The antimicrobial effect of colistin methanesulfonate (polymyxin E) on *Mycobacterium tuberculosis in vitro*.

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‘If the importance of a disease for mankind is measured by the number of fatalities it causes, then tuberculosis must be considered much more important than those most feared infectious diseases, plague, cholera and the like. One in seven of all human beings dies from tuberculosis. If one only considers the productive middle-age groups, tuberculosis carries away one-third, and often more’.

-Robert Koch, 1882

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Dedication:

I dedicate this thesis to my family. To my wife Magda for all her love and support, especially during the toughest of times. To my Father and Mother for giving me all the opportunities possible to unlock my potential and follow my dreams; without you, this would not have been possible. To my brother Jake, for all the great childhood memories. To my Oumie, thank you for all the unforgettable meals and great conversations. To my uncle Louis and cousins Ryan, and Justin; thank you for all your love.

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Summary:

Polymyxins have previously been described to have activity against *M. tuberculosis* (*M. tb*), but further research was abandoned due to systemic toxicity concerns to achieve the required MIC. Colistin methanesulfonate (CMS), a polymyxin, is well tolerated when inhaled directly into the lungs, resulting in high local concentrations. Reported here for the first time are the MIC and MBC data for CMS, CST and PST determined by the microtiter Alamar Blue® assay (MABA) against H37Ra and multi-drug-resistant (MDR) *M. tb*. Additionally determined is how the MIC of CMS would be affected by the presence of pulmonary surfactant (PS) and if any synergy with isoniazid (INH) and rifampicin (RIF) exists. The effect of CMS on the ultrastructure of *M. tb* was also determined. MICs for CMS, CST and PST were determined to be too high for systemic use. CMS can, however, be administered by inhalation allowing for high local concentrations with reduced systemic toxicity. The MIC for CMS was antagonised eight fold in PS. For synergy, indifference was determined for both H37Ra and MDR *M. tb.* Time-kill assays revealed a bactericidal killing effect when CMS was used together with INH against H37Ra *M.* tb while no enhanced effect of CMS with INH or RIF was observed against MDR *M. tb.* The resistant effects caused by *rpoB* and *katG* mutations could not be overcome. With regard to H37Ra *M. tb,* ultrastructure analysis suggests that the disruption of the capsule layer (CL) and cytoplasmic membrane (CM) by CMS may enhance the uptake of INH. These findings may provide insight for further investigations of CMS against *M. tb.*

Keywords:

Colistin sulfate, colistin mehtanesulfonate, polymyxin B, polymyxin E, polymyxin, self-promoted uptake, Alamar Blue, ultrastructure, capsule layer, tuberculosis.

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Abbreviations:

1,4-androstadien-3,17-dione	: ADD
4-androsten-3,17-dione	: AD
9-fluorenylmethyl chloroformate	: FMOC-Cl
(α, ☐)-diaminobutyric acid	: Dab
Acquired Immunodeficiency Syndrome	: AIDS
Acyl carrier protein	: ACP
Albumin, dextrose, catalase	: ADC
Antigen 5	: Ag5
Arabinogalactan	: AG
Bacille Calmette-Guérin	: BCG
Biosafety level	: BSL
Bovine serum albumin	: BSA
British Society for Antimicrobial Chemotherapy	: BSAC
Capsule layer	: CL
Clinical and Laboratory and Standards Institute	: CLSI
Colistin methanesulfonate	: CMS
Colistin sulfate	: CST
Colony-forming unit	: CFU
Cytoplasmic membrane	: CM
Diacyltrehalose	: DAT
Electron dense layer	: EDL
Electron translucent layer	: ETL
European Committee on Antimicrobial Susceptibility Testing	: EUCAST
Evaporative light scattering detection	: ELSD
Extensively-drug-resistant	: XDR
Fatty acid synthase I	: FAS I
Fatty acid synthase II	: FAS II
Forced expiatory volume in one second	: FEV_1
Sum of Fractional Inhibitory Concentration	: Σ_{FIC}
High-performance liquid chromatography	: HPLC
Human immunodeficiency virus	: HIV
Isoniazid	: INH
Leucine	: Leu
Lipoarabinomannan	: LAM
Lipomannan	: LM
Lipopolysaccharide	: LPS
Liquid chromatography tandem mass spectrometry	: LC-MS/MS
Microtiter Alamar Blue Assay	: MABA
Million International Units	: MIU
Minimum Bactericidal Concentration	: MBC
Minimum Inhibitory Concentration	: MIC
M. tuberculosis	: *M. tb*
Multi-drug-resistant	: MDR
Mycolic acids	: MA
Mycomembrane	: MM
N-acetylglucosamine	: NAG
N-acetyl/glycollylmuramic	: NAM
Nicotinamide adenine dinucleotide	: NAD(H)
Nucleotide Binding Protein	: NBD
Oleic acid, albumin, dextrose, catalase	: OADC
Para-aminosalicylic acid	: PAS

Peptidoglycan	: PG	Sensor Kinase	: SK
Periplasm	: P	Solid phase extraction cartridges	: SPE
Phenolic glycolipid	: PGL	Standard Deviation	: SD
Phenylalanine	: Phe	Substrate Binding Protein	: SBP
Phosphatidylinositol mannoside	: PIM	Sulfolipids	: SL
Phthiocerol dimycocerosate	: PDIM	Transmission electron microscopy	: TEM
Polyacyltrehalose	: PAT	Threonine	: Thr
Polymyxin B nonapeptide	: PMBN	Trehalose dimycolate	: TDM
Polymyxin B sulfate	: PST	Trehalose monomycolate	: TMM
Pulmonary surfactant	: PS	Tuberculosis	: TB
Purified protein derivative	: PPD	Ultra-performance liquid	
Pyrazinamide	: PZA	chromatography	: UPLC
Rifampicin	: RIF	World Health Organisation	:WHO
Response Regulator	: RR		

Chapter 1: Introduction

1.1 Problem statement:

It has been estimated that one-third of the world's population has been infected by *M. tuberculosis* (*M. tb*); the leading cause of adult deaths due to a single infectious microorganism and a leading killer for co-infected human immunodeficiency virus (HIV) positive patients. Multi-drug-resistant (MDR) *M. tb* cases are on the rise due to a number of reasons such as lack of access to diagnosis and programmatic management with quality controlled antibiotics, lack of adherence to antibiotic treatment, poor disease management, use of inappropriate drug combinations, use of low quality drugs, delay in diagnosis, and transmission of drug-resistant organisms. Currently, there are few new compounds with unique mechanisms and thus, there is the need to explore 'off the shelf' antibiotics with antimycobacterial action. In addition to oral agents, non-absorbable compounds delivered by inhalation should be evaluated. Inhalation delivery might allow access to otherwise unusable compounds, avert systemic toxicity, and possibly reduce transmission by local action on organisms in the airways likely to be aerosolised since delivery directly into the lungs results in local drug concentrations 40-1000 times higher than that achievable by oral or parenteral administration. Colistin or polymyxin E is a promising candidate and was first discovered in the 1940s, but later abandoned due to reports of it being nephro and neurotoxic. Recently, the antibiotic has become indispensable as a treatment against highly-resistant gram-negative MDR infections caused by *Pseudomonas aeruginosa* and *Acinetobacter baumanii.* It is commonly used by nebulisation and by dry powder inhalation to suppress *P. aeruginosa* in patients with cystic fibrosis. Two forms of the antibiotic exist, colistin sulfate (CST) and colistin methanesulfonate (CMS), the latter being a prodrug of colistin that is administered by inhalation, and is less toxic when compared to CST. The antibiotic is a cyclic peptide containing a fatty acid tail and interacts strongly with phospholipids in the cell wall of gram-negative bacteria causing a disruption in its integrity that is similar to a detergent effect. It is very likely that the antibiotic might have a similar effect on *M. tb* and has already shown antimycobacterial activity against the organism; however, the antibiotic alone might have a relatively low killing effect on the bacteria, but when used in combination with other antibiotics, the disruption of the cell wall may allow certain drugs to move easily across the hydrophobic barrier leading to a reduction in minimum inhibitory concentrations (MIC). CMS has shown to have a considerable synergistic effect when used

in combination with rifampicin (RIF) against various gram-negative MDR infections and thus, together with isoniazid (INH), could prove to be an effective local treatment for various forms of *M. tb*, assuming that the drug reaches organisms in abnormal lungs by inhalation, since absorption is limited and systemic levels are not expected.

1.2 *Mycobacterium tuberculosis*:

1.2.1 *M. tuberculosis* in general:

In 1882, Robert Koch became the first person to describe *M. tb* as the causative agent of tuberculosis (TB) by testing his four postulates with various techniques such as microscopy (acid-fast staining), staining of tissues, pure culture isolation and animal model systems (guinea pigs) [Madigan and Martinko 2006]. He was subsequently awarded the Nobel Prize for Physiology and Medicine in 1905 of his revolutionary discovery [http://www.nobelprize.org/].

Other *Mycobacteria* that have the ability to infect humans and cause TB, but are much rarer than *M. tb*, are part of the *M. tb* complex, i.e., *M. africanum*, *M. canetti,* and *M. bovis*. *M. bovis* infects a variety of mammals such as cattle and an attenuated form known as Bacille Calmette-Guérin (or BCG) is used as a vaccine against TB. *M. microti* is also part of the complex and is known to infect voles [Cole 2002; Madigan and Martinko 2006].

M. tb (Figure 1.1) can be described as being a slow growing (24-hour doubling time) pleomorphic, rod shaped (~ 1-4µm), aerobic, non-spore forming and acid-fast bacillus. When *M. tb* is stained with Ziel-Neelsen; a mixture of basic fuchsin and phenol, *M. tb* stains red while other non-acid-fast bacteria stain blue after an acid-alcohol wash and counter stain with methylene blue. The reason for this phenotypic property is due to its unique cell wall containing mycolic acids (MA); a hydrophobic chemical wax contained within the cell wall of *M. tb* that can retain the dye. Due to the high hydrophobic nature of *M. tb*, tight, compact and wrinkled colonies are observed when grown on solid media. The virulence of *M. tb* has been linked to the formation of cordlike structures often observed when cultured or viewed under a microscope, reflecting the presence of a glycolipid known as the cord factor. With regard to pigmentation, *M. tb* is considered to be non-pigmented [Madigan and Martinko 2006].

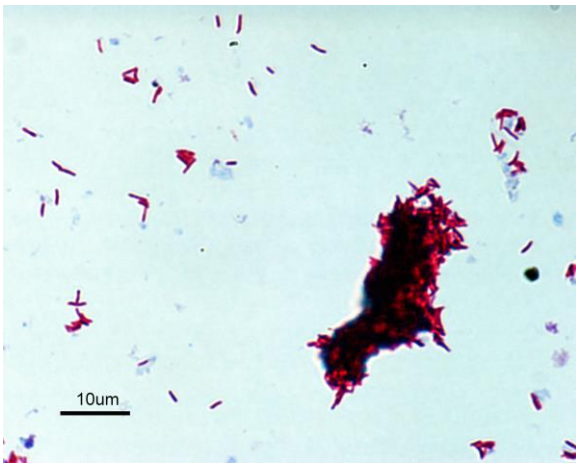


Figure 1.1: *M. tb* H37Ra ATCC 25177 stained with Ziel-Neelsen indicating single rod shaped bacteria. Shane Vontelin van Breda, 2014.

Even after the discovery of *M. tb* as the causative agent of TB, effective treatment of the disease did not appear until the 1950s with the discovery of streptomycin; the first antibiotic with proven activity against *M. tb*. This was the pioneering work at Rutgers University by Albert Schatz and Nobel Laureate Selman Waksman (1952) [http://www.nobelprize.org/]. For more than 50 years before chemotherapy, patients were treated by a variety of generally ineffective methods, including months to years of bed rest and improved nutrition, surgery, and various methods of collapsing lungs in order to close lung cavities. Once various combinations of INH, RIF, streptomycin, pyrazinamide (PZA) or para-aminosalicylic (PAS) were used in therapy, there was a 98% chance of cure during the 1980s [Herzog 1998]. However, TB has reappeared to be one of the world's most deadliest communicable diseases with an estimated 13% of all cases reported (nine million) to be co-infected with HIV. The African region accounts for four out of five TB cases and TB deaths amongst patients co-infected with HIV, because HIV/AIDS puts patients at more susceptibility to opportunistic infections such as TB. The emergence of MDR *M. tb* is currently threatening global TB control. MDR *M. tb* is an extremely dangerous form of *M. tb* resistant to at least both INH and RIF; the two most effective anti-TB drugs. Globally, during 2013, there were an estimated 480 000 cases of MDR-TB, with an estimated 210 000 deaths [World Health Organisation 2014].

1.2.2 Multi-drug-resistant *M. tuberculosis*:

Diagnoses of MDR *M. tb* between 2009 and 2013 have tripled and reached an estimated 136 000 patients worldwide, in part due to new rapid diagnostics such as Xpert® MTB/RIF. Approximately 97 000 patients were started on treatment in 2013, a three-fold increase compared to 2009. Unfortunately, between 2012 and 2013, the gap between diagnoses and treatment for MDR *M. tb* has widened, and at least 39 000 patients were on waiting lists [World Health Organisation 2014]. New analysis of MDR *M. tb* trends suggest that at global level, the number of cases of MDR *M. tb* remains unchanged (2008-2013); however, epidemics in certain countries are hampering progress [World Health Organisation 2014]. For example, in South Africa, a drug-resistant outbreak occurred around a rural hospital in Tugela Ferry, KwaZulu-Natal [Kana and Warner 2008; Wise 2006]. It has been suggested that inadequate infection control was the main reason for MDR *M. tb* originating and being transmitted in the hospital [Basu *et al* 2007; Gandhi *et al* 2006]. Clearly correct infection control and reduction in transmission are a priority not only in the health-care setting but also in the community and home [Fox *et al* 2013; Gandhi *et al* 2010], especially in Sub-Saharan Africa, the region most affected by HIV [Alland *et* al 1994; Gandhi *et al* 2010; http://www.who.int/hiv/en/; Sahai *et al* 1997; Weiner *et al* 2005].

Resistance to at least, INH and RIF are a man-made occurrence [Gandhi *et al* 2010] due to spontaneous mutations (not horizontal gene transfer) within the genome of *M. tb,* i.e., INH at the rate of 10^{-6} and RIF at the rate of 10^{-8}, and thus, under drug-selection pressure (i.e., mono therapy) during active disease, resistant strains can arise and become dominant. This is known as acquired resistance and these drug-resistant organisms can easily be transmitted by coughing [David 1970; Gandhi *et al* 2010; Kaplan *et al* 2003; Post *et al* 2004; Supply *et al* 2003]. Multiple reasons such as inappropriate treatment, incorrect use of anti-TB drugs, use of poor-quality anti-TB drugs, poor disease management, delay in laboratory testing and diagnosis, and issues with drug supply are all contributing to the rise of drug-resistant *M. tb* due to selection pressure and acquired resistance compounded by transmission [Gandhi *et al* 2010; World Health Organisation 2013].

Drug-resistance acquired by *M. tb* can be attributed to five mechanisms; (1) decreased uptake or impermeability by the cell wall due to complex lipids acting as permeability barriers; (2) increased drug efflux systems; (3) enzymatic inactivation, for example, that of β-lactamases; (4) modification of the antibiotic target, i.e., the binding site and (5) inability to activate the prodrug [Green and Gameau-Tsodikova 2013; Wade and Zang 2004]. Mechanisms of drug-resistance concerning INH and RIF are discussed in more detail later.

The WHO has outlined the following in order to prevent MDR *M. tb*; (1) prevent the development of drug-resistance through high-quality treatment of drug-susceptible TB; (2) expand rapid testing and detection of drug-resistant TB cases; (3) provide immediate access to effective treatment and proper care; (4) prevent transmission through infection control and (5) increase political commitment with financing [World Health Organisation 2014]. DOTS (Directly Observed Treatment, Short-Course) is a community-based program suggested by the WHO for the treatment susceptible TB and DOTS-Plus, for the treatment of MDR *M. tb*; the basis of the Stop TB strategy. However, DOTS-Plus is extremely costly and if DOTS-Plus is associated with minimal decreases in DOTS success, more patients would die under DOTS-Plus than under DOTS alone [Sterling *et al* 2003].

Treatment of MDR *M. tb* is complicated, especially when HIV co-infection is present. In general, up to five antibiotics are suggested, i.e., PZA, a fluoroquinolone, a parenteral agent (kanamycin, amikacin or capreomycin), ethionamide (or prothionamide), and either cycloserine or PAS if cycloserine cannot be used, with an intensive phase of 8 months and a treatment of up to 20 months long [Falzon *et al* 2011]. The drugs used for these treatments are toxic, costly and not always as effective as first-line anti-TB drugs [Shah *et al* 2007]. Bedaquiline and delamanid have been approved for MDR *M. tb* treatment and there are 10 new or re-purposed anti-TB drugs in the late phase of clinical development [World Health Organisation 2014]. It's important that when investigating the possibility of a new anti-TB drug, the drug should have a unique mechanism of action, i.e., act directly on the cell wall and can be administered in a unique way such as by inhalation, which ultimately may lead to decreased transmission of the organism. Treatment should not be expensive, and the drug should also have low toxicity with good bioavailability. It is also important that the new anti-TB drug should not have any antagonistic effects with other anti-TB drugs, i.e., INH and RIF when used in combinational therapy. If the new anti-TB could act in a synergistic manner with INH and RIF so that resistance in MDR *M. tb* may be ‘reversed’, it would be a great advantage.

1.2.3 *M. tuberculosis* cell wall:

With regard to a unique mechanism of action for anti-TB drugs, understanding the complex nature of the *M. tb* cell wall is of utmost importance. A general structure of the *M. tb* cell wall is displayed in Figure 1.2.

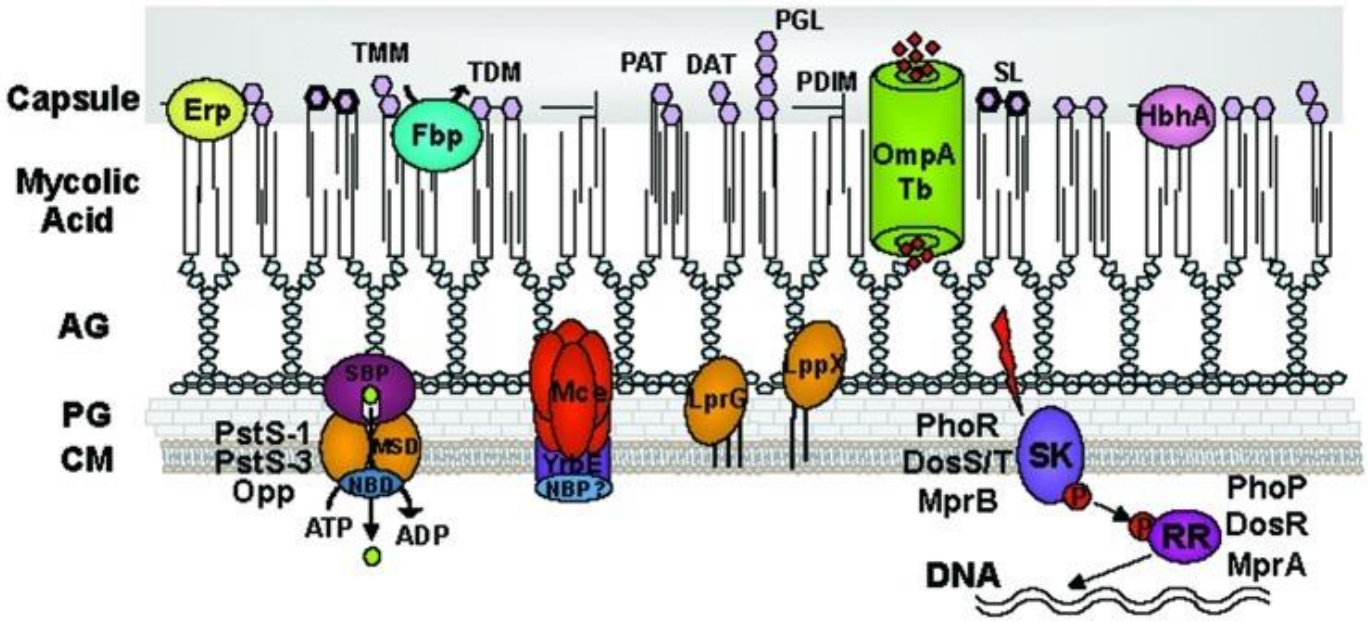


Figure 1.2: A schematic diagram of the *M. tb* cell wall, displaying its composition of the cytoplasmic membrane (CM), peptidoglycan (PG), arabinogalactan (AG), MA, the capsule layer (CL) including and various cell envelope proteins [Forrellad *et al* 2013].

M. tb contains a cytoplasmic membrane (CM) that is encapsulated by peptidoglycan (PG). The CM of Mycobacteria has been suggested to be not much different from other biological CMs. It generally contains polar lipids, many phospholipids and proteins that assemble to form a lipid bilayer [Daffé 2015].

PG, arabinogalactan (AG) and MA are all covalently linked, forming what is considered a giant macromolecule known as the cell wall skeleton. PG is composed of repeating unts; N-acetylglucosamine (NAG) and N-acetyl/glycollylmuramic acid (NAM) cross linked by short peptide bridges [Daffé 2015]. The PG is heavily cross linked and majority contains 3-3 peptide crosslinks, which are different to traditional PG 4-3 crosslinks. NAM residues are glycolated and amidation of D-Glu, and meso-diaminopimelic acid of the peptide side chain occur, which may mask recognition by

certain innate immune receptors [Kieser and Rubin 2014]. AG contains a galactan chain with alternating 5- and 6- linked D-galactofuranosyl residues [Daffé 2015] synthesised by galactofuranosyl transferases [Kieser and Rubin 2014]. In *M. tb*, three D-arabinan chains substitute the D-galactan chain [Daffé 2015], although some may remain free of arabinan. Arabinan chain termini is branched, and addition of succinyl or non-N-acetylated galactosamine may occur [Kieser and Rubin 2014]. Within CM, PG and AG are the ABC transporters PstS-1, PstS-3, Opp and the Mce proteins. The proteins reside in the substrate binding proteins (SBP), the permeases (MSD) and the nucleotide binding protein (NBD). Residing in the sensor kinase (SK) is the two component systems: PhoR-P, DosS/T-R and MprAB. It senses stimuli and the response regulator (RR), which induces the gene transcription [Forrellad *et al* 2013]. Within the membranes are lipoprotein LprG and LppX, lipoproteins known for their possible role in virulence [Forrellad *et al* 2013].

MA contain up to 90 carbon atoms that are α-branched and β-hydroxylated fatty acids esterified at the four hydroxyl groups at position five of both terminal and 2-linked D-arabinofuranosyl of the pentaarabinosyl motifs [Daffé 2015]. FAS (fatty acid synthase) I and FAS II complexes are responsible for these branches. Once processing by cascade enzymes is complete, α-meroacids, methoxy-meroacids and keto-meroacids exist, which have varying levels of saturation, cyclopropanation and oxygenation. An outer membrane exists known as the mycomembrane (MM). The inner leaflet contains MA covalently linked to AG while the outer leaflet has been suggested to contain proteins, lipids and various complex lipids, i.e., phthiocerol dimycocerosate (PDIMs), trehalose dimycolate (TDM) (cord factor), trehalose monomycolate (TMM), phosphatidylinositol mannoside (PIM), sulfolipids (SL), di and poly acyltrehalose (DAT, PAT) and phenolic glycolipid (PGL) [Daffé 2015; Forrellad *et al* 2013].

PDIMs contain 35 carbons as methyl or methylene groups, with two reactive hydroxy groups esterified to two fatty acids. Thus, PDIMs are highly apolar/hydrophobic and also considered to play a role in virulence but is also responsible for cell wall architecture and permeability [Camacho *et al* 2001]. TDM, formed from TMM via Fbp, is another important glycolipid that contains trehalose sugar esterified to two MA residues and is responsible for being immunogenic, granulomagenic and adjuvant-active [Brennan 2003; Daffé 2015; Forrellad *et al* 2013]. Sulfolipids contain a sulphur functional group, and there is a possibility that they play a role in virulence [Brennan 2003]. DAT and PAT also contain the trehalose moiety, are biologically active and unique to the *M. tb* complex [Brennan 2003; Daffé 2015; Forrellad *et al* 2013]. Many strains are deficient in PGL, i.e., H37Rv, but has been identified in W-Beijing strains. PGL is considered to play a role in virulence [Sinsimer *et al* 2008]. Overall, the MM is highly hydrophobic and restricts the movement of small polar molecules

from the exterior; however, this is solved by the presence of porins such as OmpATb, allowing for the passage of small hydrophilic molecules [Brennan 2003; Daffé 2015; Forrellad *et al* 2013].

A capsule layer (CL) is also observed in *M. tb*. This capsule contains polysaccharides, proteins (Erp, Fbp, HbpA [Forrellad *et al* 2013]) and specialised glycolipids embedded within. The composition of the capsule is largely dependent on the environment of *M. tb*, i.e., *in vitro* vs *in vivo* and if grown *in vitro*, the composition of the culture media and conditions could largely affect its composition, and it has been determined that *M. tb* capsular proteins are a mixture of complex polypeptides. Glucan is the major component of the capsule, composed of repeating units of five or six 4-(-D-glucosyl residues substituted at position six with a mono- or oligoglucosyl residues [Daffé 2015].

Other lipids known as lipomannan (LM) and lipoarabinomannan (LAM) are interspersed in the cell wall, being anchored to the PM and extending to the exterior of the cell wall (not shown). PIMs (essential precursors for LM and LAM) are phosphatidylinositol with one to six mannoses attached to inositol, with a two mannose attachment the most common in *M. tb*. LM and LAM are extensions of PIM. In *M. tb*, LAM is attached to the CM via the phosphatidylinositol anchor and has short mannose containing oligosaccharide caps, believed to be responsible for various host responses in TB [Brennan 2003]. LAM is also believed to neutralise cytotoxic oxygen-free radicals produced by macrophages [Chan *et al* 1991].

Thus, it can be seen that the Mycobacterial cell wall is an impermeable structure that controls access to the interior of the bacterium [Daffé 2015]. Therefore, disruption of this structure may allow an influx of hydrophilic drugs such as INH and even hydrophobic drugs such as RIF, lowering concentrations required to kill *M. tb* and possibly MDR *M. tb*. Since *M. tb* can differ at various stages of growth, understanding the life cycle will be of benefit.

1.2.4 *M. tuberculosis* life cycle and active vs latent infections:

A general life cycle of *M. tb* is displayed in Figure 1.3. Firstly, droplet nuclei *M. tb* bacilli are inhaled from the surrounding environment/atmosphere. It is estimated that a minimum infectious dose could be as little as one bacterium.

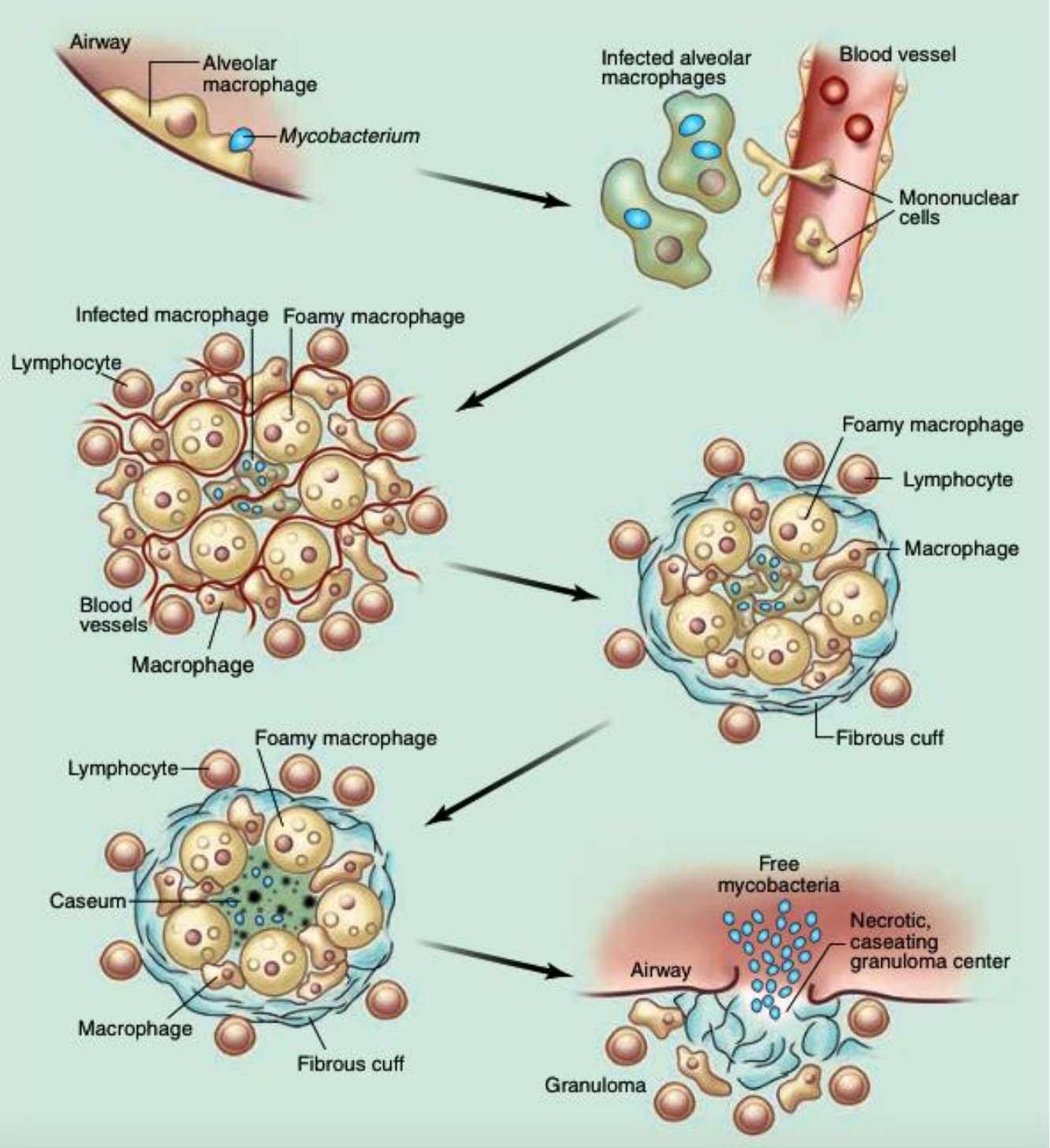


Figure 1.3: A schematic diagram displaying the general life cycle of *M. tb.* Infection is initiated when *M. tb* bacilli are inhaled from the surrounding environment, are taken up by macrophages and granulomas are formed. Eventually granuloma cavities collapse and infectious *M. tb* bacilli are released into the surrounding environment via the airways [Russell *et al* 2010].

