enzyme(s) as INH is another small piece of knowledge to complete the puzzle to understanding the mode of virulence and pathogenesis of this pathogen and develop of new ways to fight the old enemy.

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