These droplet nuclei are so small that they can remain airborne for hours. Once inhaled, bacteria are phagocytosed by alveolar macrophages leading to an inflammatory response once infiltration of the subtending epithelial layer occurs.

From the neighbouring blood vessels, mononuclear cells are recruited to assist with infected alveolar macrophages, but this ultimately allows for expansion of the existing bacterial population. The defining pathological feature of the disease is the granuloma (interaction of macrophages, lymphocytes, and release of cytokines); initially an amorphous mass of macrophages, monocytes and neutrophils. Macrophages extricate into various cell types, i.e., multinucleated giant cells, foamy macrophages (filled with lipid droplets) and epithelioid macrophages, which allow for the granuloma to develop a stratified structure due to the acquired immune response and arrival of lymphocytes. A few week's post-infection sees an end of increased *M. tb* replication and the beginning of a containment stage. When this occurs, the granuloma is extensively vascularised with active deployment of cells to the site of infection.

Eventually, a fibrous cuff (extracellular matrix material) is formed, marking the perimeter of the structure outside the macrophage area with lymphocytes being restricted to the periphery. The fibrous cuff becomes more defined with a reduction of blood vessels present within the structure. An increase in caseous debris is observed in the centre of the granuloma, likely due to an increase of foamy macrophages and necrosis. This caseous debris becomes hypoxic, that may lead to the development of latent, dormant or persistent *M. tb*. The fate of each granuloma is determined locally and not systemically, and the granuloma will eventually rupture spilling thousands of active bacteria into the airways, which are spread by coughing. Neutrophil influx in the late-stages of the disease may also contribute to tissue damage and propagation of infectious bacilli into the surrounding environment [Russell *et al* 2010].

Generally speaking, the above description refers to active *M. tb* disease. It is important to note that many people can be infected with *M. tb* without progression to active disease as the immune system is able to keep *M. tb* under control [Zhang 2004]. This latent disease due to dormant *M. tb* can cause a relapse, decades after treatment for active TB. The tubercle bacilli can reside within lesions in a quiescent state until reactivation occurs when the conditions are appropriate, for example, when the host has a compromised immune system, i.e., HIV/AIDS in addition to many other factors such as silicosis [Todar 2005; Zhang 2004]. This latency is a serious problem for effective TB control and is also a reason behind the lengthy treatment regimes currently required due to persistent bacilli that cannot be killed by anti-TB drugs or cleared by the host's immune response [Zahrt 2003; Zhang 2004]. Latency is a complicated and confusing topic that is not easily understood. Latency generally refers to

an *in vivo* situation where there is a balance between the host and *M. tb* with no apparent symptoms, i.e., an equilibrium, and does not have reference to the *M. tb* bacilli growth or metabolic status [Orme 2001; Zhang 2004]. This can arise when the host's immune system or INH prophylaxis keeps the active *M. tb* population under control, or that *M. tb* is dormant within the host [Zhang 2004]. Dormancy refers to *M. tb* bacilli with low metabolic activity that are viable but cannot form viable colonies (non-CFU forming) unless they are resuscitated to CFU forming (under specific conditions with certain resuscitation factors) [Barer and Harwood 1999; Kell 1998; Zhang 2004]. A possible factor involved in resuscitation has been identified to be resuscitation promoting factor [Mukamolova *et al* 2002; Zhang 2004]. These dormant *M. tb* bacilli are highly-resistant to many stresses and anti-TB drugs with dormancy normally arising under conditions of starvation, ageing, low temperature or hypoxia and can be associated with certain morphological changes, i.e., becoming coccoid [Zhang 2004]. Persistent *M. tb* bacilli can be described as genetically drug-susceptible bacilli that are non-growing (can be considered dormancy) or slow growing that are able to survive indefinitely within mammalian tissues despite continued exposure to the appropriate antimicrobial (or stresses) and have the capacity to regrow or resuscitate and regrow under very specific conditions [Zhang 2004; Zhang 2014]. It is likely that *M. tb* secretes bacterial factors or changes its surface antigenic properties to avoid immune attack [Zhang 2004] and diverse Mycobacterial factors are involved in dormancy and persistence such as isocitrate lyase [Wayne and Lin 1982; Zhang 2004], cyclopropane synthetase [Glickman *et al* 2000; Zhang 2004], glycine dehydrogenase, etc., [Mitchison 1980; Wayne and Lin 1982; Zhang 2004].

Miliary tuberculosis (disseminated disease) may also exist in patients and is characterised by a spread of small 1-3 mm sized granulomas with secondary lesions occurring at almost every possible anatomical location [Sharma 2012].

Clearly, an anti-TB drug that would reduce the transmission of the disease would prevent spreading of the disease and an anti-TB drug targeting the population of persisters and dormant bacilli could prevent relapses as well as shorten treatment regimes. A possible approach to treat this population of persisters and dormant *M. tb* bacilli would be to resuscitate them into their active state, thus making the bacilli susceptible to other anti-TB drugs. However, the first step before this investigation, would be to understand how the new anti-TB drug effects active *M. tb*.

1.2.5 Isoniazid and rifampicin:

INH and RIF (Figure 1.4 and 1.5) are the two most important first line anti-TB drugs [Barry 1997].

1.2.5.1 Isoniazid:

For the small hydrophilic INH molecule, radio-active labelling of the molecule revealed that INH entered *M. tb* via passive diffusion, and this was not due to facilitated diffusion or being energy-dependent [Bardou *et al* 1998]. It was also determined that the MA layer does not affect the diffusion of INH into *M. tb* [Jackson *et al* 1999]. The mechanism of action for INH is displayed in Figure 1.4. INH is a prodrug that is activated by KatG (catalase-peroxidase) [Zhang *et al* 1992] to form activated INH that then binds tightly to InhA (NADH-dependent enoyl acyl carrier protein (ACP) reductase) [Banerjee *et al* 1994].

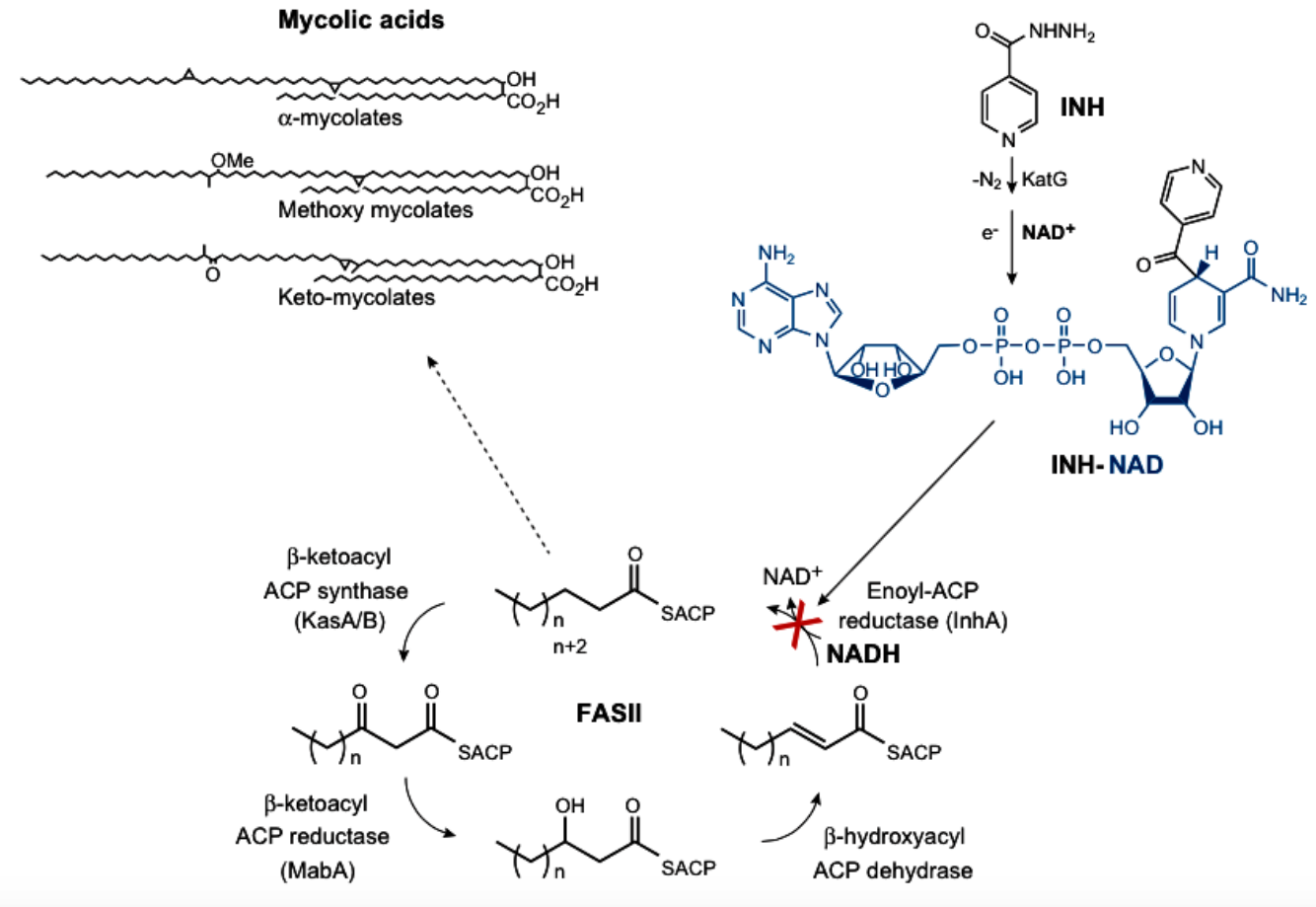


Figure 1.4: Mechanism of action for INH displaying the activation by KatG to form an INH-NAD (nicotinamide adenine dinucleotide) adduct that inhibits InhA, the enoyl-ACP reductase of FAS II that is required for MA synthesis [Vilchèze and Jacobs Jr 2007].

KatG is very important in detoxification of hydrogen peroxides or hydroxyl radicals from endogenous or exogenous sources [Raynaud *et al* 1998; Wengenack *et al* 1999]. An interesting fact to note is that KatG has been associated with culture filtrates when grown *in vitro* [Sonnenberg and Belisle 1997] and thus activation of INH might occur in surrounding environment of *M. tb*, within the cytosol of *M. tb* or possibly both. An essential component for fatty acid elongation and MA formation is InhA, and it has been shown that activated INH binds covalently to NAD that is bound within the active site of InhA causing NADH to dissociate from InhA [Kolyva and Karakousis 2012].

Majority of INH resistance associated with clinical strains is in the *katG* gene, i.e., up to 80% of cases. Mutations in *katG* reduces the ability of KatG (catalase-peroxidase) to activate the prodrug of INH. Various mutations exist, i.e., missense, nonsense, insertions, deletions, truncation and even full gene deletions with point mutations being the most commonly observed mutation of *katG*, for example, threonine (Thr) substitution for serine at residue 315 (S315T) [Abate *et* al 2001; Kolyva and Karakousis 2012; Marttila *et al* 1998]. This single point mutation is associated with significant reduction in KatG activity and higher levels of INH resistance, i.e., MICs of 5-10mg/L [Kolyva and Karakousis 2012; Rouse *et al* 1996; Saint-Joanis *et al* 1999]. However, with this mutation, KatG can retain approximately 50% of its catalase-peroxidase activity, thus retaining some form of oxidative protection [Rouse *et al* 1996; Somoskovi *et al* 2001]. INH resistance can also arise from mutations in *inhA*, affecting NADH affinity of the enzyme or mutations in the promoter region of *mabAinhA*, causing an over-expression of the wild-type enzyme. These mutations are generally associated with a low level of resistance, i.e., MICs in the range of 0.2-1mg/L. Mutations in *inhA* can also confer resistance to ethionamide [Basso *et al* 1998; Kolyva and Karakousis 2012; Musser *et al* 1996; Wade and Zhang 2004]. *M. tb* clinical isolates that confer INH resistance but do not have mutations in *katG* or *inhA* have been associated with mutations in the *ndh* gene that encodes an NADH dehydrogenase. The defective NADH dehydrogenase leads to an increased ratio of NADH/NAD that interferes with activation of INH by KatG as well as displacing the INH-NAD adduct from InhA. Other mutations such as *kasA, ahpC* have also been linked to INH resistance [Kolyva and Karakousis 2012; Lee *et al* 2001; Miesel *et al* 1998].

1.2.5.2 Rifampicin:

RIF is a broad-spectrum antibiotic and the most widely used rifamycin to treat TB [Kolyva and Karakousis 2012]. RIF contains an aromatic nucleus linked both sides by an aliphatic bridge and thus due to its lipophilic profile, RIF can easily diffuse across the hydrophobic cell membrane of *M. tb* [Wade and Zhang 2004]. RIF binds with high affinity to bacterial DNA-dependent RNA polymerase, specifically interacting with the β-subunit of RNA polymerase (RpoB), therefore inhibiting transcription [Kolyva and Karakousis 2012; McClure and Cech 1978; Somoskovi *et al* 2001]. More than 90% of RIF resistant isolates are also resistant to INH, i.e., RIF mono-resistance is relatively rare and consequently RIF resistance is a good surrogate marker for MDR *M. tb* [Felmlee *et al* 1995; Garcia *et al* 2002].

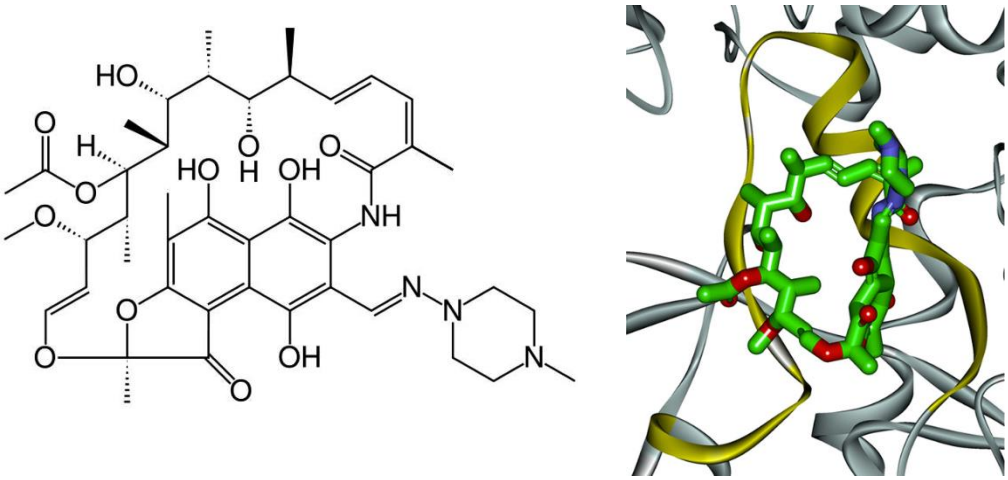


Figure 1.5: Line structure of RIF [Kolyva and Karakousis 2012] (left) and a ribbon structure (right) of the wild-type RpoB binding pocket with RIF (carbon atoms displayed in green). The ribbon highlighted in yellow is the RIF resistant-determining region [Pang *et al* 2013].

RIF resistance is most commonly associated with single point mutations in the *rpoB* gene encoding the β-subunit of RNA polymerase [Telenti *et al* 1993]. The point mutations cluster in between codons 507 and 533 of the *rpoB* gene (RIF resistant-determining region) in the majority of RIF resistant strains. Mutations in codons serine (531) and histidine (526) are more frequent [Ramaswamy and Musser 1998] and cause very high levels of resistance, i.e., MICs greater than 32mg/L, while mutations in codons 511, 516, 518 and 522 cause low levels of resistance [Mariam *et al* 2004; Ohno *et al* 1996; Somoskovi *et al* 2001]. Other high levels of resistance have been associated

with mutations in codon 176, the amino terminal region of *rpoB* (MICs in the region of 1-32mg/L) [Somoskovi *et al* 2001]. Alternative mechanisms of action such as efflux pumps, mutations in other RNA polymerase subunits and altered cellular membranes in *M. tb* could also confer RIF resistance, since a small population of resistant isolates (less than 5%) do not contain mutations in the *rpoB* gene [Louw *et al* 2009; Musser 1995].

1.3 Polymyxins:

Polymyxins are of interest in the treatment of *M. tb* for three main reasons: (1) due to systemic toxicity, they have never been used in TB treatment and in that sense represent a 'new drug class' potentially useful against highly-resistant MDR strains; (2) CMS has been routinely used by inhalation, both by nebulisation and as dry powder, for treatment of *P. aeruginosa* lung infections in cystic fibrosis patients, so its safety, pharmacokinetics and pharmacodynamics are well established in patients with advanced lung disease; and (3) because of their unique detergent-like effects on the cell wall, polymyxins have been shown to have synergistic action with other antibiotics against some pathogens and thus could be synergistic with other anti-TB drugs.

1.3.1 Polymyxins in general:

The first isolation of polymyxins occurred in 1947 from a spore-bearing soil bacillus called *Bacillus polymyxa* [Benedict and Langlykke 1947; Stansly *et al* 1947]. Although a number of different polymyxin forms exist (polymyxins A-E), only polymyxin B and E (E is also known as colistin) are used clinically and are a mixture of several major components [Nation and Li 2010]. Colistin is produced by *B. polymyxa colistinus* (nonribosomally synthesised) and was identified by Koyama *et al* [1950], but was first reported to be distinct from all other polymyxins until it was proved to be identical to polymyxin E [Komura and Kurahashi 1979; Koyama *et al* 1950; Suzuki *et al* 1965]. Colistin has been used since the 1950s, but after two decades, it was later abandoned due to concerns of nephro- [Brown *et al* 1970; Koch-Weser *et al* 1970; Ryan *et al* 1969] and neurotoxicity [Koch-Weser *et al* 1970], especially during parenteral administration, and use was subsequently replaced by less toxic antibiotics, i.e., aminoglycosides and β-lactams. Due to the toxicity concerns of polymyxin B sulfate (PST) and CST, CMS was developed, which is a chemical derivative where methanesulfonate moieties are attached through covalent bonds to the primary amines of colistin [Nation and Li 2010]. This is achieved by reacting colistin with formaldehyde and sodium bisulfate [Landman *et al* 2008]. CMS has

shown to be less toxic [Nord and Hoeprich 1964], less active [Eickhoff and Finland 1965] and is a non active prodrug of colistin as it is readily converted to colistin *in vitro* and *in vivo* (CMS loses methanesulfonate groups to form colistin)[Bergen *et al* 2006; Li *et al* 2003a; Li *et al* 2005b]. In recent years, there has been a rapid increase in highly-resistant gram-negative MDR bacteria, i.e., *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*. Thus, polymyxin B and less toxic colistin (administered as CMS) are being increasingly used as a salvage therapy for isolates resistant to nearly all other alternative antibiotics [Falagas and Kasiakou 2005; Landman *et al* 2008; Li *et al* 2005a; Li *et al* 2006; Zavascki *et al* 2007]. In the clinical setting, polymyxin B is administered as its sulfate salt (PST) parenterally, while colistin is administered as CMS parenterally and in general, these products are in powder form requiring reconstitution before administration [Nation and Li 2010]. A very attractive trait of CMS for possible use as an anti-TB drug is the extensive experience with inhalation administration that exists [Nation and Li 2010].

1.3.2 Chemistry of polymyxins:

The structures of colistin, polymyxin B and CMS are displayed in Figure 1.6. As it can be deduced by their native structures, colistin and polymyxin B are cationic at physiological pH, whereas CMS is anionic.

The general structures of polymyxins are an arrangement of D and L amino acids with a cyclic heptapeptide ring, with this cyclic ring being very important for its antimicrobial activity [Kline *et al* 2001], which is attached to a tripeptide side chain that is covalently bound via an acyl group to a fatty acid [Nation and Li 2010].

The two main components of the polymyxins are the A and B forms, with the ratio of the two forms differing between suppliers and even batches of the product [Decolin *et al* 1997]. As described in Figure 1.6, the fatty acid tails are 6-methyloctanoic acid and 6-methylheptanoic acid. These fatty acids (hydrophobic moiety) together with five cationic groups (hydrophilic moiety), make colistin and polymyxin B amphiphatic [Nation and Li 2010], and its basic pKa is approximately 10 [Li *et al* 2005a].

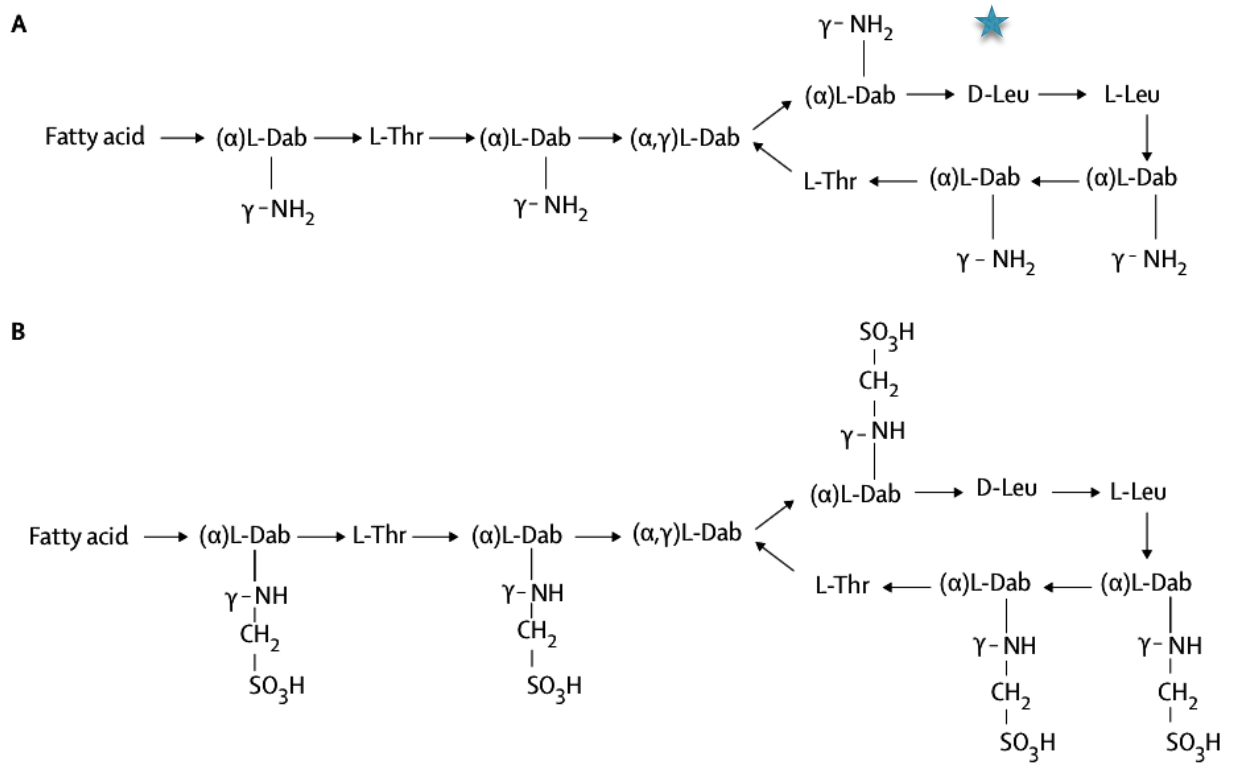


Figure 1.6: (A) Structures of colistin A and B, and polymyxin B_1 and B_2. In polymyxin B, D-phenylalanine (Phe), replaces D-leucine (Leu); note the star in A. (B) Structures of CMS A and B; note the methanesulfonate groups. For colistin A, polymyxin B_1 and CMS A, the fatty acid present is 6-methyloctanoic acid and for colistin B, polymyxin B_2 and CMS B, the fatty acid present is 6-methylheptanoic acid. Thr and (α, ☐)-diaminobutyric acid (Dab) are present, with α and ☐ indicating the $-NH_2$ involved in the peptide linkage [Nation and Li 2010].

Thus, due to this nature of polymyxins, they can easily distribute in environments such as water and prokaryotic membranes. Apart from the A and B forms being major components, minor components also exist, such as polymyxin E_3, E_4, E_7, norvaline-polymyxin E_1, valine-polymyxin E_1, valine-polymyxin E_2, isoleucine-polymyxin E_1, isoleucine-polymyxin E_8 [Li *et al* 2005a]. It is important to note that the presence of H_2SO_4 with colistin and polymyxin B forms a sulfate salt, i.e., CST and PST (the packaged product), while for CMS, Na^+ is present so it can be administered in Na^+ salt form (its packaged product) [Nation and Li 2010]. To avoid confusion throughout this thesis, packaged product forms refer to CST, PST and CMS, but their active forms are referred to as colistin and polymyxin B (Figure 1.6 A), i.e., no salts or methanesulfonate groups present.

As mentioned, CMS is not stable *in vitro* or *in vivo* and is rapidly hydrolysed to form colistin in various aqueous solutions. Considering that two major forms exist (A and B) as well as five methanesulfonate groups that are lost to form -NH_2 groups, essentially $2^5 = 32$ different forms could exist in solution [Li *et al* 2005a].

Colistin is also resistant to pepsin (pH 2.2-4.8), trypsin (pH 4.4-7.5), pancreatin (pH 4.4-7.5), erepsin (pH 6.1-7.8) and is inactivated by lipase [Li *et al* 2005a].

The different molecular weights for polymyxins are the following; for colistin A, 1170g/mol, for colistin B, 1155g/mol, for polymyxin B_1, 1204g/mol, for polymyxin B_2, 1190g/mol [Kwa *et al* 2008], for CMS A, 1750g/mol and for CMS B, 1736g/mol [Li *et al* 2003b]. For colistin and polymyxin B, H_2SO_4 needs to be factored in order to calculate the molecular masses of CST and PST.

Various other chemical properties that make polymyxins very complicated need to be considered during *in vitro* susceptibility testing and are discussed in more detail in Chapter 2.

1.3.3 Antimicrobial properties of polymyxins:

1.3.3.1 Mechanism of action and resistance:

Currently the main mechanism of action for polymyxins is described using its effects against gram-negative organisms. Since polymyxins are polycationic in their active form, the initial interaction with the outer membrane of gram-negative organisms is of an electrostatic nature [Nation and Li 2010].

Cationic charges from the polymyxins interact with phosphate groups of lipid A (a component of lipopolysaccharide (LPS)) via their anionic charges, displacing Ca^{++} and Mg^{++} [Nation and Li 2010]. The second interaction involves the fatty acid tail of polymyxins with the lipid components of the outer membrane [Nation and Li 2010]. Once this initial interaction occurs, ‘cracks’ are formed in the membrane that alters the membrane permeability and the self-promoted uptake mechanism is hypothesised to occur [Hancock and Chapple 1999]. This allows for polymyxins to transit the outer membrane and interact with the CM, i.e., cationic charges from polymyxins will now interact with the anionic charges of head groups of phosphatidylglycerol and cardiolipin while the fatty acid tail can interact with fatty acyl chains of the phospholipids. When this occurs, it is possible that polymyxins might aggregate into micelle-like structures spanning the CM.

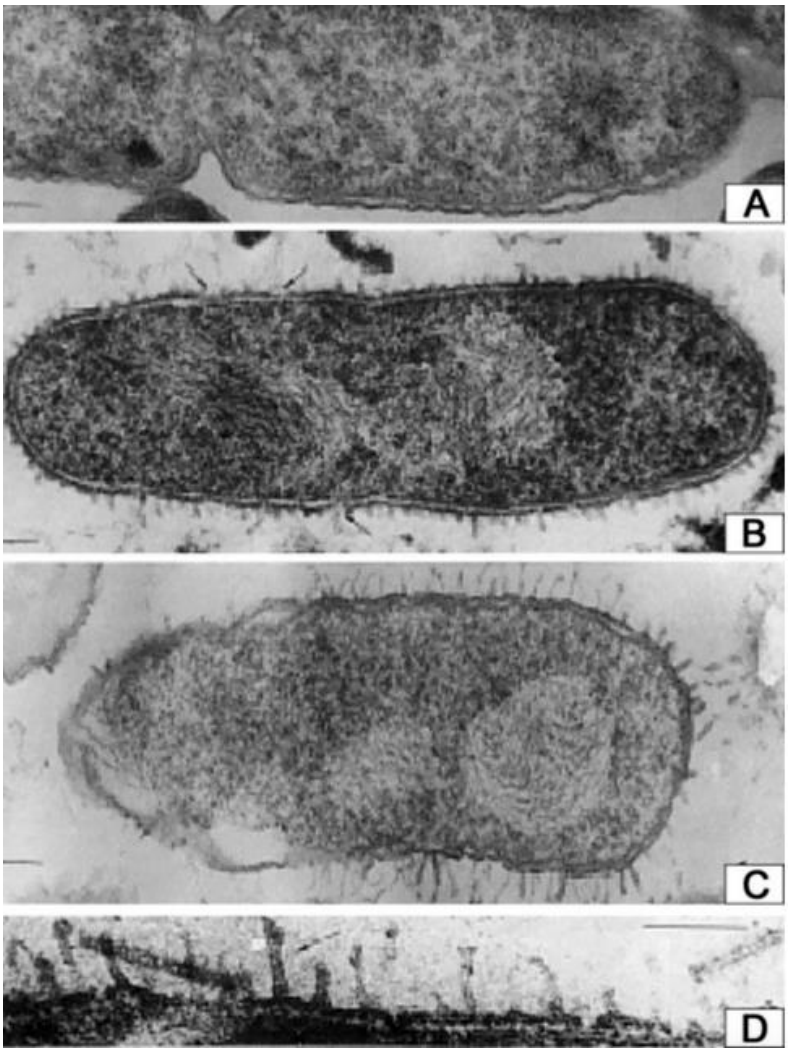


Figure 1.7: Transmission electron microscopy (TEM) images displaying the effect and mechanism of action for polymyxins against *P. aeruginosa*. A, untreated cell; B, treatment with polymyxin B; C, treatment with CMS; D, higher magnification of B. Note the projections on the cell wall. Scale bar = 0.1µm [Falagas and Kasiakou 2005; Koike *et al* 1969].

These micelle-like structures can act like pores, since they are associated with water, allowing the movement of ions across the membrane and other hydrophilic molecules. Over time the micelle structure will dissociate, and monomers will form that can be translocated into the cytoplasm, binding to cellular polyanions, i.e., DNA or RNA [Hancock and Chapple 1999]. Figure 1.7 displays a damaged CM with part of the cytoplasmic material being released through 'cracks' into the surrounding environment [Falagas and Kasiakou 2005; Koike *et al* 1969]. This entire process eventually results in cell death, manly via lysis [Falagas and Kasiakou 2005]. In addition to the above mechanism of action described, a new mechanism of action against *A. baumannii* was also observed [Sampson *et al* 2012]. It

is believed that polymyxins produce hydroxyl radicals via the Fenton reaction, allowing for rapid killing. Polymyxins activate an envelope stress response, causing a shift in the metabolic state and abnormal electron transport, promoting the production of superoxide that can take place in the Fenton reaction leading to hydroxyl radical production [Sampson *et al* 2012], i.e., produced superoxides (O_2^-) are converted to peroxides (H_2O_2) by superoxide dismutases within cells. Peroxides then interact with ferrous iron (Fe^{2+}), oxidising iron (Fe^{3+}) and thus forming hydroxyl radicals ($OH^{\cdot}$); cell death is achieved through oxidative damage to DNA, lipids and proteins. Interestingly, drug-sensitive and resistant *M. tb* have been shown to be highly sensitive to the Fenton reaction driven by vitamin C [Vilchèze *et al* 2013].

It's worthy to note that due to polymyxins strong interaction with lipid A, very potent anti-endotoxin activity exists, as it is easily neutralised. Thus, the endotoxin, i.e., lipid A, cannot induce shock via the release of cytokines and use of direct hemoperfusion using an adsorbent column with polymyxin B-immobilised fiber improves the clinical state of patients with septic shock [Falagas and Kasiakou 2005; Gough *et al* 1996; Uriu *et al* 2002].

Development of resistance to polymyxins is slow, and the main reason behind this is due to its mechanism of action, i.e., it is not dependent on bacterial metabolic activity [Li *et al* 2005a; Lorian 1971]. Rather, resistance has been associated with changes in the outer membrane LPS such as lipid alterations, reduction in LPS, reduced levels of specific membrane proteins and reduction in Ca^{++} and Mg^{++} content [Denton *et al* 2002; Falagas and Kasiakou 2005; Gunn *et al* 1998; Lim *et al* 2010; Macfarlane *et al* 1999; Moore *et al* 1984; Moskowitz *et al* 2004; Nation and Li 2010]. Reduction of LPS negative charges by modification of lipid A have also been associated with resistance in *P. aeruginosa*, *Escherichia coli* and *Salmonella enterica serovar Typhimurium* [Helander *et al* 1994; Lim *et al* 2010; Moskowitz *et al* 2004; Nation and Li 2010; Wang *et al* 2004]. These mechanisms of resistance arise via adaptation or mutations within the organisms [Falagas and Kasiakou 2005]. An efflux pump may also be associated with polymyxin resistance in *Yersinia* spp [Bengoechea and Skurnik 2000]. The above adaptations and mutations may also have an effect on the Fenton reaction, as well as species that limit intracellular iron, which is required for the Fenton reaction to take place [Sampson *et al* 2012].

1.3.3.2 Spectrum of activity:

Polymyxins display a narrow spectrum of activity as an antimicrobial; most common susceptible organisms are clinically relevant gram-negative isolates, i.e., *A. baumannii, P. aeruginosa, K. pneumoniae.* Others include *Enterobacteriaceae, Stenotrophomonas maltophilia, E. coli, Salmonella* spp, *Shigella* spp, *Haemophilus influenzae, Bordetella pertussis, Citrobacter* spp, some *Aeromonas* spp and *Legionella pneumophila* [Lim *et al* 2010; Li *et al* 2005a; Zavascki *et al* 2007]. Polymyxins display no activity against gram-positive organisms and anaerobes [Li *et al* 2005a; Zavascki *et al* 2007]. Known resistant strains are *Burkholderia cepacia* complex, *Burkholderia pseudomallei, Proteus* spp, *Providencia* spp, *Morganella morganii* and *Serratia marcescens, Aeromonas jandaei, Aeromonas hydrophila* has inducible resistance, *Neisseria* spp, *Moraxella catarrhalis, Helicobacter pylori, Vibrio* spp and *Brucella* spp are intrinsically resistant and *Campylobacter* spp vary in their susceptibility to polymyxin B, and the susceptibility of *Bartonella* spp is borderline [Li *et al* 2005a; Zavascki *et al* 2007].

1.3.3.3 *In vitro* susceptibility testing:

A complicated and confusing topic regarding polymyxins is *in vitro* susceptibility testing. Many factors can affect the outcome of the determined MIC. A recent review by Humphries [2014] sufficiently covers the intricate topic.

Table 1.1: CLSI and EUCAST MIC breakpoints for colistin [Humphries 2014].

	CLSI breakpoints (mg/L)[a]			**EUCAST breakpoints (mg/L)[b]**		
Organism	**Susceptible**	**Intermediate**	**Resistant**	**Susceptible**	**Intermediate**	**Resistant**
Enterobacteriaceae	-	-	-	≤ 2	-	> 2
Acinetobacter	≤ 2	-	≥ 4	≤ 2	-	> 2
Pseudomonas spp	≤ 2	4	≥ 8	≤ 4	-	> 4
Other non-*Enterobacteriaceae* gram-negative bacilli	≤ 2	4	≥ 8	-	-	-

[a]CLSI breakpoints include polymyxin B, which are the same for colistin.

[b]The BSAC breakpoints are the same as those published by EUCAST.

Table 1.1 displays the Clinical and Laboratory and Standards Institute (CLSI), European Committee on Antimicrobial Susceptibility Testing (EUCAST) and The British Society for Antimicrobial Chemotherapy (BSAC) breakpoints for colistin. The breakpoints for colistin are continually being evaluated for possible revision.

Different susceptibility tests for polymyxins exist, i.e., broth microdilution, agar dilution, disk diffusion and use of Etest® produced by bioMerieux, Durham, NC. Broth microdilution produces the most consistent results when done according to standardised protocols [Clinical and Laboratory and Standards Institute 2012; Humphries 2014; ISO 2006]. A number of conditions will affect the outcome of the MIC and need to be understood; (1) to determine the MIC for polymyxin B or colistin, only PST or CST should be used because CMS yields inaccurate MICs *in vitro* due to being a prodrug of colistin [Humphries 2014; Landman *et al* 2008; Li *et al* 2006]; (2) it is important the define the activity of PST or CST when used, i.e., how many IU/mg (and the manufacturer)? This is due to PST and CST being heterogeneous, with manufacturers and even batches producing polymyxins with differing active components that lead to possible MIC variability [Decolin *et al* 1997; Humphries 2014; Landman *et al* 2008]; (3) polymyxins readily adhere to polystyrene of microdilution plates, with the coating of the plates affecting the outcome of the MIC, i.e., approximately 92.5% of colistin was lost using a polystyrene microdilution plate but only 54% was lost when addition of 0.002% v/v polysorbate 80 (Tween 80) was added [Humphries 2014], leading to lower MICs. MICs using 0.002% v/v polysorbate 80 in polystyrene microdilution plates agree with MICs determined using glass (considered to adsorb less polymyxins) without 0.002% v/v polysorbate 80 [Hindler and Humphries 2013; Humphries 2014]. Lower concentrations of colistin seem to be more affected than higher concentrations since the adsorption effect to polystyrene is believed to be saturable. The CLSI has voted against the use of polysorbate 80 and any other detergents over concerns of potential synergy between the two agents [Humphries 2014]. There are concerns that this ruling will have an impact on MIC interpretations due to adsorption effect; (4) Divalent cations such as Ca^{++} and Mg^{++} can produce variable MICs, and their concentrations should be noted with standard cations being supplemented [Falagas and Kasiakou 2005; Landman *et al* 2008].

Agar dilution is a second reference point for MIC determinations and has shown a 94% categorical agreement with broth microdilution (containing 0.002% v/v polysorbate 80) MICs. Agar dilution is used less than broth microdilution due to its labor-intensive nature [Hindler and Humphries 2013; Humphries 2014].

Disk diffusion is used as a third reference point. The method is readily performed by most clinical laboratories because the method is not technically challenging. The disadvantage is that high

error rates exist, which is likely due to the poor diffusion rates of polymyxins through agar [Falagas and Kasiakou 2005; Gales *et al* 2011; Humphries 2014; Hogardt *et al* 2004; ISO 2006]. These zones of inhibition are small making it difficult to identify susceptible or resistant organisms [Humphries 2014]. It is important to confirm results with another MIC method such as broth microdilution and EUCAST recommend only MIC methods should be used for susceptibility testing [Falagas and Kasiakou 2005]; Humphries 2014].

The Etest® is a commercial susceptibly test that is available. The colistin Etest® is not FDA approved and recommended only for research use [Humphries 2014]. Very major errors exist when compared to agar dilution [Behera *et al* 2010; Hindler and Humphries 2013; Humphries 2014; Tan and Ng 2007]. Likely due to the same effect observed for disk diffusion; polymyxins have poor rates of diffusion through agar.

Extrapolating the susceptibly and resistant data for PST from colistin MICs is not suggested [Humphries 2014].

1.3.3.4 Synergistic activity:

Polymyxins display synergy with a number of compounds such as rifampin, azithromycin, ceftazidime, trimethoprim-sulfamethoxazole, imipenem, cefepime, tigecycline. The synergy is well documented against highly-resistant gram-negative MDR bacteria [Chen and Kaye 2011]. RIF is the most commonly studied antibiotic with colistin and is widely discussed as alternative treatment for highly-resistant MDR gram-negative infections [Biswas *et al* 2012; Nation and Li 2009]. Combinations of colistin and RIF have shown 100% synergy against MDR *A. baumannii* infections, and variable synergy has been observed against *P. aeruginosa* [Biswas *et al* 2012; Li *et al* 2007; Liang *et al* 2011; Sheng *et al* 2011].

One study revealed the combinational use of CMS with RIF in ten patients with ventilator associated pneumonia MDR *A. baumannii* benefited 70% of patients receiving treatment. Treatment for up to 11 days was deemed safe in patients without underlying renal disease [Song *et al* 2008]. Lee *et al* [2013] showed minimal killing of MDR *A. baumannii* when RIF was used alone due to resistance. However, when colistin was used in combination with RIF, substantially enhanced killing and synergy were observed at lower concentrations, even suppressing the emergence of *de novo* colistin resistance. This is likely due to the ability of colistin to disrupt the outer membrane of gram-negatives, allowing an influx of hydrophobic RIF into the cytoplasm of *A. baumannii.*

Other drugs that have been suggested for combinational use or observed to have synergistic activity are sulbactam, meropenem, minocycline, teicoplanin and even glycopeptides, i.e., vancomycin [Biswas *et al* 2012; Gordon *et al* 2010; Kempf *et al* 2012; Kempf and Rolain 2012; Liang *et al* 2011; Wareham *et al* 2011].

Zeidler *et al* [2013] showed colistin to have synergistic activity with the echinocandin family against all *Candida* species. Synergy was only observed when strains were known to be susceptible to echinocandin. The authors suggested that echinocandin-mediated weakening of the cell wall thus facilitating colistin targeting of fungal membranes, reinforcing the antifungal activity of echinocandin.

Synergistic activity of polymyxins with various antimicrobials may assist in lowering of MICs, especially with polymyxins and thus reducing drug toxicity [Landman *et al* 2008].

1.3.4 Inhalation of polymyxins (pharmacokinetics, pharmacodynamics and toxicity):

Extensive knowledge exists with the inhalation of CMS in patients with cystic fibrosis. This mode of administration is very successful in reduction of lower respiratory samples containing early colonisation of *P. aeruginosa* [Nation and Li 2010]. There is less experience in patients not suffering from cystic fibrosis, i.e., critical ill patients with nosocomial pulmonary infections caused by MDR *P. aeruginosa* or *A. baumannii* [Nation and Li 2010]. Methods of inhalation have mainly used nebulisation as a means [Ratjen *et al* 2006] until recently where dry powder inhalation has been investigated as a more optimised method of administration [Conole and Keating 2014; Schuster *et al* 2013]. Inhalation of CMS has the advantage of reducing systemic exposure and toxicity but simultaneously administers high drug concentrations directly into the lungs [Conole and Keating 2014; Geller 2009; Gibson *et al* 2003]. If inhalation of CMS would be used to treat *M. tb* infections, dry powder CMS would likely be used and is thus discussed in detail.

Dry powder CMS for inhalation is known as Colobreathe® or CMS DPI, which is micronised to be administered using a hand-held device known as Turbospin® and is approved in the EU for treatment of patients older than 6 years of age [Conole and Keating 2014; Schuster *et al* 2013]. This method of inhalation device allows for portability by patients, faster administration times with no need for expensive equipment requiring maintenance [Conole and Keating 2014]. Inhalation using Turbospin® and CMS DPI has also been described as ‘very easy to use’ and a preferred treatment when compared to tobramycin inhaled solution [Schuster *et al* 2013]. With regard to pharmacodymics, i.e., mechanism of action, antibacterial activity, mechanisms of resistance, etc., are the same as those that have been discussed for polymyxins.

Major concerns of toxicity are nephro and neurotoxicity when administered intravenously, which is dose dependent and reversible on discontinuation of treatment. Manifestations such as albuminuria, presence of urinary casts, reduced urine output, increased blood urea nitrogen and increased creatinine levels have been observed [Beringer 2001; Conole and Keating 2014; Falagas *et al* 2005; Hartzell *et al* 2009]. Manifestations such as paresthesia, visual alterations, neuromuscular blockade, weakness and paralysis, vertigo and ataxia have been observed concerning neurotoxicity. Likely due to inhibition of acetylcholine release at somatic nerve endings in skeletal muscle [Beringer 2001; Conole and Keating 2014; Falagas *et al* 2006; Koch-Weser *et al* 1970; Parisi and Kaplan 1965]. These effects are mainly concerned with intravenous administration since inhalation can reduce systemic exposure and toxicity. This was noted, where during the administration of CMS DPI, no relevant changes in weight, BMI and growth were observed in patients. Furthermore, no significant changes in haematological, biochemistry or urinalysis parameters were observed [Schuster *et al* 2013]. It is important to note that CMS DPI should not be co-administered with other forms of polymyxins, i.e., intravenously or be co-administered with other potential nephro or neurotoxic agents, i.e., amphotericin B deoxycholate, including non-depolarising muscle relaxants [Conole and Keating 2014; European Medicines Agency 2011]. In certain cases, patients might develop bronchoconstriction and if bronchospasm or coughing develops upon inhalation, administration of β_2-agonists before or after inhalation of CMS DPI will relieve these side effects [Conole and Keating 2014; European Medicines Agency 2011].

High doses are well tolerated and currently a regime of twice daily dosing using 125mg (1.6625 million international units (MIU)) CMS DPI was adopted for clinical trials [Conole and Keating 2014; European Medicines Agency 2011; Schuster *et al* 2013]. Table 1.2 displays the percentage of the administered dose deposited in the lungs. Approximately 80mg of CMS was used in a nebuliser while approximately 125mg of CMS DPI was administered using the Turbospin® with or without pre-treatment of salbutamol. Radio-labelled Tc_{99}-CMS was used to compare the lung deposition and distribution (Table 1.2).

Table 1.2: Percentage of administered dose deposited in the lung regions [European Medicines Agency 2011].

	Percentage of dose deposited (mean % ± SD)			
Regimen	**Whole lung**	**Central lung**	**Intermediate lung**	**Peripheral lung**
Nebulised CMS	5.9 ± 3.4	1.4 ± 0.8	2.0 ± 1.1	2.4 ± 1.6
CMS DPI (+ salbutamol)	19.3 ± 8.5	5.6 ± 3.1	6.4 ± 3.0	7.3 ± 2.7
CMS DPI (- salbutamol)	18.7 ± 6.9	5.9 ± 2.7	6.2 ± 2.2	6.6 ± 3.0

From Table 1.2, it can be seen that inhalation of CMS DPI using the Turbospin® is better than using CMS and a nebuliser for depositing the dose in the lung.

Using lung sputum as a representative of local drug concentrations, Ratjen *et al* [2006] determined that after inhalation of nebulised CMS (158mg = 2 MIU one dosage, once daily), maximum colistin sputum concentrations reached were approximately 40mg/L; way above the breakpoint for various infections (Table 1.1) and remained above the susceptibly breakpoint after 12-hour post-administration. Quantitative analysis was conducted using colistin A as a marker and thus, if colistin B was also included during quantification, a better estimate of local drug concentrations would have been determined. Inhalation of dry powder CMS DPI using the Turbospin® (125mg = 1.6625 MIU; one dosage, twice daily) determined that 38% of patients had sputum colistin concentrations > 128mg/L [Schuster *et al* 2013]. It is important to keep these values in consideration as they must be above the determined MIC against the organism causing a pulmonary disease.

Metabolism and elimination of inhaled CMS has not been studied extensively [Conole and Keating 2014]. Intravenously, CMS is eliminated renally while formed colistin undergoes renal tubular reabsorption and eliminated via non-renal routes [Conole and Keating 2014; European Medicines Agency 2011]. Pulmonary clearance via sputum has been suggested as an overall clearance when nebulised CMS is administered [European Medicines Agency 2011]. *In vitro,* analysis also revealed that colistin and CMS did not alter the activity of the cytochrome P450 enzymes [Conole and Keating 2014; European Medicines Agency 2011].

Comparing dry powder inhalation of CMS DPI to nebuliser use of tobramycin, is non-inferior for treatment of patients with cystic fibrosis and *P. aeruginosa* infections as measured by the change of Forced expiatory volume (FEV_1%) at 24 weeks [Schuster *et al* 2013]. Adverse events are experienced with dry powder inhalation of CMS DPI, the main events being experienced are cough, abnormal taste, throat irritation and this is normal for inhalation of dry powders [Schuster *et al* 2013]. Other events

experienced were respiratory, thoracic and mediastinal disorders [Conole and Keating 2014; Schuster *et al* 2013]. Most adverse events diminished after 28 days of administration [Schuster *et al* 2013].

1.4 Mycobacteria and polymyxins:

Broad antimycobacterial activity for CST has been known for many years with the first major systematic investigation being conducted in 1986 [Rastogi *et al* 1986b]. Unfortunately, further investigations of CST were abandoned due to a high MIC and systemic toxicity concerns if colistin were to be used intravenously. Luckily, as discussed, the use of inhaled CMS can circumvent these issues.

Initial investigations of polymyxins were that of colimycin (colistin) being used as an agent to prevent contamination in media for the isolation of various Mycobacteria [Lorian and Maddock 1966]. Later, the first paper describing an inhibitory effect of polymyxins against Mycobacteria was published [Camargo *et al* 1974]. The authors tested various chemotherapeutic agents against *M. lepraemurium* (a model for *M. leprae*) by monitoring metabolism radiometrically, measuring $^{14}CO_2$ produced by bacterial metabolism of acetate-U-^{14}C in a K-36 buffer. A dosage of 5mg/vial (20ml) was used, and the authors observed inhibition of metabolism by polymyxin B, that was greater than vancomycin but less than cycloserine after 15 days of incubation.

The use of polymyxin B in differentiation of *M. fortuitum* and *M. chelonei* was determined by Welch and Kelly [1979]. This method of species identification was later confirmed by Wallace *et al* [1982] and Rastogi *et al* [1986b] with minor differences.

David *et al* [1980] investigated the use of Mycobacteriophage $D29R_1$ for determining the MIC of certain antituberculosis and antileprosy drugs against *M. aurum.* Concentration of drug that reduced the final phage yield by 50% was defined as the IC_{50}, which was determined to be 0.78mg/L for colistin, while the actual MIC was determined to be 1mg/L, thus giving a IC_{50}/MIC ratio of 0.78. It was observed that colistin inhibited the lytic cycle of the phage. This effect on *M. aurum* was further investigated by David and Rastogi [1985]. Using RVB_{10} media, 10^8 CFU/ml and surface plate counts, the MIC for colistin was determined to be 5mg/L and bacteriostatic, while lethal concentrations were determined to be above 50mg/L. Lethal concentrations were associated with gross alterations of the cell envelope, serious CM damage, CM invaginations, CM lesions, peripheral deformations, polar accumulation of membrane-like structures and unusual intracytoplasmic membrane structures. A patchy and diffused outer CL was also observed. Interestingly, sublethal concentrations (15mg/L) caused synchronised cell division once cells were rinsed and resuspended in new media; likely due to

separation of the bacterial chromosome from the CM. Ultrastructurally this was associated with an increase of polysaccharide-like substances, which decreased upon removal of colistin, after which an increase in amounts of mesosome-like structures were observed. Based on these observations, colistin appeared to interact with outer CL as well as the CM of *M. aurum.*

Table 1.3 displays the systematic investigation by Rastogi *et al* [1986b] of colistin against 15 Mycobacteria species. Using 7H9 supplemented with ADC (albumin, dextrose, catalase) and 0.05% v/v/ Tween 80, the broth dilution method (5ml) was used with 0.1ml of 10^6 CFU/ml. The MIC was defined as the concentration of colistin causing at least 50% inhibition of growth (by turbidity measurements at 650nm). The minimum bactericidal concentration (MBC) was defined as the concentration of colistin that caused less than 10^{-2} survivors as determined by viable cell counts. The most important result is that of *M. tb* (H37Ra strain) as it displayed more sensitivity compared to all other species.

As discussed, previous data showed that sublethal colistin concentrations caused synchronised cell division (separation of the bacterial chromosome from the CM) as well inhibiting the lytic cycle of Mycobacteriophage D29 in *M. aurum*, i.e., in order to complete the final stages of bacteriophage assembly, binding of structural proteins to the CM is required by the phage. It was hypothesised by David *et al* [1986] that colistin caused molecular displacements of the CM architecture, preventing interaction of intracellular macromolecules with the internal aspect of the inner leaflet of the CM. The authors investigated the effect colistin would have on the lytic cycle of Mycobacteriophage D29 in *M. tb* (H37Ra) to investigate their hypothesis. The adsorption of D29 in the presence of colistin was not affected, only the lytic cycle of D29 was inhibited in *M. tb*. The author's data revealed that inhibition by colistin was at phage maturation, which was related to ultrastructure observations of an altered inner leaflet of the CM. Mycobacteria have an asymmetric staining profile of their CM, especially when using Thiéry cytochemical staining, i.e., the outer leaflet will accumulate the stain but not the inner leaflet.

Table 1.3: Antibacterial action of colistin against type strains of 15 Mycobacterial species [Rastogi *et al* 1986b].

Species	MIC (mg/L)	MBC (mg/L)	Survivors with 100 mg/L of colistin
M. tuberculosis	5	50	8.0×10^{-3}
M. africanum	15	> 100	1.7×10^{-2}
M. bovis	> 100	> 100	1.5×10^{-1}
M. avium	> 100	> 100	2.0×10^{-2}
M. intracellulare	25	50	2.0×10^{-6}
M. scrofulaceum	25	> 100	2.4×10^{-2}
M. szulgai	> 100	> 100	4.3×10^{-1}
M. xenopi	100	100	3.0×10^{-3}
M. kansasii	100	> 100	2.1×10^{-1}
M. simiae	> 100	> 100	4.3×10^{-1}
M. fortuitum	50	60	3.0×10^{-4}
M. chelonei	> 100	> 100	7.0×10^{-1}
M. marinum	> 100	> 100	3.3×10^{-1}
M. phlei	50	60	5.0×10^{-6}
M. smegmatis	30	60	5.0×10^{-5}

Thus, under the presence of colistin, the antimicrobial penetrates these membranes making the inner leaflet available for staining; making substrates normally hidden in the molecular architecture of the CM available for staining. These alterations of colistin to the inner leaflet of CM therefore can be related to inhibition of the lytic cycle of D29 at phage maturation, i.e., binding sites in the inner leaflet of the CM for phage structural proteins were not available. The inhibition was reversed when colistin was rinsed off, suggesting the binding sites were not chemically blocked. Addition of Ca^{2+} restored the lytic cycle of D29, suggesting simple topological changes at the binding sites. Addition of Mg^{2+} affected the antimicrobial effect of colistin but did not restore the lytic cycle of D29, suggesting that there is a preference of Mg^{2+} for the outer leaflet of the CM and a preference of Ca^{2+} for the inner leaflet. Even though the main mechanism of action of colistin seems to be upon the CM, leading to leakage and death, the molecular rearrangement of the CM by colistin must affect a variety of other functions vital to the cell. This was confirmed for *M. aurum* as well by observing that colistin induced

Mg^{2+} efflux, which preceded cell death by at least 15 min, proving colistin interacted with the CM, causing cell leakage [Rastogi *et al* 1987]. Similar experiments were conducted with *M. intracellulare* (colistin susceptible, MIC = 25mg/L) and *M. avium* (colistin resistant, MIC > 100mg/L). Efflux of Mg^{2+} was observed for both strains; however, loss of viability for *M. intracellulare* occurred after six hours with a plateau of efflux at 24 hours while for *M. avium*, loss of viability only occurred after 48 hours with a plateau of efflux at six hours that was three times less than *M. intracellulare*. Thus, for *M. avium*, an additional mechanism of action is likely and not solely Mg^{2+} efflux, i.e., cell leakage from the CM. It is possible that growth rates and cell division could play a role, as *M. intracellulare* and *M. aurum* (above) are faster growing than *M. avium* [Rastogi *et al* 1988]. The authors [Rastogi *et al* 1988] also observed that colistin did not increase the β-lactam (amoxicillin, carbenicillin, clavulanic acid) susceptibly of *M. avium in vitro* or intracellularly-multiplying bacilli (in J-774 macrophages). No increase in susceptibility for INH, streptomycin or RIF was observed when these drugs were used in association with colistin or polymyxin B nonapeptide (PMBN) (polymyxin B lacking the fatty acid tail) [Rastogi *et al* 1988]. Confirmation was observed when using 5mg/L colistin with either INH (2mg/L), RIF (2mg/L), ofloxacin (1mg/L) or ciprofloxacin (1mg/L). No enhancement of drug activity was observed against *M. avium* ATCC 15769 using BACTEC radiorespirometry as well as viable cell counts [Rastogi *et al* 1990].

Using *M. vaccae*, Korycka-Machała *et al* [2001] attempted to increase the permeability of the cell envelope using PMBN. Increased permeability was investigated by observing enhanced intracellular bioconversion of β-sitosterol (hydrophobic) to 4-androsten-3,17-dione (AD) and 1,4-androstadien-3,17-dione (ADD) as quantified by chromatography, i.e., the rate of AD and ADD formation is a measure for sterol penetration into the cell. Sensitisation to compounds such as erythromycin and RIF were also investigated. PMBN was used at concentrations that did not affect growth of *M. vaccae*, i.e., comparable growth to the control. During the investigation, the authors observed an enhanced formation of AD and ADD when sub-inhibitory concentrations of PMBN were used (two fold higher than control cells), furthermore, an enhanced antibiotic activity of erythromycin and RIF was observed when used together with PMBN, i.e., synergy. This enhanced permeability of the cell envelope was hypothesised to be likely due to positive charges on polycationic compounds such as PMBN binding to negative charges on the outermost cell wall layer, causing disorganisation of the native structure of non-covalently bound lipids. Similar investigations using *Mycobacterium* sp. DSM 2966 confirmed the observation made by Korycka-Machała *et al* [2001] [Malaviya and Gomes 2008].

Polymyxin B has also been identified as an inhibitor of alternative NADH dehydrogenase (IC_{50} = 1.6mg/L) and malate: quinone oxidoreductase (IC_{50} = 4.2mg/L) in *M. smegmatis* [Mogi *et al* 2009]. These enzymes are essential for the viability of *M. smegmatis* and even *M. tb,* especially in hypoxic conditions. Quinone-binding site was shown to be the primary site of action by polymyxin B. Polymyxin E (colistin) had a better bactericidal action on *M. smegmatis* compared to polymyxin B (8mg/L vs 21mg/L) due to destruction of the bacterial membrane, but polymyxin E never displayed inhibiting effects on alternative NADH dehydrogenase (IC_{50} = 2.6g/L) or malate: quinone oxidoreductase (IC_{50} = never reached) in *M. smegmatis*. This is due to the one amino acid difference in polymyxin E vs polymyxin B. One can speculate that PST can induce lower MICs compared to CST due to disruption of the hypoxic or aerobic respiratory chain. But ultimately, CST will produce lower MBCs due its bactericidal action on the bacterial membrane. It is unknown if the same effects reported for *M. smegmatis* can be extrapolated for *M. tb*.

Many other publications exist that include Mycobacteria and polymyxins. Polymyxins, however, play very small roles within these papers that are out of the scope of this thesis [Adams *et al* 1993; Barnes *et al* 1990; Chacon *et al* 2009; Giri *et al* 1998; Liu *et al* 1997; Molloy *et al* 1990; Ramasesh *et al* 1989; Toossi *et al* 1995; Valone *et al* 1988; Vouret-Craviari *et al* 1997; Wallis *et al* 1990].

1.5 Aims:

- Determine the MIC and the MBC of CMS, CST and PST against sensitive (H37Ra) and MDR *M. tb* using the Microtiter Alamar Blue Assay (MABA).
- Observe if the MIC of CMS is antagonised in the presence of pulmonary surfactant (PS) when used against *M. tb* by use of the MABA.
- To determine the synergistic effect CMS may have, using various combinations of INH and RIF on sensitive (H37Ra) and MDR *M. tb* using the MABA and Sum of Fractional Inhibitory Concentration calculations (Σ_{FIC}).
- To study the dynamics of the best synergistic combination determined (based on Σ_{FIC}) using a time-kill assay on sensitive (H37Ra) and MDR *M. tb*.
- To determine the base colistin activity in CMS at the determined MIC using Ultra-Performance Liquid Chromatography (UPLC).
- Observe the effect CMS has on the cell membranes of *M. tb* using TEM.

1.6 Hypotheses:

- H1: There is a statistical significant difference between the determined MIC and the MBC of CMS when compared to CST and PST at the 95% level of confidence against sensitive (H37Ra) and MDR *M. tb* by use of a Student's t-test.
- H2: There is no statistical significant difference of the CMS MIC and MBC determined for sensitive (H37Ra) *M. tb* compared to the CMS MIC and MBC for MDR *M. tb* at the 95% level of confidence by use of a Student's t-test.
- H3: There is a statistical significant difference of the CMS MIC determined in PS against *M. tb* when compared to the control CMS MIC (without PS), by use of a Student's t-test indicating antagonism by PS.
- H4: The lowest determined Σ_{FIC} would be for CMS used in combination with INH and RIF for sensitive (H37Ra) and MDR *M. tb.*
- H5: There is a statistical significant difference in the rate at which $\log_{10}$ viable CFU/ml units decrease when CMS is used in combination with INH and RIF versus when any two of the antibiotics are used in combination at the 95% level of confidence against sensitive (H37Ra) and MDR *M. tb* by use of a one-way ANOVA.
- H6: There is a statistical significant difference between the amount of base colistin formed from CMS at the MIC when compared to CST at the 95% level of confidence by use of a Student's t-test.
- H7: CMS has an observable damaging effect on the CL and CM of *M. tb* that is similar to that of CST.

1.7 Null hypotheses:

- $H1_0$: There is no statistical significant difference between the determined MIC and the MBC of CMS when compared to CST and PST at the 95% level of confidence against sensitive (H37Ra) and MDR *M. tb* by use of a Student's t-test.
- $H2_0$: There is a statistical difference of the CMS MIC and MBC determined for sensitive (H37Ra) *M. tb* compared to the CMS MIC and MBC for MDR *M. tb* at the 95% level of confidence by use of a Student's t-test.

- $H3_0$: There is no statistical significant difference of the CMS MIC determined in PS against *M. tb* when compared to the control CMS MIC (without PS), by use of a Student's t-test indicating no antagonism by PS.
- $H4_0$: The lowest determined Σ_{FIC} would be for CMS used in combination with INH or RIF, not INH and RIF for sensitive (H37Ra) and MDR *M. tb.*
- $H5_0$: There is no statistical significant difference in the rate at which $\log_{10}$ viable CFU/ml units decrease when CMS is used in combination with INH and RIF versus when any two of the antibiotics are used in combination at the 95% level of confidence against sensitive (H37Ra) and MDR *M. tb* by use of a one-way ANOVA.
- $H6_0$: There is no statistical significant difference between the amount of base colistin formed from CMS at the MIC when compared to CST at the 95% level of confidence by use of a Student's t-test.
- $H7_0$: CMS has no observable damaging effect on the CL and CM of *M. tb* that is not similar to that of CST.

1.8 Ethical clearance:

All research was conducted with approval from The Faculty of Health Sciences Research Ethics Committee, University of Pretoria (appendix A), which complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. The Faculty of Health Sciences Research Ethics Committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

1.9 Research outputs:

Parts of this study have been presented as an oral presentation at the following conferences respectively:

van Breda, S.V., van der Merwe, C.F., Nardell, E.A. and Stoltz, A.C. Preservation of *Mycobacterium tuberculosis* cell membrane integrity, after chemical fixation. Microscopy Society of Southern Africa 51st annual conference, 3-6 December 2013, Pretoria, South Africa. Oral presentation

van Breda, S.V., Buys, A.V., Nardell, E.A. and Stoltz, A.C. Effects of colistin methanesulfonate on *Mycobacterium tuberculosis* as determined by atomic force microscopy. Microscopy Society of Southern Africa 52nd annual conference, 2-5 December 2014, Stellenbosch, South Africa. Oral presentation

Awards:

Wirsam scientific prize for best student paper submitted at MSSA 51st annual conference.

Accepted publication:

The following article has been reviewed and accepted for publication at Tuberculosis (impact factor = 3.503)(Appendix B):

van Breda, S.V., Buys, A.V., Apostolides, Z., Nardell, E.A. and Stoltz, A.C. The antimicrobial effect of colistin methanesulfonate on *Mycobacterium tuberculosis in vitro*. *Tuberculosis* 2015. **95**: 440-6.

Chapter 2: MIC, MBC, synergy and time-kill studies of CMS against *M. tb*

2.1 Introduction:

Understanding the theory and background of MIC, MBC, synergy and time-kill studies using polymyxins such as CMS against *M. tb* are of importance in order to determine the most accurate results.

2.1.1 Considerations for polymyxin test conditions:

As discussed under section 1.3.3.3, CST or PST should be used for MIC determinations instead of CMS [Humphries 2014; Landman *et al* 2008; Li *et al* 2006]. The main reason being that CMS yields inaccurate MICs *in vitro* (under various conditions) due to being a prodrug of colistin. However, this rule is mostly concerned with routine susceptibly testing done in pathological labs. These labs are generally following guidelines set out by the CLSI, BSAC and EUCAST for accurate MIC determinations for gram-negative infections. This rule does not suggest that for drug exploration studies, i.e., this thesis, that CMS should not be investigated. Since CMS would be used in the treatment, i.e., Colobreathe® (CMS DPI) rather than CST, it is highly significant to rather focus on investigating the effects of CMS. CST and even PST play an important role as controls.

Considering the factors outlined under section 1.3.3.3 for experimental design and methodology for investigating the effects of polymyxins against *M. tb* are important. Broth microdilution such as the MABA should be used as it produces the most consistent results [Clinical and Laboratory and Standards Institute 2012; Humphries 2014; ISO 2006]. Growth media such as 7H9 OADC (oleic acid, albumin [BSA{bovine serum albumin}], dextrose, catalase) should be avoided since 7H9 OADC would antagonise the polymyxins mechanism of action for the following reasons; (1) OADC enrichment: polymyxins and BSA form complexes [Liu *et al* 2012], antagonising its effect. (2) Presence of Mg^{2+} and Ca^{2+} antagonise the effect of polymyxins [Chen and Feingold 1972; D'Amato *et al* 1975; section 1.4], and it was important to use a media where physiological concentrations of divalent cations can be controlled, i.e., cation-adjusted [Falagas and Kasiakou 2005; Landman *et al* 2008]. (3) Na^{+} (at 100 mM, present in OADC media) antagonises the effect of cationic antimicrobial

peptides such as polymyxins [Hancock and Sahl 2006; Ramón-Garcia *et al* 2013] (this might not apply to polymyxins [Newton 1954]). (4) It has been proposed that polymyxins also induce cell death through hydroxyl radical production, i.e., Fenton's reaction [Sampson *et al* 2012], and the presence of catalase in OADC would antagonise this mechanism of action.

Due to these reasons, Sauton media was used as the test media of choice as it would likely avoid variable and false results. When using Sauton media it was important to substitute the use of glycerol with glucose, since lower than normal MICs have been previously observed for *M. tb* when glycerol has been used as the only carbon source. This phenomenon is known as glycerol dependance [Pethe *et al* 2010]. As mentioned in section 1.3.3.3, use of polysorbate 80 (also known as Tween 80) is not suggested as there might be possible synergy between polymyxins and the detergent [Humphries 2014]. Tween 80 also has been known to potentiate the MIC of drugs such as RIF against *M. tb* [Franzblau *et al* 2012], possibly by altering cell membranes due to this detergent effect. However, since Tween 80 forms part of the Sauton media recipe and no evidence of this synergy with polymyxins has been proven, it was decided to include the detergent throughout the investigations. As an additional reason for its use, Tween 80 prevents the adsorption of polymyxins to the walls of microdilution plates [Hindler and Humphries 2013; Humphries 2014], thus preventing non-specific adsorption of polymyxins and allowing *M. tb* to come into contact with the true polymyxin concentration. The use of Tween 80 in the test media has also been suggested, since it allows for accurate determination of polymyxin potencies [Sader *et al* 2012]. Furthermore, Rastogi *et al* [1986b] observed no enhanced effect of Tween 80 with polymyxins against various Mycobacteria (including *M. tb*) during their investigations. Tween 80 is also important in minimising clumping of Mycobacteria [Franzblau *et al* 2012], allowing for accurate potency determinations. All this data leans in favour of including Tween 80 in the test media.

Section 1.3.3.3 also mentions that due to the amphiphatic-cationic nature of polymyxins, they non-specifically bind to polystyrene material [Humphries 2014]. Less non-specific adsorption of polymyxins have been observed when using polypropylene material [Hindler and Humphries 2013]. Thus using polypropylene material whenever polymyxins are to be used as well as Tween 80 in the test media, should allow for the least amount of non-specific binding of polymyxins, providing accurate potencies and data. For example, use of Eppendorf® Protein LoBind tubes are specifically designed to prevent non-specific adsorption by using a special polypropylene manufactured material.

When investigating antimicrobial effects of different drugs against *M. tb* it is a standard practice to filter sterilise antibiotic solutions. Due to the various nature of the filter membranes that are used, i.e., cellulose, etc., it is likely that polymyxins would non-specifically adsorb to these membranes.

Thus, filter sterilisation should be avoided and monitoring of possible contamination should be done through various controls.

2.1.2 MIC determinations using MABA:

Various formats are used to determine the MIC of polymyxins (section 1.3.3.3) and since broth microdilution produces the most consistent results [Clinical and Laboratory and Standards Institute 2012; Humphries 2014; ISO 2006], this was the format that was adopted.

Different reference strains for *M. tb* exist that are used for initial drug discovery, i.e., H37Rv and H37Ra. According to Franzblau *et al* [2012], the majority of labs use H37Rv ATCC 27294 (virulent) for *in vitro* evaluation of drug susceptibilities, although a number of labs do employ H37Ra ATCC 25177 (attenuated), i.e., 20 % vs 5 % of labs surveyed respectively. According to the survey by Franzblau *et al* [2012], one lab noticed differences in MIC for H37Rv vs H37Ra, and another noticed H37Ra is more sensitive than H37Rv to RIF. The H37Ra and H37Rv genomes are highly similar with respect to gene content and order. Variations in the PE/PPE/PE-PGRS genes have been determined, which encode proteins with diverse functions possibly related to virulence. These genes can also affect fibronectin binding and cell-surface antigenic variations to evade the immune system. Missense/nonsense mutations in *mazG* (stress response), *phoP* (transcriptional activation), *ilvD* (amino acid biosynthesis), *pks12* and *nrp* (polyketide synthesis) may contribute to the loss of virulence in H37Ra. H37Ra also has promoter region mutations that can be up regulated *in vitro* and down regulated *in vivo*, relative to H37Rv, which could be linked to H37Ra attenuated virulence [Dokladda *et al* 2015; Zheng *et al* 2008]. Protein differences with mutations also exist between H37Ra and H37Rv [Jena *et al* 2013] and due to polymyxins primary action on cell membranes, it is important to understand the differences between H37Ra and H37Rv. Målen *et al* [2011] reported that majority of proteins have the same amount of relative abundance between the two strains. Difference in abundance of 29 membrane-associated proteins was observed with at least a five-fold difference. H37Rv had more abundance in 19 membrane proteins and lipid proteins while a different group of 10 membrane protein were more abundant in H37Ra. Målen *et al* [2011] suggested that the bacterial secretion system and trans membrane transport system may be responsible for virulence in H37Rv. Other differences between H37Ra and H37Rv include membrane lipids. Compared to H37Rv, H37Ra is deficient in SL, DAT, PAT and PDIMs [Brennan 2003; Chesne-Seck *et al* 2008]. H37Ra also has a different mycolate profile compared to H37Rv; (1) unusual dicyclopropyl methoxy- and keto- mycolates are present. (2) H37Ra can only synthesise seven distinct homologous mycolate series vs 15 for H37Rv, and (3)

deficiencies in mycolate elongation and the transformation of cis-double bonds to trans-double bonds with an adjacent methyl branch exist. H37Ra also has an enhanced synthesis of mycolates with an additional cyclopropane ring [Watanabe *et al* 2001]. LAM from H37Ra vs H37Rv also differs, i.e., the termini are capped differently, being without mannose [Chatterjee *et al* 1992]. Thus, all these differences contribute to the attenuation of H37Ra.

However, H37Ra was chosen over H37Rv as the reference strain for sensitive *M. tb* throughout this investigation for a number of reasons; (1) our collaborator Ampath Pathology Laboratory Support Services (Centurion, Gauteng, South Africa) uses H37Ra as a reference strain. (2) Previous investigations of polymyxins against *M. tb* used H37Ra as a reference strain [David *et al* 1986; Rastogi *et al* 1986b]. (3) *M. tb* H37Ra has been used as reference strain when investigating activity of various peptides against *M. tb* [Davis *et al* 2004; Miyakawa *et al* 1996; Santos *et al* 2012; Vermeer *et al* 2012] and (4) no accessibility to H37Rv was available at our facilities.

As a representative of clinical isolates, an MDR *M. tb* strain was decided upon. Since up to 80% of clinical strains contain *katG* gene mutations concerning INH resistance and majority of clinical strains contain *ropB* mutations (as much as 95%) concerning RIF resistance (section 1.2.5.1 and 1.2.5.2), an MDR *M. tb* strain with such a characteristic would be representative of the MDR *M. tb* population.

MABA can be considered to be a broth microdilution test that incorporates the use of Alamar Blue® (also known as resazurin [blue]) as an indictor within the assay that dramatically reduces turn-around time for MIC determinations of compounds against *M. tb*. Since Alamar Blue® is non-toxic to cells, the assay can be adopted for MBC determinations [Collins and Franzblau 1997; Franzblau *et al* 1998; Parish and Brown 2008]. According to Franzblau *et al* [2012], the labs included in their survey most frequently used Alamar Blue® for endpoint readouts of MIC. The most common readouts are of monitoring Alamar Blue® reduction using fluorimetric or spectrophotometric measurements. Alamar Blue® can be described as a reagent that is cell wall permeable, non-toxic and weakly fluorescent. The compound (resazurin) is an oxidation-reduction indicator that changes its colour in response to metabolic activity reduction (Figure 2.1). It forms a highly fluorescent pink compound called resorufin, with reduction being proportional to the amount of living cells and thus Alamar Blue® can quantitatively measure cell viability [https://www.abdserotec.com/alamarblue-cell-proliferation-assay.html].

The advantages of using Alamar Blue® are numerous, i.e., (1) water soluble; (2) suitable for spectrophotometric measurements; (3) non-toxic to cells, user and environment; (4) high sensitivity; (5) extremely stable allowing for continuous monitoring; (6) extremely reliable and widely referenced [https://www.abdserotec.com/alamarblue-cell-proliferation-assay.html].

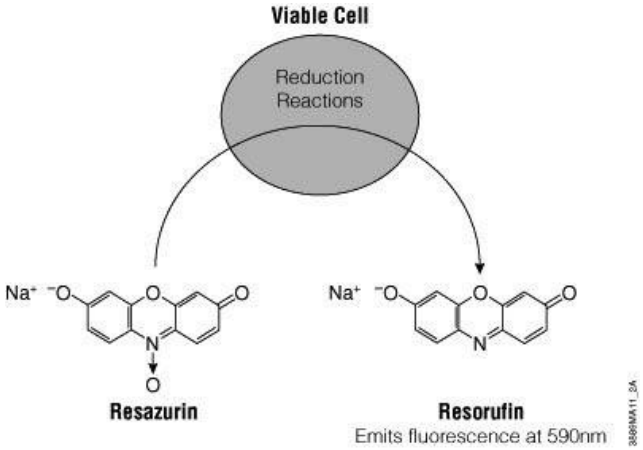


Figure 2.1: Reduction of resazurin (blue) to resorufin (pink) in living cells [https://www.promega.com/].

Disadvantages include possible signal to noise ratio being affected by the long incubation periods (up to 24 hours). Spectral overlapping of resazurin with resorufin may affect spectrophotometric measurements and should be noted during analysis, even though the majority of labs using Alamar Blue® use this method as a possible measurement [Franzblau *et al* 2012]. Other indicator dyes such MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide] exist but don't offer the same advantages as Alamar Blue®, i.e., MTT has the ability of interrupting the electron transport chain and requires a solubilisation step [https://www.abdserotec.com/alamarblue-cell-proliferation-assay.html].

Based on these facts and that MABA is widely used for MIC determinations of *M. tb*, having Chapters dedicated to the procedure in text books [Parish and Brown 2008], being used to determine MIC of peptides against *M. tb* [Rivas-Santiago *et al* 2013a; Rivas-Santiago *et al* 2013b; Ramos *et al* 2015] and Alamar Blue® has been used in conjunction with polymyxins [Wareham *et al* 2011], thus, MABA was determined to be the best format for the investigations.

Due to the Biosafety level (BSL) laboratory (2 and 3) only allowing access to spectrophotometric measurements for Alamar Blue®, Equation 1 is used to determine the % reduction of Alamar Blue®;

$$\% \text{ Reduction} = \frac{(O_2 \times A_1) - (O_1 \times A_2)}{(R_1 \times N_2) - (R_2 \times N_1)} \times 100 \qquad \text{Equation 1}$$

where O_2 = molar extinction coefficient (E) of oxidised Alamar Blue® at 600nm; A_1 = absorbance of test wells at 570nm; O_1 = E of oxidised Alamar Blue® at 570nm; A_2 = absorbance of test wells at 600nm; R_1 = E of reduced Alamar Blue® at 570nm; N_2 = absorbance of sterile control well at 600nm; R_2 = E of reduced Alamar Blue® at 600nm; N_1 = absorbance of sterile control well at 570nm.

It is significant to note that many factors will affect the outcome of the determined MIC, i.e., components used in media, pH of media, inoculum size, compound solubility, batch of compound, growth stage of cells [Franzblau *et al* 2012]. Thus, it is important to consider all these factors and standardise the format throughout initial drug screening of MIC, MBC, synergy and time-kill assays, as the slightest differences could affect results.

The MIC was defined as the well containing the lowest concentration of the antimicrobial that corresponds to $\geq$ 90% prevention in Alamar Blue® reduction. Definitions for INH and RIF breakpoints when using Alamar Blue® in this format where used as previously described by Palomino *et al* [2002], where INH has a breakpoint of 0.25mg/L and RIF has a breakpoint of 0.5mg/L.

2.1.3 MBC determinations:

MBC can easily be determined using the same broth microdilution method as the MABA [Franzblau *et al* 2012]. The MABA is carried out as normal but cells are subcultured from broth microdilution onto drug free solid media such as 7H10 OADC agar. By determining CFU/ml of drug treated cells to control cells at the beginning of the incubation, the MBC can be defined as the lowest concentration of antimicrobial that affects a $\geqq$ 2 $\log_{10}$ (MBC_{99}) reduction in CFU/ml, relative to the starting inoculum [Franzblau *et al* 2012].

If the determined MBC is at least four times the MIC, the antimicrobial can be considered to be bactericidal. If it is larger than four, then the antimicrobial is considered to be bacteriostatic [French 2006].

The best method for determining CFU/ml in such a situation is to use the pour plate method, i.e., a small amount of cells from the broth microdilution wells are added to a petri dish using a pipette. Cooled but molten 7H10 OADC agar is poured into the petri dish and gently mixed by swirling the plate in clockwise and anticlockwise directions. After incubation, bacterial enumeration is possible.

2.1.4 PS MIC investigations:

As outlined in section 1.3.4, CMS in the form of Colobreathe® (CMS DPI) is inhaled directly into the lungs. The pharmacology of the lungs differs from the rest of the body and can have various effects on antibiotic efficacy when inhaled [Pennington 1981]. An important component of lung physiology is epithelial lining fluid, which contains PS as a primary component. PS is rich in various proteins (surfactant-associated proteins A, B, C, D) and lipids (phospholipids such as DPPC, phosphatidylcholine, phosphatidylglycerol as well neutral lipids and cholesterol) that coat the interior surface of the airway that assists in reducing surface tension within the alveoli [Goerke 1998]. Daptomycin has been shown to interact with PS *in vitro*, resulting in inhibition of antimicrobial activity, which helped explain the change in daptomycin efficacy in *in vivo* pulmonary infection models [Silverman *et al* 2005]. Determining MIC for CMS in the presence of PS *in vitro* can assist with predicting its efficacy in an *in vivo* situation. Porcine PS has shown superiority over other mammalian PS for the treatment of respiratory distress syndrome [Knudsen *et al* 2012; Singh *et al* 2011], and has successfully been used *in vitro* as a surrogate marker for determining the affect PS would have on various antimicrobial MIC [Schwameis *et al* 2013]. PS is generally used at a concentration of 1mg/ml, but concentrations of PS have been described as being low as 0.01mg/ml in epithelial lining fluid. Higher concentrations have been used to ensure that even slight effects on antibiotic activity can be observed [Schwameis *et al* 2013].

Schwameis *et al* [2013] did observe porcine PS from Curosurf at 1mg/ml in cation-adjusted Mueller-Hinton broth to antagonise the antimicrobial effect of colistin against *P. aeruginosa*. The inhibitory effects of PS on colistin were overcome at concentrations ≧ 64 times the MIC of *P. aeruginosa* in Mueller-Hinton broth with PS.

Due to the composition of PS [Goerke 1998], it is important to consider if *M. tb* could use PS as a possible energy source. This could possibly lead to falsely elevated MIC of CMS that might be interpreted as antagonism of CMS by PS.

It is important to note that a concentration of 0.1% Triton X-100 (detergent) could reverse the inhibitory effect PS had on daptomycin [Silverman *et al* 2005]. Thus care needs to be taken when interpreting data generated with media that contains Tween 80.

It is important to determine if PS has any inhibitory effect on the MIC for CMS against *M. tb* because the investigation by Schwameis *et al* [2013] cannot be extrapolated since different organisms, media, conditions and MIC values were used.

Since porcine PS only needs to be added to media at 1mg/ml [Schwameis *et al* 2013], MABA can be modified to accommodate the addition of PS in determining the change in MIC. Thus the MIC can be defined as the well containing the lowest concentration of the antimicrobial that corresponds to ≥ 90% prevention in Alamar Blue® reduction (section 2.1.2).

2.1.5 Synergy and time-kill assays:

As discussed in section 1.2.5.1 and 1.2.5.2, INH and RIF are the most important first-line antimycobacterial agents used in the combat against *M. tb* infection and is the defining factor for MDR *M. tb* infections. Since CMS would not be used as a mono therapy, but rather polytherapeutically, the interaction of CMS with INH or RIF, or both in combination is of importance. There is a possibility of antagonistic or synergistic interactions between these drugs. In section 1.3.3.4, the evidence is clear that polymyxins display considerable synergistic activity with RIF against MDR gram-negative infections. This synergistic effect is likely due to disruption of the outer membrane of MDR gram-negative organisms; a barrier preventing the influx of hydrophobic RIF. Disruption increases the amount of RIF influx into the cytoplasm of the MDR gram-negative organism, providing a synergistic effect, lowering of MICs, reducing drug toxicity [Landman *et al* 2008; Lee *et al* 2013; Song *et al* 2008].

No synergistic or enhanced activity for INH or RIF was observed when used in combination with colistin against *M. avium* [Rastogi *et al* 1988; Rastogi *et al* 1990]. These studies cannot be extrapolated for *M. tb* since *M. avium* is a very different Mycobacterium. The investigation using BACTEC radiorespirometry as well as viable cell counts could be questionable as this might lead to antagonistic effect on colistin (section 2.1.1). However, for *M. vaccae*, synergistic activity of RIF with PMBN was observed [Korycka-Machała *et al* 2001; Malaviya and Gomes 2008]. In addition, no investigation of all three drugs, i.e., colistin, INH and RIF were conducted in combination or in variable concentration combinations.

Thus, considering all the facts outlined in section 2.1, together with evidence that polymyxins disrupt the various aspects of Mycobacterial (including *M. tb*) cell wall membranes leading to an increase in permeability and cell leakage [David *et al* 1986; David and Rastogi 1985; Korycka-Machała *et al* 2001; Malaviya and Gomes 2008; Rastogi *et al* 1987], it can be hypothesised that synergistic activity will be observed. Disruption of the hydrophobic barrier, together with the self-promoted uptake mechanism [section 1.3.3.1] will allow the influx of hydrophilic INH into the cytoplasm of *M. tb*. Influx of RIF will also occur for the same reasons due to the disruption of the hydrophobic barrier. Thus, just as discussed for MDR gram-negative organisms [section 1.3.3.4], use of CMS in combination with INH or RIF, or both INH and RIF will lead to a lowering of MICs, reducing drug toxicity. This is an extremely important concept for MDR *M. tb*, as this synergistic effect might overcome the resistance mechanisms, allowing for lower INH and RIF MICs. For example, *katG* gene mutations cause INH MIC in the range of 5 - 10 mg/L and *rpoB* gene mutations cause RIF MIC of 32 mg/L. With an increased influx of INH and RIF into the cytoplasm of the organism; breakpoints for INH and RIF could be reached.

Synergy investigations using two or three dimensions, i.e., two or three drugs in combinations for *M. tb* have been described Bhusal *et al* [2005] and Caleffi-Ferracioli *et al* [2013]. These assays known as checkerboards, are in broth microdilution format and have been used with Alamar Blue® (resazurin). This method is extremely attractive as the same conditions and procedures outlined for MABA in section 2.1.1, and 2.1.2 can be adopted for synergy investigations of CMS with INH or RIF, or both. Since a 96 well microtiter plate is used for both microdilutions, the three dimensions can be divided into x, y and z-axis. For example, two dimensions are first prepared by diluting antimicrobial A along the x-axis, i.e., wells number 1-12 on a microtiter plate. Next, antimicrobial B is diluted along the y-axis, i.e., wells labelled A-H. If a third dimension is required, antimicrobial C is added on the z-axis as an overlay, i.e., the entire plate wells 1-12 and A-H get a one standard concentration of antimicrobial C. Different concentrations of antimicrobial C are added over different plates providing a dilution range [Bhusal *et al* 2005]. Using spectrophotometric measurements of Alamar Blue®, Equation 1 and the same definition for MIC determinations (section 2.1.1), the best combinations of CMS with INH or RIF, or both can be determined. Determining the Σ_{FIC} (Equation 2) allows for insight if synergism, indifference or antagonism occurred [Bhusal *et al* 2005; Caleffi-Ferracioli *et al* 2013].

$$\Sigma_{FIC} = \frac{\text{MIC [CMS] combination}}{\text{MIC [CMS] alone}} + \frac{\text{MIC [INH] combination}}{\text{MIC [INH] alone}} + \frac{\text{MIC [RIF] combination}}{\text{MIC [RIF] alone}} \quad \text{Equation 2}$$

Where the definition of synergy is; synergy $\Sigma_{FIC} < 0.5$, indifference Σ_{FIC} 0.5-4 and antagonism is $\Sigma_{FIC} > 4$.

Unfortunately as with MABA MIC determinations, synergy studies employing this format are more qualitative than quantitative. A time-kill assay can, however, provide quantitative data. By plotting $\log_{10}$ CFU/ml and days of incubation, insight into the dynamics of killing such as kinetics and bactericidal effects can be observed for CMS in combination with INH or RIF, or both. The strategy to employ the time-kill assay was by selecting the combinations of CMS, INH or RIF that produced the best Σ_{FIC}, i.e., the lowest possible value. Using the same format as determining synergy, but without the addition Alamar Blue®, but rather determining viable cells, a combination of CMS, INH or RIF was considered to be bactericidal if a > 3 $\log_{10}$ (> 99.9%) reduction of viable cells relative to the starting concentration was determined [National Committee for Clinical Laboratory Standards 1999].

It is important to note that since it would be likely that an MDR *M. tb* strain would have INH MIC of 5-10 mg/L with *katG* gene mutations and RIF MIC of 32 mg/L with *rpoB* gene mutations, the definitions for breakpoints outlined in 2.1.1 were used, i.e., INH has a breakpoint of 0.25mg/L and RIF has a breakpoint of 0.5mg/L for synergy investigations. If a combination of CMS with INH or RIF could produce INH or RIF MICs below the breakpoint, this would allow concentrations likely achievable during treatment. Anything above will prevent the formulation of clinically relevant treatments.

2.2 Material and methods:

2.2.1 Preparation of strain and working stocks:

M. tb H37Ra ATCC 25177 and an MDR *M. tb* clinical strains were obtained from Ampath Pathology Laboratory Support Services (Centurion, Gauteng, South Africa). The strains were swabbed onto Middlebrook 7H10 agar (Becton Dickinson, Woodmead, Gauteng, South Africa), supplemented with 0.5% v/v glycerol (Saarchem, Krugersdorp, Gauteng, South Africa), and enriched with 10% v/v OADC (Becton Dickinson). After three weeks of incubation for *M. tb* H37Ra and six weeks for MDR *M. tb* at 37ºC, a cell suspension of McFarland 3 was prepared in 1 x PBS (Sigma-Aldrich, Kempton Park, Gauteng, South Africa) containing 0.05% v/v Tween 80 (Saarchem). Aliquots of 1ml were stored at -80°C in cryovials, containing 20% v/v glycerol (Saarchem). Presence of *M. tb* was confirmed by using the TB Ag MPT64 Device (KAT Medical, Roodepoort, Gauteng, South Africa) and purity was determined by swabbing 100µl of culture media onto tryptic soy agar (Merck, Darmstadt, Germany), and incubating at 37°C for 24 to 48 hours. Additionally, both strains were tested using *Mycobacterium* CM/AS, MTBC and MTBDRplus assays (Hain Life-Science, Nehren, Germany). Before any of the investigations took place, a cryovial of the stored aliquot was allowed to thaw to room temperature, was vortexed and swabbed onto Middlebrook 7H10 OADC agar. Plates were sealed in Ziploc bags (preventing drying up) and incubated at 37ºC until active growth (approximately two weeks) was reached for both strains, i.e., H37Ra and MDR strains were used at the same growth stage (actively growing/metabolically active) throughout all experimentation. It is important to note that no more than three culture passages were used for cultures throughout the investigations.

2.2.2 Antimicrobials:

All antimicrobials used were prepared fresh on the day of experimentation. All stock solutions were dissolved in dH_2O (RIF was dissolved in DMSO (Sigma-Aldrich)), filter sterilised using 0.2µm filters (except for polymyxins; due to possible interactions with the filter membrane) and further diluted in dH_2O. The final concentration of DMSO was always < 1% v/v. For polymyxins; all dilutions were prepared using Eppendorf (Hamburg, Germany) Protein LoBind tubes.

2.2.3 MIC determinations using MABA:

Sauton liquid medium was used as the test media to better control parameters and avoid false polymyxin MIC and MBC. The media was prepared as previously described [Larsen *et al* 2007]; glycerol was substituted with 0.2% w/v glucose (Saarchem) to avoid the possibility of lower than normal MICs due to glycerol dependence [Pethe *et al* 2010], cation-adjusted to provide Mg^{2+} (10-12.5mg/L) and Ca^{2+} (20-25mg/L), and supplemented with 0.05% v/v Tween 80. MABA was adopted from previously established protocols [Collins *et al* 1997; Franzblau *et al* 1998]. Sterile polypropylene, flat bottomed, 96 well microtiter plates with lids were used (Eppendorf). Using a actively growing culture grown from a stored aliquot (section 2.2.1), i.e., actively growing/metabolically active H37Ra and MDR strains, a McFarland 1 cell suspension was prepared using 1 x PBS containing 0.05% v/v Tween 80 and adjusted using a spectrophotometer. A 1:25 dilution of the cell suspension was made using Sauton media. Next (Figure 2.2), 250 µl dH_2O was added to the outer perimeter wells to prevent evaporation of the media from the test wells. In row B, columns 2-11, 195µl media was added while 100µl of the media was added to the rest of the test wells. The final concentration of the antimicrobials tested was as follows: INH (Merck): 0.016-0.256mg/L (H37Ra) and 0.5-8mg/L (MDR *M. tb*), RIF (EMD Millipore, Billerica, MA, USA): 0.0016-0.0256mg/L (H37Ra) and 2-32mg/L (MDR *M. tb*), PST (7986IU/mg)(EMD Millipore, Billerica, MA, USA), CST (≥ 15000U/mg)(Sigma-Aldrich) and CMS (12500IU/mg)(Sanofi-Aventis, Midrand, Gauteng, South Africa): 2-32mg/L. Antimicrobials (5µl) were added to row B, column 2-11 and serially diluted using a multichannel pipette by mixing and transferring 100µl from row B to row C. Identical serial dilutions were continued through to row F. Test wells then received 100µl of the cell dilution, approximately yielding 1.5 x 10^5CFU/ml (previously determined by serial dilutions and the pour plate method). Row G contained alternating wells of growth controls (containing the same as the test wells without antimicrobials, i.e., cells only) and sterile controls (containing the same as test wells without cells, i.e., media only). Plates were sealed with Parafilm or Ziploc bags and incubated for five days at 37ºC. On day five, 50 µl of 1:1 Alamar Blue® (AbD Serotec, Kidlington, Oxford, UK) and 10% v/v Tween 80 was added to one growth and one sterile control well, resealed with Parafilm or Ziploc bags and incubated in the dark. After 24 hours, if the Alamar Blue® solution remained blue in the sterile well and changed to pink in the growth

well, 50µl of 1:1 Alamar Blue® and 10% v/v Tween 80 was added to all wells and incubated in the dark for a further 24 hours, after which results were determined spectrophotometrically.

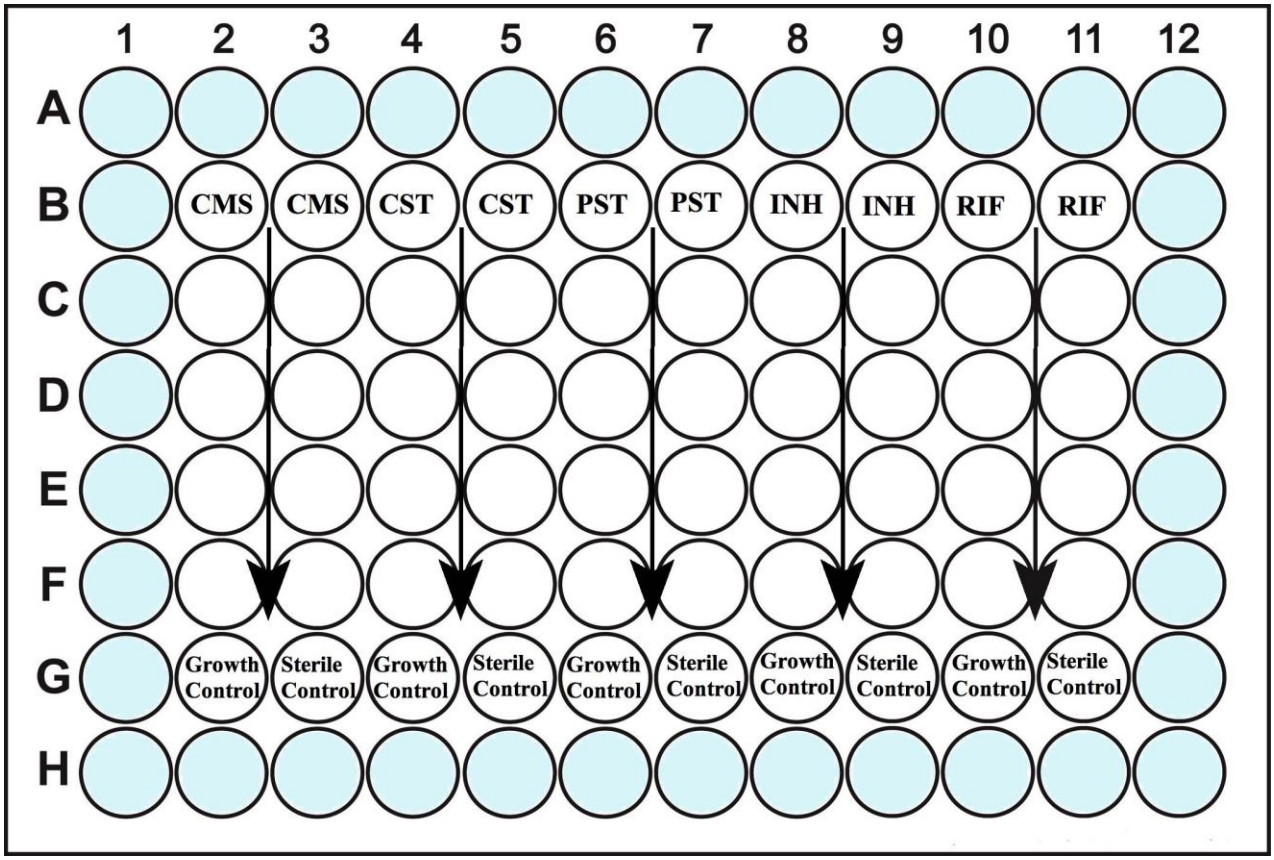


Figure 2.2: Schematic diagram of MABA design. Blue wells indicate dH_2O addition. Respective drugs, i.e., CMS, CST, PST, INH and RIF are also displayed with black arrows indicating the direction of serial dilution. Growth and sterile controls are also displayed.

If no change was observed in the growth control, plates were incubated for two more days, and the process was repeated until positive growth was indicated. On day five, the addition of Alamar Blue® solution to one growth and one sterile control only served as an indicator of sufficient growth and sterility, without being included in the analysis. Only wells that received the Alamar Blue® solution at the same time were evaluated together. Sterility was further confirmed by using TB Ag MPT64 Device and tryptic soy agar as described (section 2.2.1). If sterility was compromised, the experiment was halted. The MIC was defined and calculated as described (section 2.1.2). Loss in buffer capacity for Alamar Blue® was factored in by subtracting the percentage reduction for the sterile

control well from the percentage reduction for the test wells. MIC was completed in duplicate on the day, and three independent experiments were conducted for both H37Ra and MDR *M. tb*.

2.2.4 MBC determinations:

For MBC, the same method as MIC determinations were used (section 2.2.3), except that (1) concentrations investigated for PST, CST and CMS were 16-256mg/L, and (2) the Alamar Blue® and Tween 80 solutions were only added to growth and sterile control wells to monitor growth and possible contamination (section 2.2.3). For starting inoculum, cells were serially diluted in 1 x PBS containing 0.05% v/v Tween 80 and 100µl was plated onto Middlebrook 7H10 agar containing glycerol and OADC. CFU/ml was determined by the pour plate method. After growth and sterility was indicated by Alamar Blue® addition, 10µl of each test well were plated out onto Middlebrook 7H10 OADC agar. CFUs were counted at three to five weeks after incubation at 37ºC until no more increase in viable cells were observed.

The MBC was defined and determined as outlined (section 2.1.3). The MBC was completed in duplicate on the day, and three independent experiments were conducted for both H37Ra and MDR *M. tb*.

2.2.5 PS MIC investigations:

Determination of CMS MIC in PS was adopted from Schwameis *et al* [2013] and Silverman *et al* [2005]. MIC for CMS was determined using the MABA (section 2.2.3), except that the test media in the plates contained porcine surfactant (Curosurf, Chiesi Farmaceutici SpA, Parma, Italy) to a final concentration of 1mg/ml. The MIC was defined and calculated as described (section 2.2.3). As a control, MIC was conducted in media without PS. CFU/ml of growth controls for media with and without PS were monitored by the pour plate method. Sterility was also monitored as described (section 2.2.3). MIC was completed in duplicate on the day, and three independent experiments were conducted for H37Ra.

2.2.6 Synergy and time-kill assays:

Synergy investigations using a modified checkerboard MABA were adopted from Bhusal *et al* [2005] and Caleffi-Ferracioli *et al* [2013]. Culture, antimicrobials, media and 96 well microtiter plates were prepared as described (section 2.2.3) except for certain differences. For three-dimensional investigations, CMS (0.25-32mg/L) was serially diluted along the x-axis, RIF (0.0001-0.0032mg/L [H37Ra] and 0.03125-1 mg/L [MDR *M. tb*]) was serially diluted along the y-axis and as an overlay (z-axis), INH (0.008-0.064mg/L [H37Ra] and 0.032-0.256mg/L [MDR *M. tb*]) concentrations were dispensed over all wells of four separate plates. Two-dimensional investigations were also investigated with CMS (0.25-32mg/L) serially diluted along the x-axis, with RIF (0.0001-0.0032mg/L [H37Ra] and 0.03125-1mg/L [MDR *M. tb*]) or INH (0.002-0.064mg/L [H37Ra] and 0.008-0.256mg/L [MDR *M. tb*]) serially diluted along the y-axis. As a control for INH and RIF, INH (0.0005-0.064mg/L [H37Ra] and 0.002-0.256mg/L [MDR *M. tb*]) was serially diluted along the x-axis while RIF (0.0001-0.0032mg/L [H37Ra] and 0.03125-1mg/L [MDR *M. tb*]) was serially along the y-axis. Column 11 contained growth controls (containing the same as the test wells without antimicrobials, i.e., cells only) and column 10 contained sterile controls (containing the same as test wells without cells, i.e., media only) in all plates. Plates were inoculated with approximately 1.5 x 10^5CFU/ml, sealed with Parafilm or Ziploc bags and incubated at 37ºC for five days. After five days, the same strategy of MIC (≥ 90% prevention in Alamar Blue® reduction) determination using Alamar Blue® was used (section 2.2.3), and synergy calculations were conducted using Σ_{FIC} and the same synergy definitions (section 2.1.5). Synergy investigations were conducted using three independent experiments for both H37Ra and MDR *M. tb*.

The best Σ_{FIC} combinations determined from synergy investigations were subjected to a time-kill assay. Time-kill assay was adopted from Bhusal *et al* [2005] and de Steenwinkel *et al* [2010]. Combinations of antimicrobials, Sauton media and preparation of microtiter plates were prepared as described for synergy investigations. Cultures (1.5 x 10^5CFU/ml) were prepared (section 2.2.3) and incubated with the relevant synergistic antimicrobial combination. Row G in the microtiter plates contained alternating growth and sterile controls for each antimicrobial combination test. Plates were sealed with either Parafilm or in Ziploc bags and incubated at 37ºC. Part of the culture was collected on day zero, one, two, three and six for CFU determinations by serial dilution in 1 x PBS containing 0.05% v/v Tween 80 and plating out 100µl of each dilution using the pour plate method with Middlebrook 7H10 OADC agar. Agar plates were incubated for three to five weeks, and colonies were counted every week until no more increase was determined to occur. Time-kill investigations were conducted using three independent experiments for H37Ra and MDR *M. tb*.

2.2.7 Statistical analysis:

Statistical analysis was conducted using JMP® Pro 11.0.0 (64 bit) software copyright © 2013 SAS institute Inc. One way ANOVA was conducted, and means were compared using Student's t-test for all statistical analysis (generally by use of letter groupings) ($\alpha = 0.05$).

2.3 Results and discussion:

2.3.1 MIC determinations using MABA:

An example of a typical MABA result is displayed in Figure 2.3. Sufficient growth is indicated in the growth control that has changed pink, while the sterility control indicates sufficient purity in the media by remaining blue. INH and RIF also serve as positive controls and provide data to conduct synergy investigations.

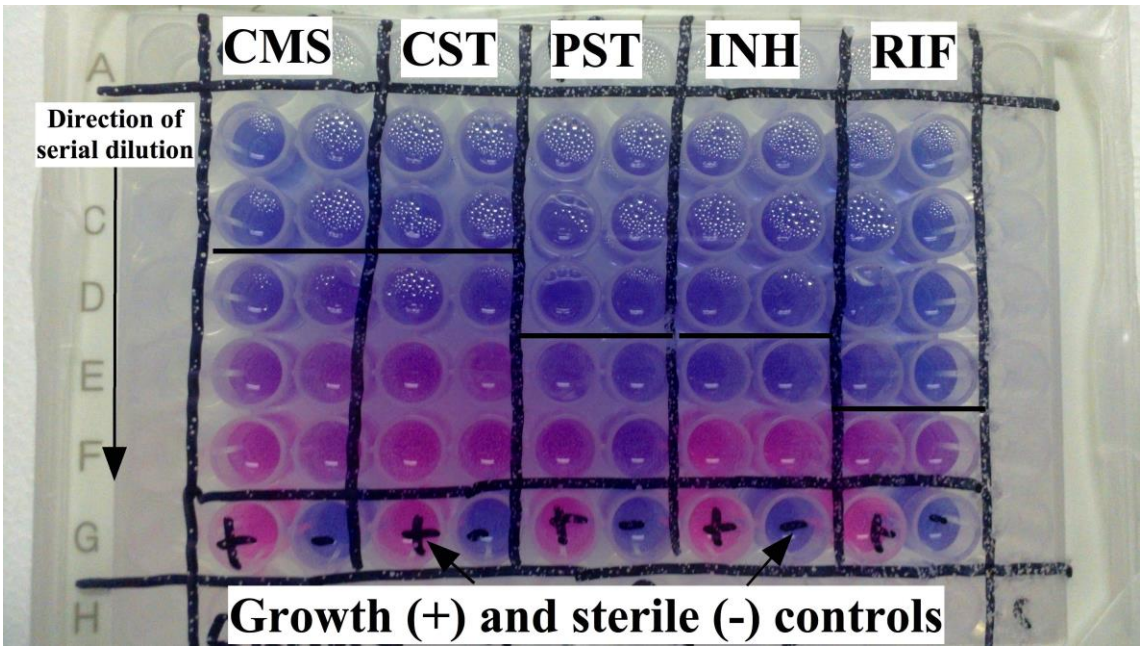


Figure 2.3: Typical H37Ra MABA result (~ 1.5 x 10^5CFU/ml) in cation-adjusted Sauton media. CMS, CST, PST, INH, RIF are displayed. Growth control = + and Sterile control = -. MICs are determined by using Equation 1. Pink colour indicates growth, blue indicates inhibition of growth. Arrow indicates direction of serial dilution. Wells above black lines indicate MICs.

The MIC for antimicrobials can be determined visibly by eye and are defined as the well with the lowest concentration of antimicrobial preventing a colour change for Alamar Blue® [Collins and Franzblau 1997; Franzblau *et al* 1998; Parish and Brown 2008]. A more sensitive method is spectrophotometric analysis using Equation 1.

At various points during culturing and experimentation, the TB Ag MPT64 Device was used to ensure the presence of *M. tb*. This is achieved by using immunochromatographic identification of the MPT64 protein found in culture fluid. This is only possible for the presence of *M. tb*. With the presence of *M. tb* being confirmed, any contaminating bacteria such as gram-negatives or gram-positives can be determined using tryptic soy agar within 48 hours, since *M. tb* will not have visible colonies on this media within 48 hours. Furthermore, analysis of H37Ra and MDR *M. tb* cultures with *Mycobacterium* CM/AS, MTBC can rule out the presence of any other Mycobacteria, but is only conducted before the storage of stock cultures. At the same time, MTBDRplus assays determined the MDR *M. tb* to be positive for *rpoB* and *katG* mutations (Figure 2.4). H37Ra was a representative for the INH and RIF

sensitive *M. tb* population while MDR *M. tb* was a representative for the INH and RIF resistant population, as discussed in section 2.1.2.

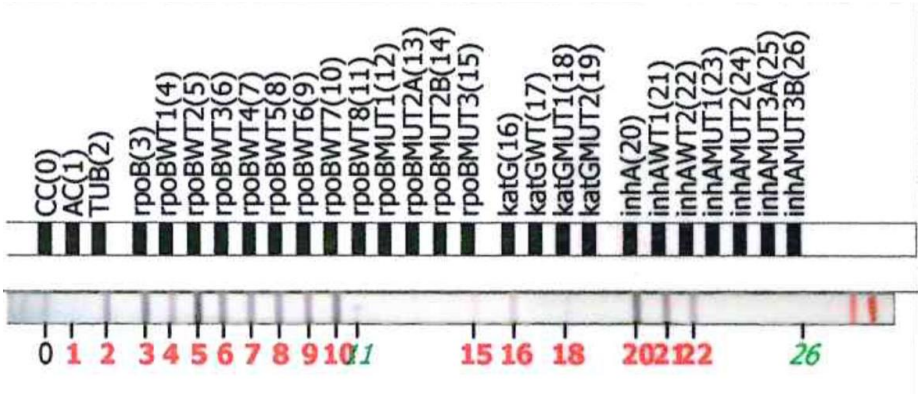
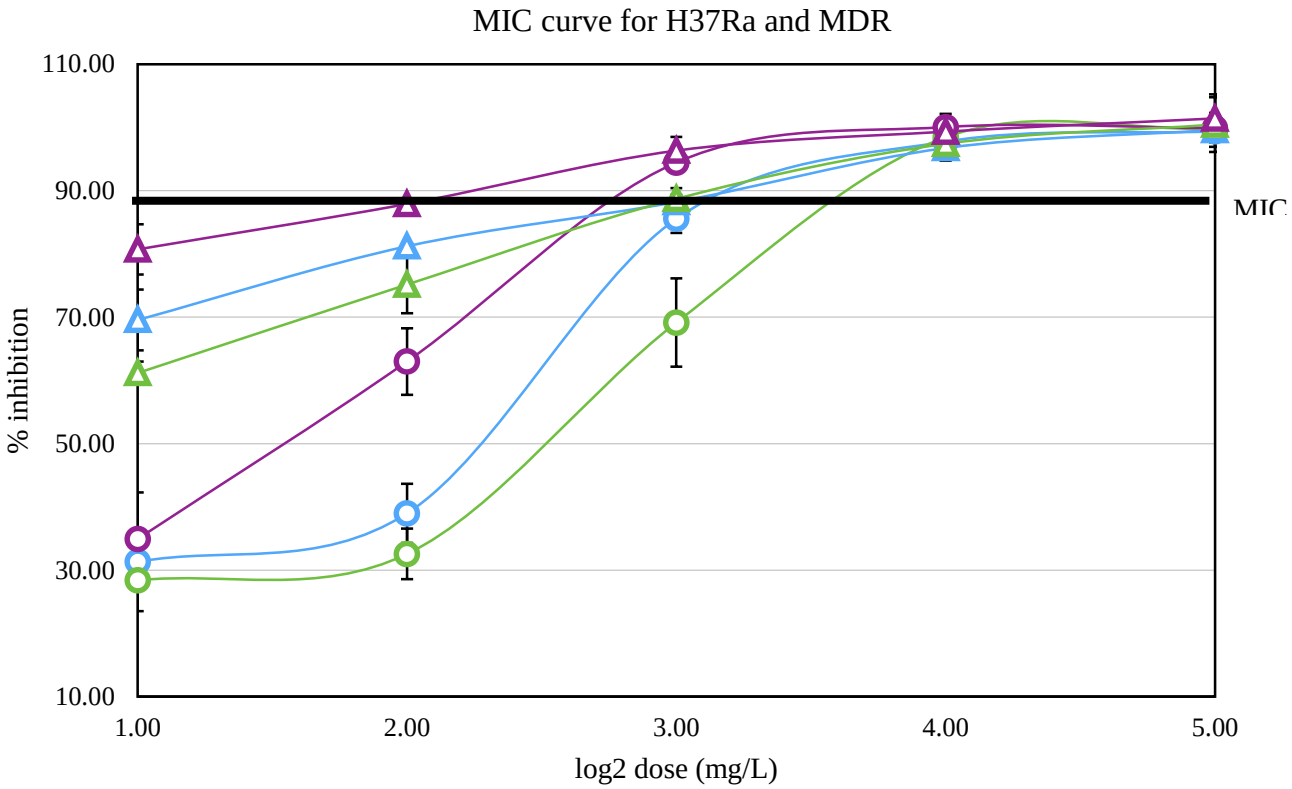


Figure 2.4: Hain Lifescience GenoType MTBDRplus assay for MDR *M. tb*. RIF resistance is determined with mutations in rpoBMUT3 (15) and INH resistance is determined with mutations in katGMUT1(18).

As mentioned in section 2.1.1, there is much controversy surrounding the use of Tween 80 in media when determining MICs for polymyxins. Even though its use of Tween 80 is sufficiently motivated by various authors [Hindler and Humphries 2013; Humphries 2014; Rastogi *et al* 1986b; Sader *et al* 2012], an attempt was made to determine if H37Ra grown in the absence of Tween 80 would give similar results as H37Ra grown in the presence of Tween 80 (0.05% v/v). Sufficient growth was always indicated by a change in Alamar Blue® (blue to pink) for H37Ra growth controls by day 7 of incubation containing Tween 80. This was associated with an increase of CFU/ml from ~1.5 x 10^5 to ~1 x 10^7. In the absence of Tween 80, growth became stunted. Growth controls of H37Ra did not change Alamar Blue® (blue to pink) even after seven days of incubation. This was associated with a < 5 x 10^5CFU/ml; significantly different to the inclusion of Tween 80 ($\alpha = 0.05$). It is highly likely that *M. tb* (H37Ra) requires Tween 80 within Sauton media as it uses the fatty acids present within Tween 80 as an energy source, allowing for sufficient growth [Schaefer and Lewis 1965; Smith *et al* 1993]. This provided further motivation to determine MICs in the presence of Tween 80 and to avoid erroneous MIC data.

Figure 2.5 describes the results obtained from MIC determinations using MABA for both H37Ra and MDR *M. tb*. MICs determined for CMS (16mg/L or $\log_2$ dose = 4), CST (16mg/L or $\log_2$ dose = 4) and PST (8mg/L or $\log_2$ dose = 3) were the same for both H37Ra, and MDR *M. tb* according to the outlined definition and using Equation 1. According to the Student's t-test (not shown)($\alpha = 0.05$), MICs ≥ 90% are statistically different to the rest of the values tested and are statistically the same for

H37Ra and MDR *M. tb.* For example, CMS (16mg/L or $\log_2$ dose = 4) is statistically different to lower



CMS concentrations tested. This applies to CST and PST.

Figure 2.5 and the Student's t-test revealed that CMS and CST share similar MICs (16mg/L or $\log_2$ dose = 4), regardless of the Mycobacteria strain tested. Since CMS is a (1) nonactive prodrug of colistin [Bergen *et al* 2006; Li *et al* 2003a; Li *et al* 2005b], (2) less active compared to CST [Eickhoff and Finland 1965], (3) the possibly that 32 different forms could exist in solution [Li *et al* 2005a] and (4) depending on the manufactures and test conditions, a maximum of 60-80% of CMS might convert to colistin [Li *et al* 2003b]; it was unexpected that CMS and CST would have the same MICs (16mg/L or $\log_2$ dose = 4). Rather, CMS would be expected to have a higher MIC than CST due to these reasons.

Interestingly, PST had a statistically lower MIC (8mg/L or $\log_2$ dose = 3) compared to CMS and CST. This is attributed to the single amino acid difference of PST compared to CMS and CST, i.e., Phe vs Leu (Figure 1.6) [Nation and Li 2010].

Figure 2.5: MIC curve displaying % inhibition vs $\log_2$ dose (mg/L) for H37Ra (O) and MDR *M. tb* (△). CMS (blue), CST (green) and PST (purple). Error bars display standard error and the MIC threshold is included as per definition.

Referring to section 1.4, PST has the ability of inhibiting alternative NADH dehydrogenase (IC_{50} = 1.6mg/L) and malate: quinone oxidoreductase (IC_{50} = 4.2mg/L) with the Quinone-binding site being the primary site of action [Mogi *et al* 2009]. Polymyxin E has no such inhibitory effects. Although this is true for *M. smegmatis*, it could explain the lower polymyxin B MIC observed for *M. tb*. This leads to the disruption of the hypoxic or aerobic respiratory chain [Mogi *et al* 2009], producing more of an inhibitory effect and lower MIC. One will however expect a larger/similar bactericidal effect (MBC) from polymyxin E than polymyxin B [Mogi *et al* 2009].

It is very interesting that specific to each polymyxin tested, the same activity against H37Ra and MDR *M. tb* is observed; according to Student's t-test (α = 0.05). If polymyxins interact with the various cell membranes of *M. tb (*H37Ra and MDR), this suggests that a commonality in the mechanism of action between the two strains could exist, resulting in the same activity observed.

An observation made was the increased sensitivity of MDR *M. tb* to polymyxins at lower concentrations, i.e., CMS at 2mg/L ($\log_2$ dose = 1) inhibited 31.33 ± 2.13% of H37Ra *M. tb* vs 69.57 ± 4.79% for MDR *M. tb*. The trend was the same for all polymyxins. This is attributed to the difference in growth for MDR vs H37Ra *M. tb*. MDR *M. tb* was determined to produce ~ 5 x 10^6CFU/ml (vs ~ 1 x 10^7CFU/ml for H37Ra). This slower growth of MDR *M. tb* is thus attributed to higher sensitivity to polymyxins at lower concentrations.

H37Ra *M. tb* had INH and RIF MICs of 0.064 and 0.0032mg/L respectively while MDR *M. tb* had INH and RIF MICs of 4 and > 32mg/L respectively, clearly displaying sensitive and resistant MICs. These results are important for synergy investigations.

Table 2.1: MIC determinations by use of MABA for H37Ra *M. tb* and MDR *M. tb*. Highlighted cells in blue indicate the respective MIC as per definition for each antimicrobial tested.

Antimicrobial tested	**H37Ra *M.tb* (% inhibition ± SD)**	**Antimicrobial tested**	**MDR *M.tb* (% inhibition ± SD)**
INH (0.256mg/L)	95.13 ± 3.88	INH (8mg/L)	96.23 ± 1.65
INH (0.128mg/L)	94.69 ± 7.35	INH (4mg/L)	93.73 ± 2.94
INH (0.064mg/L)	93.99 ± 8.23	INH (2mg/L)	67.13 ± 11.56

Antimicrobial tested	H37Ra *M.tb* (% inhibition ± SD)	Antimicrobial tested	MDR *M.tb* (% inhibition ± SD)
INH (0.032mg/L)	59.61 ± 1.69	INH (1mg/L)	35.53 ± 4.50
INH (0.016mg/L)	27.59 ± 4.09	INH (0.5mg/L)	38.28 ± 5.51
RIF (0.0256mg/L)	98.86 ± 1.07	RIF (32mg/L)	72.19 ± 8.21
RIF (0.0128mg/L)	98.80 ± 1.70	RIF (16mg/L)	58.24 ± 6.09
RIF (0.0064mg/L)	97.04 ± 5.12	RIF (8mg/L)	45.01 ± 4.88
RIF (0.0032mg/L)	93.35 ± 5.78	RIF (4mg/L)	37.89 ± 1.39
RIF (0.0016mg/L)	36.73 ± 0.24	RIF (2mg/L)	38.53 ± 4.92

2.3.2 MBC determinations:

Figure 2.6 outlines the determined MBC for H37Ra and MDR *M. tb* by monitoring reduction of viable cells, i.e., $\log_{10}$ CFU/ml. Specific to each polymyxin ($\log_2$ dose (mg/L)), the Student's t-test (α = 0.05) was applied (data not shown).

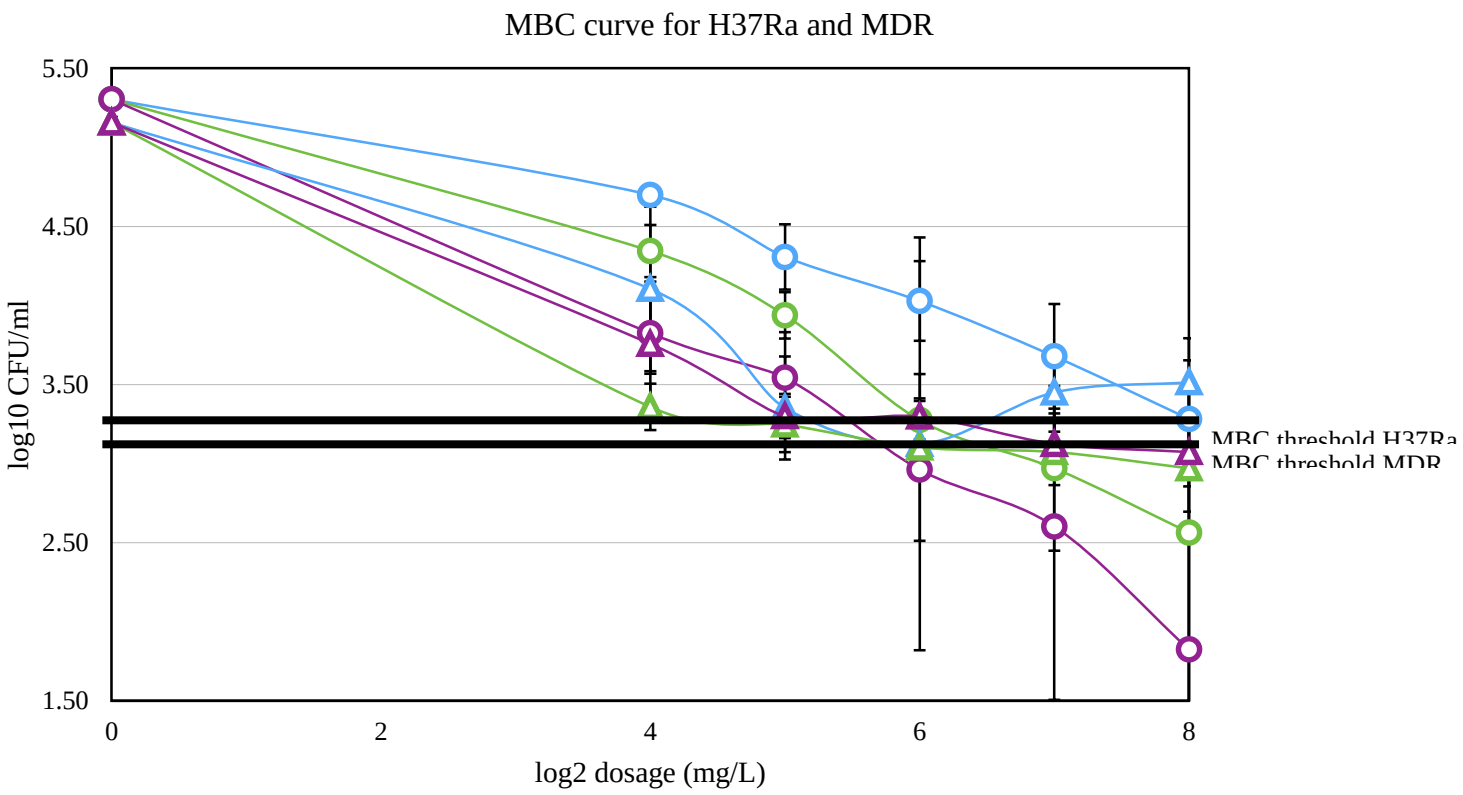


Figure 2.6: MBC curve displaying $\log_{10}$ CFU/ml vs $\log_2$ dose (mg/L) for H37Ra (O) and MDR *M. tb* (△). CMS (blue), CST (green) and PST (purple). Error bars display standard error and the MBC threshold is included as per definition with regard to starting inoculum; H37Ra $\log_{10}$ CFU/ml = 5,3 and MDR $\log_{10}$ CFU/ml = 5,16.

Referring to Figure 2.6, the starting $\log_{10}$ CFU/ml for H37Ra was 5,3 and for MDR *M. tb* was 5,16. Thus, a decrease of 2 $\log_{10}$ CFU/ml would make the MBC threshold for H37Ra at 3,3 and MDR *M. tb* at 3,16. According to the definition of MBC (≥ 99% reduction in viable cells, i.e., a reduction of ≥ 2 $\log_{10}$ CFU/ml), the MBC for H37Ra *M. tb* was determined to be 256mg/L ($\log_2$ dose = 8), 64mg/L ($\log_2$ dose = 6) and 64mg/L ($\log_2$ dose = 6) for CMS, CST and PST respectively. Interesting, CST and PST share the same MBC of 64mg/L ($\log_2$ dose = 6) for H37Ra *M. tb*.

The reason for CMS having a much higher MBC (256mg/L or $\log_2$ dose = 8) than CST (64mg/L or $\log_2$ dose = 6) and PST (64mg/L or $\log_2$ dose = 6) could be due to the reasons outlined in section 2.3.1, i.e., CMS is a nonactive prodrug of colistin, less active than CST and PST, with a difference in conversion from CMS to colistin. CST and PST are already in active forms, inducing lower MBCs.

The MBC for MDR *M. tb* was determined to be 64mg/L ($\log_2$ dose = 6), 64mg/L ($\log_2$ dose = 6) and 128mg/L ($\log_2$ dose = 7) for CMS, CST and PST respectively. For CMS at higher concentrations of 128mg/L ($\log_2$ dose = 7) and 256mg/L ($\log_2$ dose = 8) against MDR *M. tb*, a lower reduction of viable

cells than the actual MBC at 64mg/L ($\log_2$ dose = 6) was observed. However, these concentrations tested are statistically similar (α = 0.05). When statistically comparing the CMS MBC (64mg/L or $\log_2$ dose = 6), CST and PST cause an equivalent reduction in viable cells at 16mg/L ($\log_2$ dose = 4). This indicates a similar statistical trend observed for H37Ra *M. tb*, i.e., higher concentrations of CMS are required to statistically reduce the a similar amount of viable cells as CST and PST. The reason for this trend can be determined using liquid chromatography quantification. It appears that CST (64mg/L or $\log_2$ dose = 6) has a lower MBC than PST MBC (128mg/L or $\log_2$ dose = 7) for MDR *M. tb*. However, this must be interpreted with care, because the PST MBC at 64mg/L ($\log_2$ dose = 6) statistically causes the same reduction of viable cells as the determined CST MBC (64mg/L or $\log_2$ dose = 6). Since CST and PST share similar MBC values, it is similar to what was described for *M. smegmatis* [Mogi *et al* 2009], i.e., polymyxin B has larger inhibitory effects against *M. smegmatis* compared to polymyxin E, but polymyxin E has greater bactericidal effects than polymyxin B.

Using the MBC definition, H37Ra and MDR *M. tb* do not share similar CMS MBC values. However, statistically (α = 0.05), H37Ra and MDR *M. tb* can be considered to be similar (Figure 2.6), i.e., CMS at 64mg/L ($\log_2$ dose = 6) for H37Ra *M. tb* statistically causes the same reduction in viable cells as MDR *M. tb* determined at CMS MBC (64mg/L or $\log_2$ dose = 6). MDR *M. tb* has a thicker cell membrane compared to sensitive *M. tb* such as H37Ra (17.1nm vs 15.6nm) [Velayati *et al* 2009]. It appears that this trait might not affect the ability of CMS to reduce viable *M. tb*.

As observed for MIC determinations, there is a higher sensitivity of MDR *M. tb* at lower concentrations to polymyxins when compared to H37Ra *M. tb*. This once again can be explained by MDR *M. tb* growing slower when compared to H37Ra *M. tb*.

As mentioned by Rastogi *et al* [1986b], the MIC and MBC for CMS, CST and (determined in this thesis) against both H37Ra and MDR *M. tb* remain much higher than that achievable in serum during treatment and raise concerns of systemic toxicity [Nation and Li 2010]. CST and PST fail as likely drug candidates for further development. Since CMS had an MBC/MIC ratio of $\geq$ 4 for both H37Ra and MDR *M. tb*, CMS also appeared to fail as a likely drug candidate as it is bacteriostatic. The reason for the bacteriostatic property of CMS can be explained by the persister hypothesis. Since the probable mechanism of action of reducing viable CFU/ml is by acting on the cell wall and causing cell lysis, it is plausible that H37Ra and MDR *M. tb* contain a group of difficult to lyse persisters. However, inhaled CMS is commonly used in patients with cystic fibrosis and Ratjen *et al* [2006] determined the pharmacokinetics of inhaled colistimethate-Na (CMS) (administered using a nebuliser) in cystic fibrosis patients. Local concentrations in sputum were much higher than that of systemic concentrations. Maximum sputum concentrations (~ 40mg/L) were ten times higher than the MIC

breakpoint for *P. aeruginosa* proposed by the BSAC. After 12 hours, mean colistin concentrations were still above 4mg/L. Thus, inhalation of CMS dry powder (without a nebuliser/CMS DPI) could possibly lead to concentrations well above the determined MICs in the proximal airways [Schuster *et al* 2013], especially when used polytherapeutically, as this can reduce the required MIC of CMS to become bactericidal. Thus the need for MIC determinations in the presence of PS, synergy and time-kill assays.

Similarly to Rastogi *et al* [1986b], CMS concentrations were not tested above 256mg/L because with current methods, these concentrations are unattainable in the clinical setting.

2.3.3 PS MIC investigations:

Table 2.2 displays the determined MICs of CMS at various concentrations against H37Ra *M. tb* in media containing PS (1mg/ml porcine surfactant), with and without Tween 80. A control without PS was also included. Letter groupings are displayed throughout. Means annotated with different letters are significantly different ($\alpha = 0.05$).

Table 2.2: PS MIC determinations by use of MABA for H37Ra *M. tb*. Highlighted cells in blue indicate the respective MIC as per definition.

Antimicrobial tested	Control (without PS) (% inhibition ± SD)	With PS, with Tween 80 (% inhibition ± SD)	With PS, without Tween 80 (% inhibition ± SD)
CMS (256mg/L)	96.28 ± 1.29 (A, B)	98.21 ± 1.04 (A)	93.84 ± 4.38 (A, B)
CMS (128mg/L)	98.24 ± 2.76 (A)	94.99 ± 4.59 (A, B)	83.28 ± 7.81 (B, C)
CMS (64mg/L)	98.91 ± 3.87 (A)	74.34 ± 8.35 (C)	57.00 ± 17.25 (D)
CMS (32mg/L)	98.14 ± 2.61 (A)	44.80 ± 6.34 (D, E)	37.28 ± 16.76 (E)
CMS (16mg/L)	90.44 ± 0.62 (A, B)	35.81 ± 5.76 (E)	32.45 ± 15.79 (E)

The control used generated the same CMS MIC (16mg/L) as per definition as the initial investigation carried out (Figure 2.5). When PS was included in media, the MIC was antagonised by eight fold, from 16mg/L to 128mg/L. Due to concerns that 0.1% Triton X-100 has previously been observed to reverse antagonism for daptomycin [Silverman *et al* 2005], the same investigations were conducted without Tween 80. The MIC for H37Ra *M. tb* in the presence of PS without Tween 80 was determined to be 256mg/L. Thus, comparing to the control, the MIC can be suggested to be have been antagonised 16 fold. Unfortunately a control without PS and without Tween 80 cannot be used as a

comparison since growth is stunted without the presence of Tween 80, as outlined and discussed in section 2.3.1.

Due to the nature of PS, it was a concern that H37Ra *M. tb* may use PS as an energy source, producing falsely elevated MICs. It was important to determine CFU/ml at the time of analysis of the growth controls for each condition tested. The determined CFU/ml were as follows; 6.8 x 10^6CFU/ml for the control, 8.27 x 10^6CFU/ml for media containing PS and Tween 80, and 5.83 x 10^6CFU/ml for media containing PS without Tween 80 (three independent experiments). It was determined that there was no statistical significant difference ($\alpha = 0.05$) for the CFU/ml determined at time of analysis for the growth controls under the different conditions tested. In section 2.3.1 it is mentioned that growth of H37Ra *M. tb* in media without Tween 80 is stunted, however, when PS is substituted for Tween 80, sufficient growth is obtained. Porcine surfactant (Curosurf, Chiesi Farmaceutici SpA, Parma, Italy) contains 99% polar lipids (phospholipids such as phosphatidylcholine and dipalmitoylphosphatidylcholine) and 1% hydrophobic low molecular weight proteins (SP-B and SP-C) [http://curosurf.com]. Thus, H37Ra *M. tb* has to use certain components of PS as an energy source in order to explain the growth observed in absence of Tween 80. The likely energy source present would be the polar lipids as it makes up 99% of the PS used.

The MIC determined for H37Ra *M. tb* in media, containing PS without Tween 80 was rejected for the following reasons; (1) no suitable control to compare MICs, i.e., media without PS and Tween 80. (2) Antagonism of CMS may be taking place, such as interaction with the walls of the microtiter plate. (3) CMS MICs were antagonised with the inclusion of Tween 80 and not mitigated. (4) The MIC determined at 256mg/L statistically had the same reduction at 128mg/L. It can thus be concluded that CMS is antagonised in the presence of PS.

This is consistent with the findings of Schwameis *et al* [2013], since the authors determined that at concentrations 64 times the determined MIC for CST against *P. aeruginosa*, the inhibitory effects of PS were overcome. Antagonism is due to CMS/colistin forming complexes with the components of PS, i.e., phospholipids such as phosphatidylcholine and dipalmitoylphosphatidylcholine in a similar way that CMS would form complexes with the outer membrane of gram-negative bacteria [Schwameis *et al* 2013]. Inhalation of CMS DPI using the Turbospin® might overcome the inhibitory effects of PS [Schuster *et al* 2013]. Using sputum as a representation of local drug concentrations after inhalation of dry CMS powder using, sputum of 38% patients had colistin levels $\geq$ 128mg/L while the remaining patients had levels $\leq$ 128mg/L [Schuster *et al* 2013]. It appears that if CMS is to be used as a mono therapy with this method, overcoming 128mg/L in order to produce a satisfactory inhibitory effect would not always be a successful, and if it was achieved, would likely be bacteriostatic. However, it is

unlikely that CMS would be used as a mono therapy, but rather polytherapeutically. Using CMS with INH or RIF, or both might allow for much lower synergistic MICs, thus allowing for CMS to overcome the antagonistic effect of PS. Importance of conducting synergistic and time-kill assays will allow for insight to this data. The most important factors being the reduction of CMS concentrations to overcome inhibitory effects of PS as well as the possibility of reducing MDR *M. tb* INH and RIF concentrations to clinically relevant therapeutic levels.

It must be noted that concentrations of PS used were 100 times higher than that found in epithelial lining fluid according to Schwameis *et al* [2013], i.e., 1mg/ml vs 0.01mg/ml. It is possible that this antagonistic effect might be lower *in vivo* compared to the *in vitro* result due to this reason and should be considered in future investigations.

PS was not included in any further investigations due to the expense, limited supply and because INH, and RIF would not be inhaled. Rather, using the antagonistic MIC result for CMS against H37Ra *M. tb*, it can serve as a general result for the overall *M. tb* population and that in all further investigations, it must be considered that CMS might be antagonised when inhaled due to the interaction with the various polar lipids and the hydrophobic proteins present in PS.

As with MBC, concentrations were not tested above 256mg/L as this would not be currently achieved in the clinical setting. Due to the inhibitory effect observed, the MBC value of CMS in PS would be considerably higher than that of the MIC concentration.

Of course PS is only one factor of interaction for CMS. Other factors such sputum and caseum might also cause a possible antagonism of CMS during inhalation and should be investigated using *in vivo* models. Since *in vitro* investigations are concerned here, and PS can be obtained as a sterile product, PS was used over sputum and caseum.

2.3.4 Synergy and time-kill assay:

Using a modified checkerboard MABA (section 2.2.6), the best combinations of CMS, INH and RIF were determined. The same definition of ≥ 90% Alamar Blue® reduction and Equation 1 were used to define the MIC. In order to determine synergy, indifference or antagonism, the Σ_{FIC} was calculated using Equation 2 where the definition of synergy is; synergy $\Sigma_{FIC} < 0.5$, indifference Σ_{FIC} 0.5-4 and antagonism is $\Sigma_{FIC} > 4$.

Table 2.3 displays the best synergistic combinational results determined for H37Ra and MDR *M. tb* with the Σ_{FIC} displayed. When conducting the assay for MDR *M. tb*, certain assumptions were made. Since the determined MICs for INH and RIF were 4 and > 32mg/L respectively, it cannot be included in the modified checkerboard MABA. Instead, the breakpoints for clinically relevant INH and RIF concentrations [Palomino *et al* 2002] were used, and the MICs were set as 0.256 and 1mg/L respectively (section 2.1.2). If synergy was observed above these breakpoints, it would be meaningless since the result cannot be extrapolated to a possible clinical setting.

Table 2.3: Various synergy concentration combinations of CMS, INH and RIF as determined by the modified checkerboard MABA (≥ 90% Alamar Blue® reduction) with displayed calculated Σ_{FIC} (Equation 2). Highlighted cells in blue indicate the combinations with the lowest Σ_{FIC}.

Synergy treatment	H37Ra *M. tb*				MDR *M. tb*			
	CMS (mg/L)	INH (mg/L)	RIF (mg/L)	Σ_{FIC}	CMS (mg/L)	INH (mg/L)	RIF (mg/L)	Σ_{FIC}
1	4	0.064	0.0004	1.375	16	0.256	0.0625	2.063
2	16	0.032	0.0016	2.000	16	0.128	0.313	1.531
3	16	0.016	0.0032	2.080	16	0.064	0.125	1.445
4	16	0.008	0.0032	2.125	16	0.032	0.0625	1.186
5	16	-	0.0032	2.000	32	-	0.313	2.031
6	2	0.064	-	1.125	16	0.032	-	1.125
7	-	0.064	0.0032	2.000	-	> 0.256	>1	> 2.000

In Table 2.3, each synergy treatment refers to a different plate tested with varying concentrations of CMS, INH and RIF. In each plate, the lowest concentrations that generated a ≥ 90% Alamar Blue® reduction were used as the combinational MIC for the Σ_{FIC} calculations. When deciding to choose an optimal combination, the most important factor was that of CMS; the lower the

concentration of CMS, the better. This was due to the following reason; CMS is antagonised in the presence of PS, thus having a lower MIC would likely allow CMS to overcome the antagonistic effects of PS when used in combination with INH, RIF or both. It will also be an advantage if the combination had a bactericidal effect rather than a bacteriostatic one. The next important factor would be selecting the lowest concentrations of INH and RIF, especially for MDR *M. tb*, as this may allow for lowering of INH and RIF breakpoints to clinically relevant concentrations.

For H37Ra *M. tb*, all combinations revealed an Σ_{FIC} between 0.5-4 indicating indifference when using CMS in combinations with INH and RIF. CMS (2mg/L) and INH (0.064mg/L) displayed the lowest Σ_{FIC} with 1.125. For MDR *M. tb*, the results obtained could be misleading, i.e., INH and RIF MICs are lower than the described breakpoints (section 2.1.2). This data required closer scrutinising. No statistical difference ($\alpha = 0.05$) in Alamar Blue® reduction was observed for the lowest concentrations of INH, RIF or both used in combination with CMS compared to the highest concentrations of INH, RIF or both with CMS. Thus, INH and RIF had no effect on Alamar Blue® reduction, only change in the concentration of CMS lead to statistical differences in Alamar Blue® reduction ($\alpha = 0.05$). Thus, it was concluded that the inhibition of MDR *M. tb* was due to CMS and not INH or RIF, indicating no mitigation of resistance. As with H37Ra *M. tb*, all combinations tested against MDR *M. tb* revealed an Σ_{FIC} between 0.5-4 indicating indifference when using CMS in combinations with INH and RIF. CMS (16mg/L) and INH (0.032mg/L) displayed the lowest Σ_{FIC} with 1.125 for MDR *M. tb*.

It is likely that even though indifference was determined for H37Ra *M. tb*, some of the combinations might cause a bactericidal effect. As for MDR *M. tb*, since it is likely that CMS is the only compound responsible for the inhibition observed, and not INH or RIF, it was likely that no bactericidal effect would be present, stressing the importance of a time-kill assay.

Figure 2.7 displays the time-kill assay. The best synergy treatments from Table 2.3 were selected (lowest Σ_{FIC}), for example, synergy treatment 1 and 5-7 were used for H37Ra *M. tb*, and synergy treatments 4-7 were used for MDR *M. tb* in the time-kill assays.

For H37Ra, the only statistical difference in killing kinetics was for RIF (0.0032mg/ L) + INH (0.064mg/L) within 24 hours. Until day six, no statistical differences in any other combinations were observed. On day six, the lowest amount of killing was that of RIF (0.0032mg/ L) + INH (0.064mg/L); only reducing 1.20 $\log_{10}$ CFU/ml with respect to the starting inoculum.

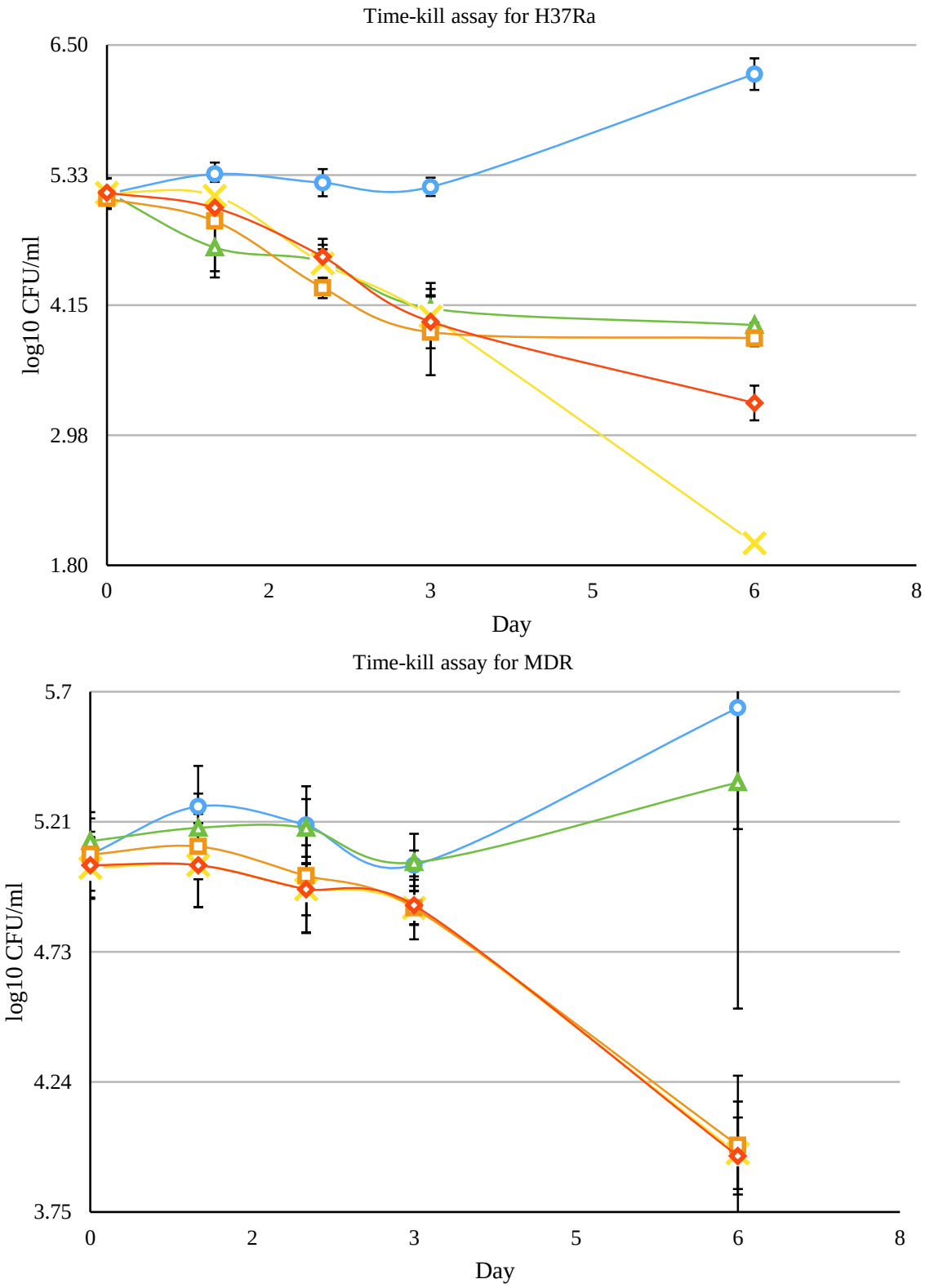


Figure 2.7: Time-kill assay displaying $\log_{10}$ CFU/ml vs day for H37Ra and MDR *M. tb*. Growth control (O), INH + RIF (△), CMS + INH (×), CMS + RIF (□), CMS + INH + RIF (◇). Error bars display standard error.

Next, CMS (16mg/L) + RIF (0.0032mg/L) caused a 1.26 $\log_{10}$ CFU/ml decrease. This combination was not statistically different from RIF (0.0032mg/ L) + INH (0.064mg/L). CMS (4mg/L) + INH (0.064mg/L) + RIF (0.0004mg/L) reduced CFU/ml by 1.90 $\log_{10}$ CFU/ml and was determined to be statistically different from RIF (0.0032mg/ L) + INH (0.064mg/L) and CMS (16mg/L) + RIF (0.0032mg/L). CMS (2mg/L) + INH (0.064mg/L) caused a reduction of 3.16 $\log_{10}$ CFU/ml (> 99.9% reduction) indicating a bactericidal effect [National Committee for Clinical Laboratory Standards 1999]. This combination was statistically different from all other combinations tested on day six. It must be noted that RIF and INH cause additive killing when used in combination [Almeida *et al* 2009]. A reduced killing effect was observed when CMS is used together with RIF, compared to CMS and INH.

For MDR *M. tb*, no statistical difference in reduction of $\log_{10}$ CFU/ml between the control and RIF (1mg/L) + INH (0.256mg/L) was observed at day six. There was a statistical significant difference for the combinations of CMS (16mg/L) + INH (0.032mg/L), CMS (32mg/L) + RIF (0.313mg/L) and CMS (16mg/L) + INH (0.032mg/L) + RIF (0.0625mg/L) when compared to the control and RIF (1mg/ L) + INH (0.256mg/L).

However, no statistical significant difference in $\log_{10}$ CFU/ml reduction was observed for either combination of CMS (16mg/L) + INH (0.032mg/L), CMS (32mg/L) + RIF (0.313mg/L) and CMS (16mg/L) + INH (0.032mg/L) + RIF (0.0625mg/L). Reduction of for each combination was determined to be 1.07, 1.09 and 1.09 $\log_{10}$ CFU/ml respectively. This indicated a bacteriostatic effect similar to that observed for MIC and MBC determinations (section 2.3.1 and 2.3.2). As hypothesised, the killing effect observed during synergy investigations against MDR *M. tb* is purely due to the presence of CMS and not due to any additional interactions observed with INH or RIF. Strangely, as observed for H37Ra *M.tb*, a similar antagonistic effect of CMS with RIF was observed, i.e., a larger concentration of CMS (32mg/L) with RIF (0.313mg/L) was required to produce the same reduction of $\log_{10}$ CFU/ml as the other combinations used. It can further be concluded that at the concentrations tested using CMS, INH and RIF, there was no increased ability to overcome the resistant effects induced by *rpoB* and *katG* mutations. Thus it is unlikely that any beneficial effect would be obtained using CMS, INH and RIF in an *in vivo* situation against MDR *M. tb*.

Previous drug enhancement studies using CST with RIF or INH against Mycobacteria report contrasting data to that reported in this thesis. Using *M. avium*, Rastogi *et al* [1990] observed no enhancement of drug activity for CST with RIF or INH, but no antagonism was observed either. However, enhanced antibiotic activity for RIF was observed when used with PMBN against *M. vaccae* [Korycka-Machała *et al* 2001]. The differences reported in these manuscripts and this thesis are likely

due to the Mycobacterial strains used, i.e., *M. avium* vs *M. vaccae* vs *M. tb*. Further differences could be due to the use of CST vs PMBN vs CMS. Other differences also include bacterial media used as well as culture conditions. Another independent study on drug enhancement using CST or CMS with INH and RIF against *M. tb* would be required to confirm the data reported (discussed in Chapter 5).

Understanding the chemistry of CMS, INH and RIF can assist in developing a suitable hypothesis as to why enhancement was observed using INH and not RIF. As discussed in section 1.3.2, CMS contains a hydrophobic moiety in the form of a fatty acid tail [Nation and Li 2010]. In addition, CMS is initially anionic at physiological pH until conversion to colistin occurs. CMS is expected to have a net charge of -5, which could oscillate between -3 and +3 during its conversion to colistin [Bergen *et al* 2006]. Considering the structure of RIF (Figure 1.5), an overall hydrophobic structure is observed [Pang *et al* 2013]. In addition, at physiological pH, it is expected that RIF will be zwitterionic (hydroxyl; pKa = 1.7, piperazine nitrogen; pKa = 7.9). However, it has been predicted that up to 40% of RIF molecules at physiological pH will be negatively charged (predicted by MarvinView1 5.4.1.1 software from ChemAxon) [Pinheiro *et al* 2014a]. It is thus highly likely that hydrophobic and electrostatic interactions between the two molecules occur under the *in vitro* conditions tested. At lower concentrations of CMS with RIF, there would be an antagonistic effect due to this interaction, causing less of a killing effect observed when CMS is used with RIF. In addition, INH has been determined to be a non-charged species at physiological pH as predicted by the MarvinView1 5.4.1.1 software from ChemAxon (basic pyridine nitrogen; pKa = 1.8, hydrazide nitrogen; pKa = 3.5 and hydrazide group pKa = 10.8) [Pinheiro *et al* 2014b]. Thus no antagonistic interactions are present between INH and CMS. However, additional reasons due to the differences in INH and RIF drug enhancement exist and are further discussed in Chapter 4 and 5.

2.4 Conclusion:

MICs (≥ 90% prevention in Alamar Blue® reduction) for CMS, CST and PST were determined to be 16, 16 and 8mg/L for both H37Ra and MDR *M. tb* using Equation 1. No statistical difference was determined for the MICs against H37Ra and MDR *M. tb*. Using the definition for MBC (≥ 99% reduction in viable cells), H37Ra *M. tb* had an MBC of 256, 64 and 64mg/L for CMS, CST and PST respectively. MDR *M. tb* was determined to have an MBC of 64, 64 and 128mg/L for CMS, CST and PST. However, even though these values differed according to the MBC definition, they did not differ statistically from each other, i.e., CMS of 64mg/L for H37Ra *M. tb* was statistically the same as MDR *M. tb* CMS MBC of 64mg/L.

H1 states: there is a statistical significant difference between the determined MIC and the MBC of CMS when compared to CST and PST at the 95% level of confidence for sensitive and MDR *M. tb* by use of a Student's t-test. Since the MIC for CMS was not statistically different from CST and only statistically different for PST, for both H37Ra and MDR *M. tb*, H1 is partially accepted with regard to MIC. Concerning MBC, there was a statistical difference in killing of viable cells for CMS when compared to CST and PST for both H37Ra and MDR *M. tb*. Thus, H1 is accepted with regard to MBC. Since H1 is partially accepted for MIC and accepted for MBC, it is important to quantify the amount colistin produced from CMS (Chapter 3).

H2 states: There is no statistical difference for the CMS MIC and MBC determined for sensitive *M. tb* compared to the CMS MIC and MBC for MDR *M. tb* at the 95% level of confidence by use of a Student's t-test. H2 is accepted is because the CMS MIC (16mg/L) and MBC (64mg/L) for both H37Ra and MDR *M. tb* were statistically similar.

The similar MIC and MBC data for H37Ra, and MDR *M. tb* suggest there is common mechanism of action against the two strains. This should also remove doubts that the difference in the cell wall of H37Ra vs H37Rv (section 2.1.2) could result is different effects by CMS (see Chapter 5). CMS used as a mono therapy is unsuccessful as a candidate for further drug development due to its high MIC, bacteriostatic action and an MBC/MIC ratio greater than 4. Since these concentrations of CMS against *M. tb* are much higher than that achievable in serum, and due to systemic toxicity concerns [Nation and Li 2010], additional drug development might appear to be unsuccessful. However, use of CMS DPI using the Turbospin® could avoid systemic toxicity [Schuster *et al* 2013], whereas CST and PST cannot be used with this method. Using sputum as a representation of local drug concentrations after inhalation of CMS DPI, it was shown that 38% of patients had colistin levels ≥ 128mg/L while the remaining patients had levels ≤ 128mg/L [Schuster *et al* 2013]. It was determined that using porcine surfactant (1mg/ml) as a representative for PS, which CMS is likely to come into contact if inhaled, the MIC was antagonised by eight fold (16mg/L vs 128mg/L). This is probably due to interaction with polar lipids and hydrophobic proteins [http://curosurf.com] in a similar way that CMS/colistin would interact with the outer membrane and CM of gram-negative organisms.

A point to ask is why gram-negative organisms display higher sensitivity to polymyxins than *M. tb*. This is likely due to the CL impeding the access of colistin. The structure of the CL, which mainly consists of polysaccharides, proteins and glycolipids/complex lipids within, with glucan being a major component is very different to the outer membrane lipids within gram-negative organisms. Colistin disrupts gram-negative membranes by displacing counter ions from lipopolysaccharides and interaction of the fatty acid tail with hydrophobic components. In *M. tb*, since the main component is glucan,

colistin doesn't likely have a major effect due to the absence of these lipids. Thus majority interactions would be via the fatty acid tail and not by displacement of counter ions, due to their absence. But synergy with INH may allow for sufficient lowering of MICs to overcome this.

H3 states there is a statistical significant difference of the CMS MIC determined in PS against *M. tb* when compared to the control CMS MIC (without PS), by use of a Student's t-test indicating antagonism. Thus, H3 is accepted according to data obtained.

As mentioned, only 38% of patients had colistin levels ≥ 128mg/L after inhalation of CMS DPI using the Turbospin®. Thus, with this method, CMS is likely to produce a sufficient MIC in a small amount of patients. It is possible that through further method development that sputum concentrations of colistin after inhalation of CMS DPI could be improved. However, in the clinical setting, CMS would not likely be used as a mono therapy, but rather polytherapeutically. Use together with INH and RIF might lower concentrations significantly enough to produce bactericidal effects. These interactions might also overcome the resistant effects of *rpoB* and *katG* mutations as observed for highly-resistant MDR gram-negative infections treated with CMS and RIF [Landman *et al* 2008; Lee *et al* 2013; Tascini 2013].

When conducting synergy and time-kill assays, certain assumptions were made for MDR *M. tb.* MICs for INH (4mg/L) and RIF (> 32mg/L) were much higher than the defined breakpoints [Palomino *et al* 2002], thus clinically relevant concentrations for INH and RIF were used, and MICs were set at 0.256 and 1mg/L for INH and RIF respectively for all calculations and experiments conducted.

H4 states: the lowest determined Σ_{FIC} would be for CMS used in combination with INH and RIF for sensitive and MDR *M. tb.* In both H37Ra and MDR *M. tb,* the lowest Σ_{FIC} (1.125) was determined for the combinations of CMS + INH (Table 2.3). Thus, H4 is rejected and $H4_0$ is accepted.

Using the definition of synergy; synergy $\Sigma_{FIC} < 0.5$, indifference Σ_{FIC} 0.5-4 and antagonism is $\Sigma_{FIC} > 4$, indifference was determined for the combinations of CMS, INH and RIF for both H37Ra and MDR *M. tb*. At this point, it was noticed that even though indifference was present for H37Ra, differences in concentrations for CMS with INH and RIF were observed. A difference in kinetic killing and bacteriostatic vs bactericidal effects of the combinations were immediately hypothesised. As for MDR *M. tb,* no such trend was observed. Instead, statistical analysis revealed that CMS was solely responsible for the inhibition observed, and it was eventually hypothesised that no difference in kinetic killing or bacteriostatic vs bactericidal effects for the combinations would be observed. The combinations that produced the lowest Σ_{FIC} were selected to be investigated in a time-kill assay.

These hypotheses were correct. A bactericidal effect was observed for CMS (2mg/L) + INH (0.064mg/L) against H37Ra *M. tb,* i.e., a reduction of 3.16 $\log_{10}$ CFU/ml, which was statistically

different to the rest of the combinations. Interestingly, a slight antagonistic interaction of RIF with CMS from the assay was observed, likely due to hydrophobic and electrostatic interactions. Other reasons for these observations are further discussed in Chapter 4 and 5. No statistical difference in any of the CMS combinations were observed for MDR *M. tb*. Reduction of approximately 1.0 $\log_{10}$ CFU/ml was determined for each CMS combination used with INH, RIF or both. This indicated that the inhibitory effect was due to CMS only.

H5 states: there is a statistical significant difference in the rate at which $\log_{10}$ viable CFU/ml units decrease when CMS is used in combination with INH and RIF versus when any two of the antibiotics are used in combination at the 95% level of confidence against sensitive and MDR *M. tb* by use of a one-way ANOVA. The only statistical difference observed was against H37Ra *M. tb* on day 6, with CMS + INH being the lowest. No statistical difference was observed for MDR *M. tb*. Thus, H5 is rejected and $H5_0$ is accepted.

For H37Ra *M. tb*, CMS can be used in a lower concentration with INH to achieve a bactericidal effect. This lower concentration will allow for CMS to overcome the antagonistic properties of PS during inhalation. Especially if it is considered that the concentration of PS used *in vitro* is 100 times higher than that contained in epithelial lining fluid [Schwameis *et al* 2013]. It is also not known how the different aspects of lung anatomy could affect the antibacterial activity of CMS, i.e., sputum, caseum etc., and this needs to be investigated further using *in vivo* assays. CMS, however, is very successful as an inhalant during various highly-resistant MDR gram-negative infections. This result is also a possible indication that treatment of mono-resistant *M. tb*, i.e., only containing the *rpoB* mutation might be promising. RIF mono-resistance could affect as much as 10% of isolates [Felmlee *et al* 1995; Garcia *et al* 2002], this could further prevent the transmission and development of INH resistance. This question needs to be answered by use of a clinical trial that will determine the effectiveness of CMS against sensitive *M.tb* as well as mono-resistant *M. tb* containing resistant mechanisms such as efflux pumps, i.e., less than 5% do not contain mutations in *rpoB* [Louw *et al* 2009; Musser 1995].

Chapter 3: Quantification of colistin formed from CMS

3.1 Introduction:

As mentioned throughout this thesis, CMS is unstable and readily hydrolysed to form colistin in microbiological broth (*in vitro*) [Bergen *et al* 2006], and *in vivo* [Li *et al* 2003a; Li *et al* 2005b]. Due to the nature of CMS, it is expected that $2^5 = 32$ different species may exist *in vitro* [Li *et al* 2005a]. In addition, more than 30 minor components have been separated successfully using liquid chromatography [Orwa *et al* 2000]. Li *et al* [2003b] determined that a maximum of 60-80% of CMS might convert to colistin; depending on the manufacture and test conditions. Polymyxins are also lost through non-specific binding to polystyrene and polypropylene which the microtiter test plates contain [Hindler and Humphries 2013; Sader *et al* 2012]. Furthermore, use of CMS over CST has been suggested to yield inaccurate MICs [Humphries 2014; Landman *et al* 2008; Li *et al* 2006], due to variation in conversion to colistin. All this theory suggests that CMS should produce less colistin than CST *in vitro*. As outlined in Chapter 2, MICs for CMS were hypothesised to be higher than CST for these reasons. Thus, to understand the chemistry and dynamics of CMS in the *in vitro* model here and quantify the amount of colistin formed, and to determine if similar species as CST are present, it would ultimately help understand the concerns raised above and unanswered questions in Chapter 2.

3.1.1 Previous studies quantifying colistin from CMS:

Thomas *et al* [1980] analysed PST and CST by various microbiological and chemical assays, i.e., gas-liquid chromatography to measure fatty acids, thin-layer and high-performance liquid chromatography (HPLC) to determine the complex composition, and potencies of polymyxins. Out of all the techniques tested, it was determined that HPLC provides the most information for the polymyxin samples investigated. It was further suggested that HPLC is the best method for monitoring the composition of polymyxin samples [Thomas *et al* 1980].

Furthermore, in recent years, HPLC methods quantifying colistin in biological fluids have been developed [Li *et al* 2001] which are more accurate, sensitive and reproducible than traditional bioassays [Leroy *et al* 1989]. This accurate, sensitive and reproducible characteristic is due to use of C_{18} solid phase extraction (SPE) cartridges allowing for the concentration of colistin on the column due to hydrophobic interactions. Derivatisation on the same column with 9-fluorenylmethyl chloroformate (FMOC-Cl) can then be conducted. Since no chromophores are present in polymyxins, fluorescence

detection of the FMOC-colistin derivative is then possible (λ_{ex} = 260nm and λ_{em} = 315nm) [Li *et al* 2001]. Figure 3.1 displays the reaction FMOC-Cl would have with the amines present on polymyxins, such as colistin producing an FMOC-colistin derivative.

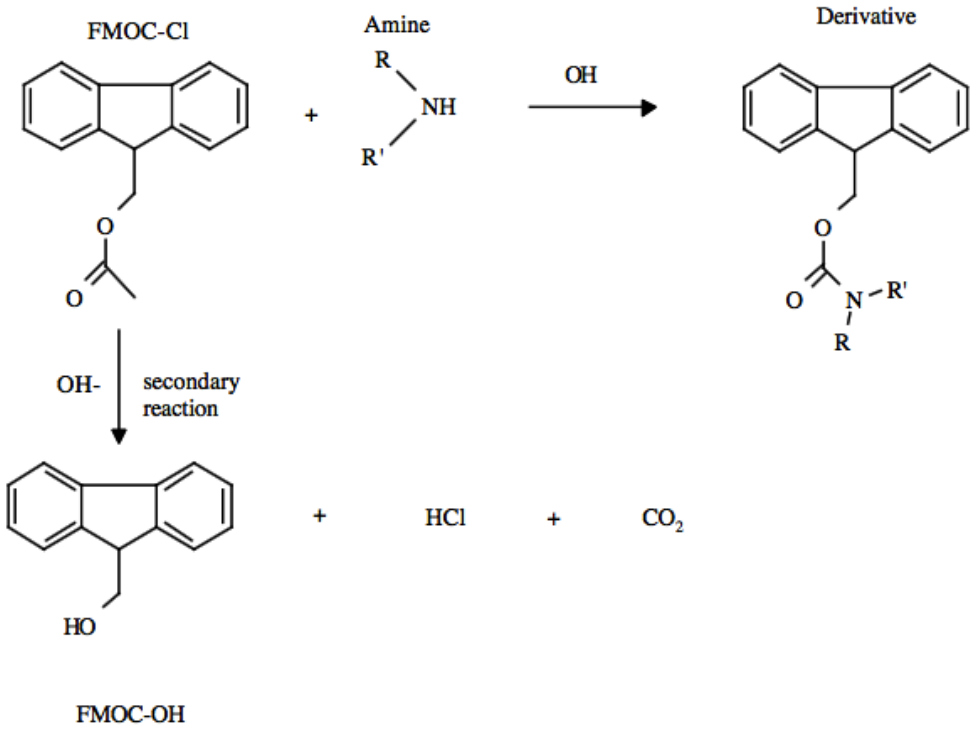


Figure 3.1: Reaction of FMOC-Cl with amines such as those present on colistin, producing a FMOC-colistin derivative that can be detected using fluorimetric or UV-VIS detection [Coppex and Walz 2000].

The use of FMOC-Cl via pre-columns such as SPE allows for derivatisation of primary and secondary amines. The reaction is via nucleophilic substitution in a borate buffer, giving very stable derivatives. Excess FMOC-Cl is required for the reaction to take place. Detection is very easily conducted via fluorimetric methods or UV-VIS (260nm), giving sensitive detection levels [Coppex and Walz 2000].

As displayed in Figure 3.1, a secondary reaction takes place causing the formation of FMOC-OH. This is a drawback to fluorescence detection [Coppex and Walz 2000] as this may cause peak overlapping. Another disadvantage of using FMOC-CL would be the following; since during hydrolysis of CMS to colistin, various amounts of methanesulfonate groups and -NH_2 groups will be present, pre-column derivatisation could produce species containing anything from one FMOC group to five FMOC groups. This might result in a variety of species during analysis using this method. Detection of FMOC-colistin derivatives have been optimised with the use of C_{18} HPLC columns [Li *et*

al 2001; Li *et al* 2003b]. Detection of colistin formed from CMS in various *in vitro* and *in vivo* situations had been successfully quantified using FMOC-Cl pre-column derivatisation [Dudhani *et al* 2010; Imberti *et al* 2012; Li *et al* 2002; Li *et al* 2003b]. It is important to note that CMS can also be quantified by use of FMOC-Cl, but this involves acid hydrolysis (1M of H_2SO_4) of the remaining CMS to colistin in *in vitro* and *in vivo* situations, and using the amount of colistin formed during acid hydrolysis as a reference for the amount of CMS remaining [Li *et al* 2002]. It must be mentioned that HPLC retention times are long in order to assay FMOC-colistin derivatives, i.e., > 10 minutes.

Colistin has also ben analysed using HPLC and UV-VIS detection at 210nm, i.e., detection of peptide bonds present [Li *et al* 2001]. In certain cases, strong anion exchange column has been used for suitable detection of colistin using this method [Li *et al* 2003b]. Detection of FMOC-colistin derivatives is still more sensitive using UV-VIS compared to UV-VIS detection of peptide bonds at 210 nm. Using fluorescence detection and FMOC-Cl, limits of quantification as low as 0.1mg/L have been described [Li *et al* 2001].

Evaporative light scattering detection (ELSD) has also been used for the detection of colistin during HPLC, avoiding the need for pre-column derivatisation, i.e., detection is by measuring light scattered from solid solute particles after nebulisation and evaporation of the mobile phase [Clarot *et al* 2009]. This method has been described as rapid and simple for simultaneous determination of tobramycin and colistin in a pharmaceutical formulation by reverse phase HPLC [Clarot *et al* 2009]. Unfortunately, ELSD is not as sensitive as FMOC derivatisation of colistin, i.e., ELSD concentrations of colistin required are 160-240mg/L; based on calibration curves. This is a major drawback in considering its use as a quantitative method.

More modern methods employ the technique of LC-MS/MS, i.e., liquid chromatography tandem mass spectrometry. This method has been described as being rapid, simple, reliable and specific for determining colistin concentrations in bacterial media [Zhao *et al* 2014]. This method has the advantage of avoiding sample pre-treatment, i.e., pre-column derivatisation and long run times. Avoiding the use of FMOC derivatisation prevents the possibility of generating multiple species due to the oscillation in the amount of methanesulfonate groups and $-NH_2$ groups. In addition, the method can be very sensitive, i.e., colistin can be detected in the range of 0.0241-2.41 and 0.0439-4.39mg/L for colistin A and colistin B respectively, based on standards [Zhao *et al* 2014]. Electron spray ionisation is used with detection in the positive ion mode which is successful for *in vitro* PK/PD studies for colistin in Mueller-Hinton broth [Zhao *et al* 2014]. Colistin and CMS have successfully been quantified using LC-MS/MS in various *in vitro* and *in vivo* situations [Gobin *et al* 2010; Jansson *et al* 2009; Ma *et al* 2008]. Retention times to complete the assay are relatively short, i.e., five minutes [Zhao *et al* 2014].

In all cases of analysis, the requirement of SPE cartridges cannot be avoided. SPE cartridges assists in isolating colistin from culture media components such as salts and allows for it to be concentrated in smaller volumes of solvent, allowing for its retention on HPLC columns and its detection. This is also very important when quantifying colistin in biological fluids such as serum.

3.1.2 Determining the method of choice:

Briefly, initial investigations involved determining the limit of quantification for colistin within Sauton media used throughout this study. Firstly, LC-MS at the University of Pretoria, Department of Chemistry was conducted according to previously described methods, followed by LC-MS/MS at the University of Stellenbosch, Department of Chemistry, also according to previously described methods [Gobin *et al* 2010; Jansson *et al* 2009; Ma *et al* 2008; Zhao *et al* 2014]. Unfortunately, the level sensitivity was not obtained, i.e., at least 10mg/L detection of colistin. Next, UPLC and ELSD quantification was conducted according to previously described protocols [Clarot *et al* 2009]. As discussed in 3.1.1, insufficient level of detection for colistin was obtained using this method of detection. Our last technique of choice was that of FMOC-Cl pre-column derivatisation using a UPLC system and UV-VIS via a PDA detector. These systems were also available for easy access and use at the University of Pretoria, Department of Biochemistry with the necessary skills and expertise available.

3.2 Material and methods:

3.2.1 Quantification of colistin formed from CMS as determined by UPLC:

CMS hydrolysis to colistin determined by UPLC was adopted from Li *et al* [2003b]. CMS and CST were incubated at 37ºC in Sauton media using the same method as described (section 2.2.3) at their determined MIC concentrations (16mg/L) without the presence of *M. tb*. Samples (600µl) were taken at days zero, one, four and six, and were stored at -20ºC pending analysis by UPLC. Samples (300µl) were passed through a Waters Oasis® HLB SPE (3cc, 60mg) (Waters, Milford, MA, USA) extraction cartridges conditioned with 1ml methanol (Sigma-Aldrich; HPLC grade) and equilibrated with 1ml carbonate buffer (pH 10) (Fluka; HPLC grade). FMOC-Cl (100µl, 30 mM; acetonitrile) (Sigma-Aldrich; HPLC grade) was reacted on the SPE column for ten minutes. It was extremely important to make sure that none of the acetonitrile was eluted during the reaction.

FMOC-colistin derivatives were eluted using 900µl of tetrahydrofuran (THF) (Sigma-Aldrich; HPLC grade) into Eppendorf Protein LoBind tubes using a vacuum manifold and disposable flow control valve liners (Supelco, Bellefonte, PA, USA), and mixed with 600µl of 0.2M boric acid (Sigma-Aldrich; ACS grade). UPLC analysis was performed on a Waters ACQUITY UPLC H-Class system (Waters, Milford, MA, USA) equipped with a binary solvent delivery pump, an auto sampler (4ºC), and a photodiode array detector (PDA) controlled by Empower-3 software. Colistin was assayed using a Waters (Milford, MA) Acquity UPLC BEH C_{18} 1.7µm 2.1 × 50mm column at 40ºC with the PDA detection set at 265nm. An isocratic mobile phase of acetonitrile-THF-dddH_2O (87:4:13 [v/v/v]) was pumped at a flow rate of 0.3ml/min. Injection volumes of 10µl were used, and quantification was conducted by combining the areas of colistin A and colistin B; the main components of CST. Standard curves ($R^2 > 0.98$) were constructed using CST (2-16mg/L) and as a control, CST (16mg/L) was injected every ten injections to confirm system stability. The run time was five minutes.

3.2.2 Statistical analysis:

Conducted according to section 2.2.7.

3.3 Results and discussion:

3.3.1 Initial experimental observations:

Theoretical plates (Equation 3), where N = number of theoretical plates, 5.54 is the coefficient used for width at half height, tr = retention time and $W_{0.5}$ = width of peak at half height can be used to determine performance and effectiveness during UPLC [Waters TWLC02-A].

$$N = 5.54 \left(\frac{tr}{W_{0.5}} \right)^2$$ Equation 3

During optimisation of the method, the shortest possible retention times, narrow peaks and adequate peak separation were desired. Various conditions were investigated, i.e., using gradient elution vs isocratic elution, change in mobile phase pH according to the pKa of colistin and column

temperature. Ultimately, the main factor affecting Equation 3 was determined to be column temperature, with the best temperature being determine do be 40ºC, thus improving quantification. All other parameters already determined by Li *et al* [2003b] could not be improved upon.

During sample collection, it was extremely important to keep all collected samples at -20ºC. This is to prevent any conversion of CMS to colistin during the storage period [Li *et al* 2003b]. Furthermore, during analysis, it was important to keep the time from derivatisation with FMOC-Cl to analysis as short as possible. When batches were being analysed in the autosampler, temperature was kept at 4ºC for increased stability of the deriviative. It was observed that decay of the FMOC-colistin species occurred if there was an increased period between derivatisation, analyses and being kept at room temperature.

As mentioned, the limit of detection for LC-MS/MS was never achieved. These reasons are likely due to inter-lab variation. Use of the UPLC system at the University of Pretoria, Department of Biochemistry allowed for direct access and plentiful time for optimisation of the method. The limit of detection was optimised to be 2mg/L for colistin. The detection limit is still much higher than that of Li *et al* [2001], i.e., 0.1mg/L. This is because Li *et al* [2001] used fluorescence detection for FMOC-colistin while the method developed for these investigations utilised UV-VIS via a PDA detector which doesn't provide the same level of sensitivity.

3.3.2 Quantification of colistin formed from CMS as determined by UPLC:

A typical chromatogram for CST is displayed in Figure 3.2

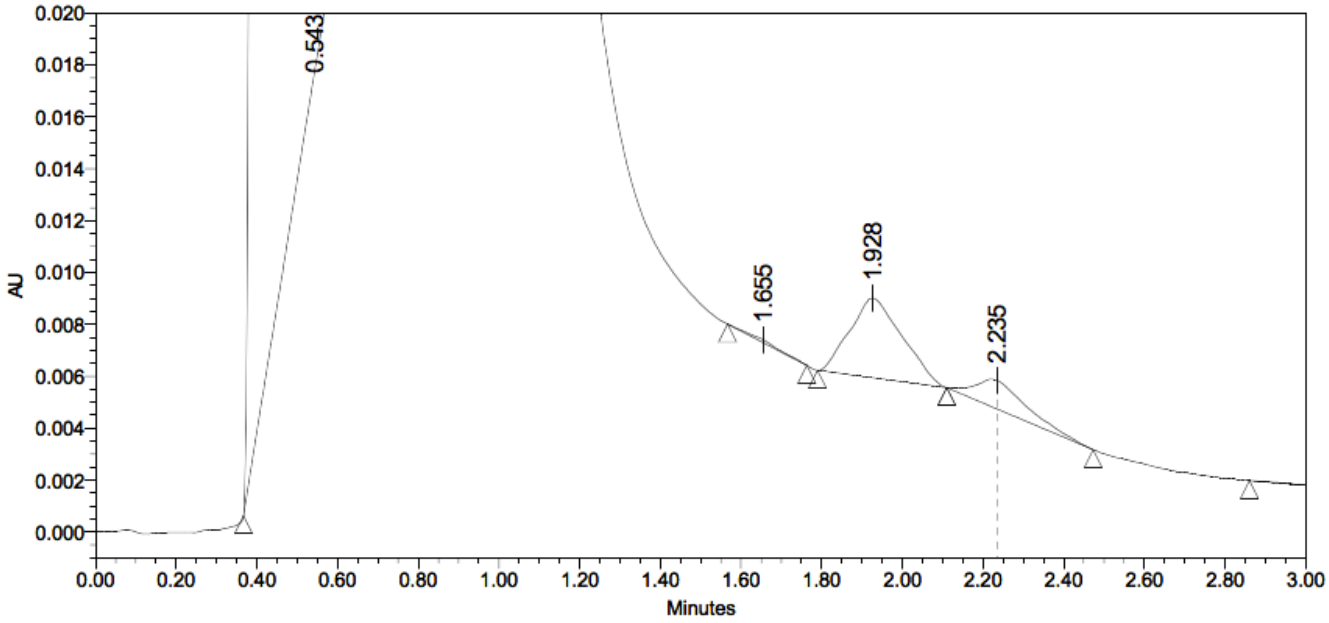


Figure 3.2: Typical chromatogram revealing the separation of FMOC-colistin species (using CST 16mg/L) in Sauton media. A column (C_{18}) temperature of 40ºC was used and detection was at 265nm (UV-VIS).

When analysis was conducted using CST as a control (Figure 3.2), two major species were also determined, i.e., 1.9 minutes and 2.2 minutes retention time. In certain instances, an additional peak at 1.6 minutes was determined, but its peak area was negligible to use during quantification. The major peak seen from ~ 0.4 minutes was attributed to the formation of FMOC-OH, due to a secondary reaction. This was determined by use of controls, i.e., Sauton media with/without CMS or CST. According to extensive literature on the topic and the chemical structures of colistin A (6-methyloctanoic acid) and colistin B (6-methylheptanoic acid) (Figure 1.6); colistin B was determined to be the peak at 1.9 minutes and colistin A was determined to be the peak at 2.2 minutes [Li *et al* 2002]. As discussed by Li *et al* [2003b], since the major components of colistin that are being resolved are the A and B form, combining the peak areas of colistin A and colistin B for quantification is sufficient. A smaller percentage of other components would include the presence of various insignificant polymyxin species [Orwa *et al* 2000], sodium counter ions, water and bisulphate salts [He *et al* 2013].

Figure 3.3 displays a typical chromatogram for colistin formed from CMS after 24 hours incubation at 37ºC in Sauton media.

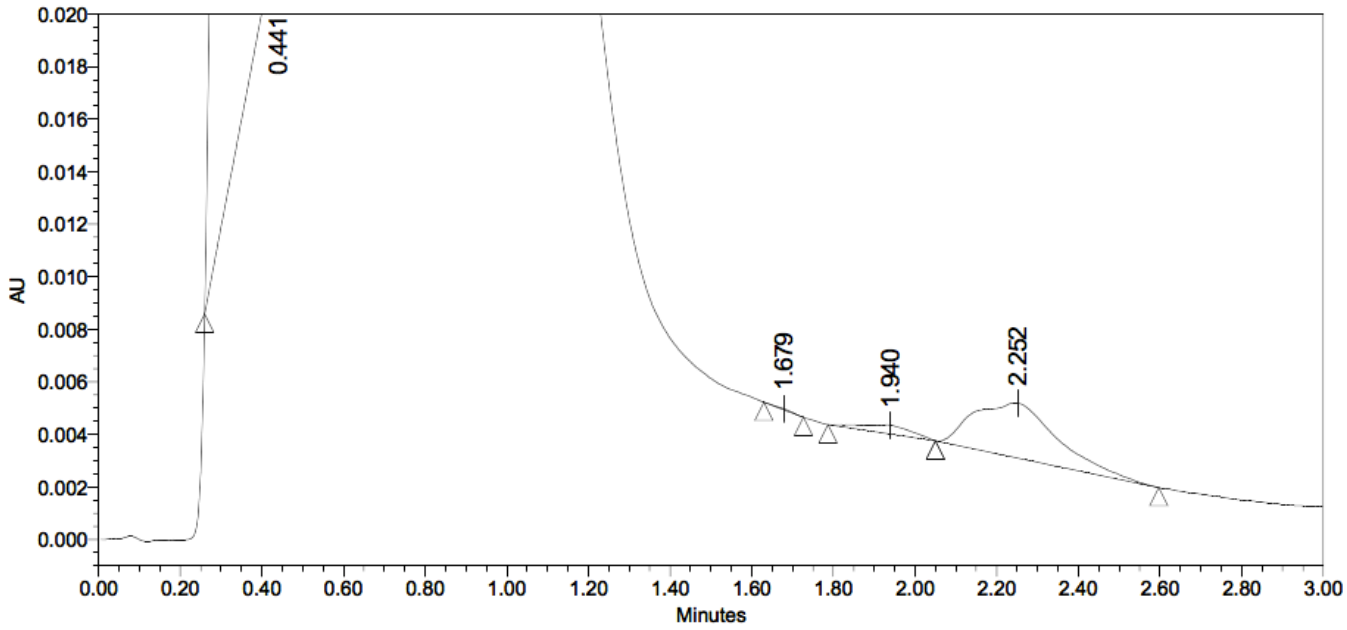


Figure 3.3: Typical chromatogram revealing the separation of FMOC-colistin species formed from CMS (16mg/L) incubated at 37ºC in Sauton media 24 hours. A column (C_{18}) temperature of 40ºC was used and detection was at 265nm (UV-VIS).

Similar to that of the results obtained for CST, two major species corresponding to colistin A (2.2 minutes) and colistin B (1.9 minutes) were resolved. In certain instances, the peak at 1.6 minutes was also observed but the area was negligible in terms of quantification. The FMOC-OH peak at ~ 0.4 minutes can also be clearly observed. Since the major species determined were colistin A and colistin B, their areas were combined for quantification [Li *et al* 2003b]. Since the overall amount of colistin formed was of concern, in relation to the amount of colistin from CST, and not the amount of CMS remaining, no acid hydrolysis of CMS sub-samples were conducted [Li *et al* 2002]. For both methods, it can be clearly observed that run times of five minutes were required, an improvement over existing methods.

Figure 3.3 does reveal one striking difference when comparing CST (Figure 3.2) and CMS. The amounts of colistin A and colistin B differed. This was observed throughout the analysis. This observation is, however, common knowledge since it has been previously described that different batches, and manufacturers contain variations in colistin species [Decolin *et al* 1997; Humphries 2014; Landman *et al* 2008]. Colistin A (2.2 minutes) from CMS in certain instances contained the presence of a shoulder. As described for CMS, during hydrolysis to form colistin, there is the presence of species containing various amounts of charges, i.e., it could oscillate between -3 and +3 [Bergen *et al* 2006] vs

CST, which contains +5. Charge is an indication of amine groups available for FMOC-Cl to bind to during derivatisation. Since the major component of CMS is colistin A, various amounts of FMOC-Cl bind to the various amine groups available, producing a shoulder due to a difference in hydrophobicity (various amounts of FMOC groups attached to the amines). This shoulder also appeared in certain instances for CST for colistin B. Thus, there was also a variation in binding of FMOC groups to colistin primary amines from CST. This is not due to the amines not being available for CMS, but rather due to incomplete reactions taking place. CMS would thus also be affected by this issue in addition to charge oscillation during conversion. This is an indication of one of the disadvantages for pre-column derivatisation, which cannot be avoided.

Table 3.1 displays the amount of colistin (A and B) present for CST and CMS determined over six days.

Table 3.1: Amount of colistin (A and B) present over six days of incubation at 37ºC in Sauton media using CMS (16mg/L) and CST (16mg/L), i.e., simulation of section 2.2.3. Letter groupings are displayed.

Day of incubation	Colistin (A and B) formed (mg/L)	
	CMS ± SD	CST ± SD
0	0 ± 0 (B)	15.97 ± 0.43 (A)
1	15.86 ± 1.39 (A)	14.8 ± 1.03 (A)
4	15.92 ± 0.69 (A)	14.73 ± 0.69 (A)
6	16.06 ± 0.47 (A)	15.04 ± 0.06 (A)

As seen in Table 3.1 and according to statistical analysis using letter groupings, the only difference in amount of colistin formed from CMS or CST is on day zero. CMS had not converted to colistin, i.e., methanesulfonate groups are present with no available amines for FMOC-Cl to react with during pre-column derivatisation. At day zero, from CST, there was 15.97 ± 0.43mg/L colistin present; 99.81% of the determined MIC (16mg/L). After 24 hours of incubation, 15.86 ± 1.39mg/L of colistin is hydrolysed from CMS; 99.13% conversion rate. Comparing colistin formed from CMS and CST using the A and B forms as references, there was no statistical difference in the amount formed from day one until day six of incubation.

This data suggests that the same amount of colistin (A and B) is delivered to *M. tb* (H37Ra and MDR) during MIC determinations and likely other investigations conducted, i.e., MBC, effect of PS, synergy and time-kill assays. For CST, the major components were determined to be colistin B then

colistin A. For CMS, the major components were determined to be colistin A then B. But since very similar MICs were determined for CST and CMS, it suggests that regardless of colistin A or colistin B being a majority component, the overall concentration (16mg/L) determines the inhibitory effect on *M. tb*. MBC data between CMS and CST differ slightly according to definitions; CMS can be higher than CST for H37Ra but the same for MDR. However, in both cases, statistically CST causes significantly larger amounts of killing at lower concentrations. Since CMS is in the process of hydrolysis to form colistin over 24 hours, it highly suggests that the first 24 hours are extremely important with regard to reduction in cell viability. CST has the advantage over CMS as it is already added in its active form, reducing viable cells from time of addition. This is an indication of the importance for colistin having positive charges from primary amines available.

It was initially expected that only 60-80% of CMS would convert to colistin [Li *et al* 2003b]. The maximal conversion of CMS to colistin occurs after 48 and 72 hours of incubation under various conditions. Concentrations of colistin then decrease after the peak has been obtained [Li *et al* 2003b; Zhao *et al* 2014]. Comparing to CST (16mg/L) it can easily be seen that a conversation rate close to 99% was achieved within 24 hours. Thus the importance of understanding the nature of polymyxins as outlined in section 1.3 and 2.1.1. The faster conversion rate for CMS to colistin is likely due to the pH used *in vitro*, as slower conversion rates have been observed at lower pH [Li *et al* 2003b]. In addition, after maximal conversion of CMS to colistin, no decrease in colistin concentrations were observed, whereas previously Li *et al* [2003b] describe colistin concentrations to decrease with time due to instability. This can be explained by the following; Li *et al* [2003b] expressed their data as levels of colistin formed from CMS based on original molar CMS. Whereas here, the actual colistin A and B concentrations are determined referring to colistin A and B in CST. In addition, the reason for sustained concentrations of colistin (A and B) is likely due to the stability of the *in vitro* environment developed (Chapter 2).

3.4 Conclusion:

Overall, the UPLC method was optimised to use similar conditions as previously described [Li *et al* 2003b], but run times were shortened, i.e., five minutes with very short retention times for colistin A and B. A limit of quantification of 2mg/L was achieved using UV-VIS detection for FMOC-colistin via a PDA detector.

H6 states: there is a statistical significant difference between the amount of base colistin formed from CMS at the MIC when compared to CST at the 95% level of confidence by use of a Student's t-

test. Using exactly the same experimental conditions as outlined for MIC determinations (as well as MBC, PS, synergy and time-kill assay) no statistical difference in the amount of colistin delivered to *M. tb* by either CMS or CST was determined from day one until day six of incubation. The only difference was at day zero when CMS is in the process of hydrolysis to form colistin. Thus H6 is partially accepted. It is not known how *M. tb* would affect the concentrations of colistin in an *in vitro* environment and should be investigated.

During analysis, CST was determined to contain larger amounts of colistin B than A and CMS was determined to contain more colistin A than B. This had no effect on the determined MICs since the two drugs had the same concentrations (16mg/L). Overall, it is likely that the combined components of colistin A and B made up the overall concentration (16mg/L), which would cause the inhibitory effect on *M. tb*. Concerning MBC, greater killing of CST at lower concentrations compared to CMS are attributed to the conversion of CMS to colistin within the first 24 hours. This conversion is also likely to be a mixture of -3 and +3 CMS and colistin species. Thus, within the first 24 hours, CST is able to cause a greater reduction of viable cells than CMS.

The fact that other species and forms of colistin present in CMS or CST were not observed using this method of quantification does not suggest that they are not present. These species are likely to be present, but in such a low concentrations related to colistin A and B, it could not be detected. Thus, it has to be hypothesised here that the major inhibitory effect on *M. tb* is due to colistin A and B and with no preference to either form, just the overall concentration.

Since CMS converts to colistin within 24 hours, it would be interesting to compare the ultrastructure effects of CMS and CST at their respective MICs on *M. tb*. Further, it would be interesting to isolate the different species of colistin formed from CMS and determine, which are more active against *M. tb*.

Chapter 4: Morphological effects of CMS on *M. tuberculosis* H37Ra studied by transmission electron microscopy

4.1 Introduction:

Since the likely mechanism of action for polymyxins would be related to disruption of cellular membranes, the best method of investigation is that of microscopy. Only two investigations concerning the ultrastructure effect of polymyxins on Mycobacteria have been conducted. David and Rastogi [1985] investigated the mode of action of colistin against *M. aurum* and David *et al* [1986] investigated the action of colistin on *M. tb* (H37Ra) by use of Mycobacteriophage D29. Both cases only used CST in their investigations. Since for this thesis, MICs were determined using a different *in vitro* method (MABA and Sauton media) and CMS converts to colistin within 24 hours, it is of interest to determine the effect CMS would have on *M. tb* within 24 hours. It would be important to compare this to CST to observe if they are similar in nature within 24 hours. Furthermore, this investigation can provide insight into the bactericidal effect of using CMS together with INH against H37Ra *M. tb*. Understanding the bactericidal effect would make it easier to predict if CMS in combination with other drugs would be bactericidal.

4.1.1 Chemical fixation vs cryopreservation for ultrastructure investigations of Mycobacteria:

Routine preparation of samples for TEM involves the following process; a buffer containing aldehydes (formaldehyde and glutaraldehyde) for fixation. Fixation of proteins, carbohydrates and lipids occur, forming an insoluble matrix. $OsO_{4(aq)}$ is used as it further allows for fixation of lipids and provides contrast for the image, i.e., the heavy metal is embedded directly into the membranes. Dehydration in solvents (ethanol) normally occurs, followed by infiltration and embedding in hydrophobic resins, which are then polymerised. The resins make ultra-thin sections possible for TEM analysis. Heavy-metal salts (uranyl acetate and lead citrate) are usually used as a counter stain, providing additional contrast [Hayat 2000].

Silva and Macedo [1983] sufficiently reviewed the unique characteristics of Mycobacteria during ultrastructure investigations, providing suggestions for its preservation during routine preparation for TEM analysis, sufficiently preserving the various membranes. Use of PIPES buffer is highly recommended; it has a pKa of 6.76, near physiological pH. In addition, PIPES has been documented to preserve lipids during fixation [Hayat 2000]. It is important to supplement buffers with 10mM Ca^{2+} or Mg^{2+}. This allows for sufficient stabilisation of membrane structures during fixation [Silva and Macedo 1983]. Addition of ruthenium red during initial fixation protocols for Mycobacteria are common [David and Rastogi 1985; Rastogi *et al* 1986a; Stokes *et al*]. Ruthenium red sufficiently

stains the CL, i.e., polysaccharides [Hayat 2000] such as glucan (section 1.2.3). Once an insoluble matrix has been formed and *M. tb* cells are sufficiently fixed, use of 1% v/v $OsO_{4(aq)}$ is possible to counter stain lipids and provide additional fixation [Hayat 2000]. The fixation process allows for adequate preservation of *M. tb* cells during dehydration with ethanol, embedding using a resin such as Epon 812 and further staining of sections with heavy-metal salts. Even though this method of sample preparation is extensively optimised, disadvantages do exist, i.e., collapse of water rich structures and even extraction of various components and introduction of various artefacts [Dubochet and Blanc 2001].

The use of cryo-electron microscopy of vitreous sections is the best method for pure structural studies of Mycobacteria, i.e., ultrastructural analysis. This is due to the organism being viewed in their vitreous state (closest to native state), avoiding the harsh environment of chemical fixation, dehydration, embedding and heavy-metal salts [Bleck *et al* 2010; Hoffmann *et al* 2008; Zuber *et al* 2008]. With regard to Mycobacteria, this method has only been applied to *M. smegmatis* and *M. bovis* BCG [Bleck *et al* 2010; Hoffmann *et al* 2008; Zuber *et al* 2008].

After cryo-electron microscopy of vitreous sections, the next-best preparation method for ultrastructural analysis is that of high-pressure freezing, freeze substitution [Bleck *et al* 2010] and variations of the techniques exist [Bleck *et al* 2010; Takade *et al* 2003; Yamada *et al* 2014]. High-pressure freezing rapidly arrests physiological processes by fixing samples via vitrification, i.e., instead of chemical fixation, samples are frozen rapidly at low temperatures and high-pressure. Next, during freeze substitution, samples are gently dehydrated at low temperatures. Samples can then be embedded in resins at low temperatures, and polymerisation is conducted by UV. Minimal artefacts are introduced using this technique [Steinbrecht and Zierold 1987].

Thus, the best technique for ultrastructural studies, based on preservation of cellular structures and presence of artefacts is cryo-electron microscopy of vitreous sections followed by variations of high-pressure freezing and freeze substitution and lastly standard sample chemical fixation.

As mentioned by Yamada *et al* [2014], *M. tb* requires BSL-3 containment. In general, samples are sterilised during chemical fixation, which allow for their removal from BSL-3 containment and further processed for ultrastructure analysis in a general laboratory. Equipment required for cryo-electron microscopy of vitreous sections, high-pressure freezing, and freeze substitution are bulky, expensive, specialised and are usually only located in a centralised multi-user laboratory, outside of BSL-3 containment. Thus, initial sterilisation by aldehydes (i.e., chemical fixation) for this thesis was unavoidable.

At the Microscopy Society of Southern Africa 51st annual conference, preservation of *M. tb* cell membrane integrity, after chemical fixation was presented. The investigation was conducted as part of this thesis. The technique involved the sterilisation of samples using chemical fixation as described, followed by high-pressure freezing and freeze substitution, which was conducted in a centralised multi-user laboratory. With regard to preservation of cellular structures and presence of artefacts, the technique proved to be much better than standard chemical fixation, dehydration and embedding of samples. This observation has also been previously been determined for mice liver tissue [Venter *et al* 2013]. However, when this method of sample preparation was applied to *M. tb* treated with CMS or CST, they were not retained in sufficient amounts and cellular material was lost at the various stages of processing. Thus, unfortunately standard chemical fixation and dehydration had to be used as it was the only method that retained sufficient amounts of *M. tb* for analysis. Since standard chemical fixation and dehydration was conducted, it is important to understand which artefacts may be present. This allows for accurate analysis of the CMS effect on *M. tb.*

4.1.2 Elucidation of Mycobacterial microanatomy:

The best method to understand the true nature of *M. tb* would be that of cryo-electron microscopy of vitreous sections. Only *M. smegmatis* and *M. bovis* BCG have only been studied using this method, thus *M. bovis* BCG can serve as a comparison [Bleck *et al* 2010; Hoffmann *et al* 2008; Zuber *et al* 2008]. Yamada *et al* [2014] sufficiently preserved the ultrastructure of pre-fixed *M. tb* using cryofixatton and freeze substitution. These can be used as comparisons to interpret chemically fixed Mycobacteria [Rastogi *et al* 1986a]. Figure 4.1 is described best with reference to section 1.2.3, focusing on the cell wall interpretation.

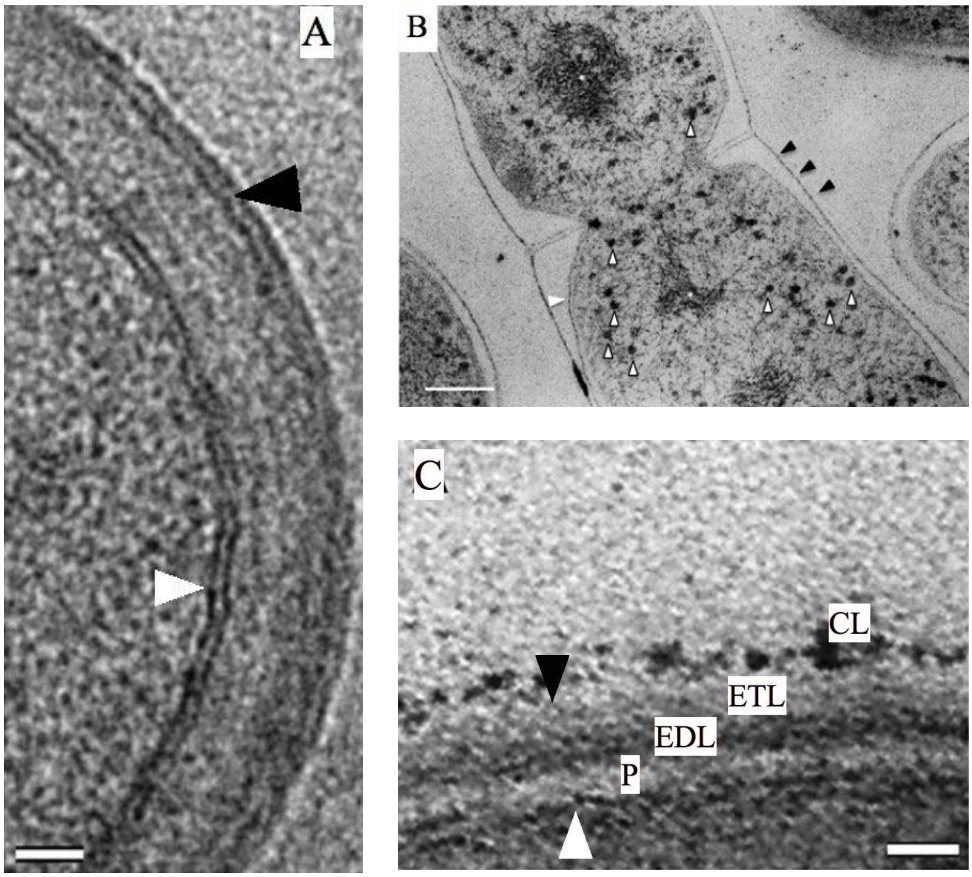


Figure 4.1: Ultrastructure comparison of (A) cryo-electron microscopy of vitreous sections (*M. bovis* BCG) [Zuber *et al* 2008], (B) cryofixatton and freeze substitution (*M. tb*) [Yamada *et al* 2014] and (C) standard chemical fixation and dehydration (*M. smegmatis*)[Zuber *et al* 2008]. White arrows indicate CM (A, B, C), white arrows with a black border indicate ribosomes (B), black arrows indicate MM (A, B, C). Periplasm (P), electron dense (EDL) and electron translucent layers (ETL), and capsule layer (CL) are indicated (C). Scale; (A) 20nm, (B) 100nm and (C) 20nm.

Using *M. bovis* BCG (Figure 4.1 A) cryo-electron microscopy of vitreous sections, a general and 'most correct' structure of the Mycobacteria cell wall is observed [Daffé and Zuber 2014; Hoffmann *et al* 2008; Zuber *et al* 2008]. A bilayer CM is clearly observed (white arrow). Beyond the CM is the periplasm (P) followed by a PG/AG layer (not shown). P is observed as a granular layer [Zuber *et al* 2008] which is different to the PG/AG layer. The black arrow indicates the bilayer structure of the MM. The inner most leaflet contains MA covalently linked to AG while the outer most layers contains proteins, lipids and glycolipids/complex lipids. Interestingly, no CL is observed (Figure 4.1 A) due to the cryoprotectant being the same density as the CL, and that culture conditions used caused the CL to be shed [Daffé and Zuber 2014; Zuber *et al* 2008]. Very similar results were obtained

by Yamada *et al* [2014] for *M. tb* (Figure 4.1 B) concerning the cell wall structure. A very different structure is observed for *M. smegmatis* analysed ultrastructurally by standard chemical fixation and dehydration (Figure 4.1 C) [Zuber *et al* 2008] and a similar ultrastructure has been observed for various Mycobacteria, including *M. tb* [Rastogi *et al* 1986a]. In Figure 4.1 C, a CM is clearly observed (white arrow). Above the white arrow, is a hypothesised area considered to be the P. In certain cases, the P might not appear to exist, this is likely due to collapse during conventional preparation, giving the outer leaflet of the CM a thicker appearance [Daffé and Zuber 2014; Zuber *et al* 2008]. Next, an EDL exists, considered to be the PG/AG layer of which AG extends into the ETL. Thus, the ETL can be considered to contain MA and is likely considered to be part of the MM [Daffé and Zuber 2014]. This theory is still under debate [Daffé and Zuber 2014]. Observable with this method of preparation is a CL, which is stained and made visible by ruthenium red [Rastogi *et al* 1986a]. Rastogi *et al* [1986a] have described the CL of *M. tb* to be continuous, 8nm thick and present in 50-80% of bacilli.

Bleck *et al* [2010] sufficiently compared the various methods of sample preparation and observed various artefacts that are encountered during ultrastructure analysis of Mycobacteria. In general, with conventionally prepared Mycobacteria, the likely artefacts encountered are the following; mesosome, a regular infolding of the CM, condensed nuclei and an accumulation of condensed rope-like DNA fibrils [Bleck *et al* 2010; Silva and Macedo 1983]. Thus, when analysing possible perturbations induced in *M. tb* by polymyxins during ultrastructure investigations, it is very important to be aware of these possible artefacts.

4.1.3 Determining the ultrastructure effects of peptides on *M. tb*:

Even though conventional preparation (chemical fixation and dehydration) for ultrastructure analysis of *M. tb* does not optimally preserve the ultrastructure, it is the most widely used method of preparation for observing the effect peptides such as polymyxins would have on the ultrastructure of *M. tb* [Rivas-Santiago *et al* 2013a; Rivas-Santiago *et al* 2013b]. Once MICs are determined, the effect peptides have on the ultrastructure on *M. tb* within 24 hours can be determined [Rivas-Santiago *et al* 2013a; Rivas-Santiago *et al* 2013b]. One complication is the starting inoculum used for MIC determinations (MABA) is generally 1.5 x 10^5CFU/ml; not a sufficient amount to use for ultrastructure analysis. Thus a higher starting inoculum is required, i.e., 1 x 10^7CFU/ml [Rivas-Santiago *et al* 2013a; Rivas-Santiago *et al* 2013b]. Using a higher inoculum may increase the MIC, with respect to the MABA. Determining MICs using the MABA at a higher starting CFU/ml has been described [Carroll *et al* 2009].

4.2 Material and methods:

4.2.1 Preparation of strain and working stocks:

M. tb H37Ra ATCC 25177 obtained from Ampath Pathology Laboratory Support Services (Centurion, Gauteng, South Africa) was used. All processing and preparation of working stocks were the same as that described in Section 2.2.1.

4.2.2 Antimicrobials:

All antimicrobials used were prepared as described for section 2.2.2.

4.2.3 MIC determinations using MABA:

MICs for CMS and CST were determined using exactly the same method as described for section 2.2.3, except for certain differences. The method used was adopted from Carroll *et al* [2009] using a final cell titre of 1 x 10^7CFU/ml, as this was required for a workable cell pellet. The final concentration of CMS and CST investigated was 8-128 mg/L. INH, and RIF were also included as controls. The MIC was determined after three or four days of incubation and was defined as the last well that prevented a pink colour change for Alamar Blue® [Collins *et al* 1997; Franzblau *et al* 1998; Franzblau *et al* 2012]. The MIC was completed in duplicate on the day, and three independent experiments were conducted for H37Ra *M. tb*.

4.2.4 Ultrastructure analysis of *M. tb* treated with CMS:

Ultrastructure investigations were adopted from Rivas-Santiago *et al* [2013a; 2013b]. TEM was used to investigate the effect CMS would have on the ultrastructure of H37Ra *M. tb*. *M. tb* was incubated in concentrations of 16, 64 and 256mg/L of CMS and CST (as a control) for 24 hours to determine the effect on the ultrastructure. After 24 hours, cell suspensions were removed and centrifuged at 10 000g's for one minute. The supernatant was subsequently removed, and the pellet was fixed for one hour at room temperature using 2.5% v/v formaldehyde and glutaraldehyde in 0.1M PIPES (Merck) (pH 7-7.2), supplemented with 5mM $CaCl_2$, $MgCl_2$ and 0.1% w/v ruthenium red (EMD

Millipore) [David *et al* 1985]. Pellets were later rinsed using PIPES buffer (3 x ten minutes) followed by fixation in 1% w/v $OsO_{4\,(aq)}$ (SPI Supplies, Philadelphia, USA) and rinsing with PIPES as described. Pellets were then dehydrated using ethanol (EtOH) (30, 50, 70, 90 and 3 x 100%) for ten minutes each. After dehydration, samples were infiltrated using 1:1 EtOH and SPI-Pon 812 (SPI Supplies) for one hour, followed by 100% SPI-Pon 812 for four hours. Pellets were then polymerised at 60ºC for 36 hours, reorientated in embedding moulds and polymerised for a further 36 hours in 100% SPI-Pon 812. Samples were sectioned to gold reflectance using a Reichert Ultracut E Ultramicrotome (Vienna, Austria) and collected on Cu formvar coated grids. Samples were post stained using uranyl acetate (three min), rinsed using dH_2O, stained with lead citrate (five min) and rinsed once again using dH_2O. Once grids were dry, samples were viewed using a Jeol 2100F TEM. Two independent experiments were conducted.

4.3 Results and discussion:

4.3.1 MIC determinations using MABA:

MICs for CMS and CST against H37Ra *M. tb* using 1 x 10^7CFU/ml were determined to be 16mg/L. This result was unexpected. Rather, a higher MIC was expected since a larger inoculum size was used, i.e., 1 x 10^7CFU/ml vs 1 x 10^5CFU/ml. However, small differences in the assay (section 4.2.3 vs section 2.2.3) are the time of incubation and MIC definition. Since section 4.2.3 has a shorter incubation period to determine the MIC and the MIC was determined by eye and not spectrophotometrically; this can explain the lower than expected MIC. Thus, using the same conditions, the effect of CMS and CST on the ultrastructure of *M. tb* within 24 hours could be investigated at 16, 64 and 256mg/L.

4.3.2 Ultrastructure analysis of *M. tb* treated with CMS:

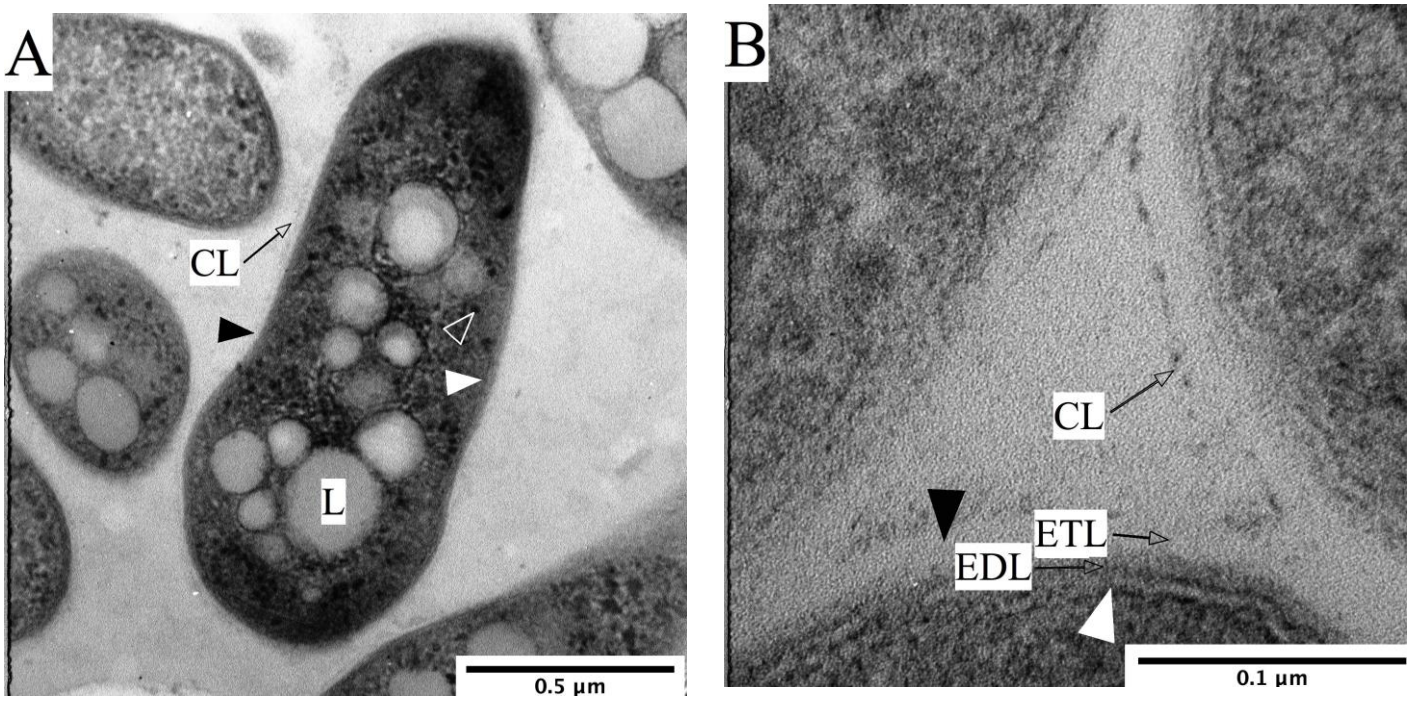


Figure 4.2: Ultrastructure of H37Ra *M. tb* control cells. White arrows display CM, black arrows display MM and CL is also displayed. (A) Lipid bodies (L) are observed within the cell as well as ribosomes (white outline arrow). (B) Magnified area of three cells displaying EDL and ETL.

Figure 4.2 displays the ultrastructure of H37Ra *M. tb* control cells (non-treated). The ultrastructure displays *M. tb* cells with a rigid structure. The CM is ably defined (Figure 4.2 A, B). On the magnified area (Figure 4.2 B), the EDL and ETL can clearly be seen. In both cases, the hypothesised area of the MM is displayed according to the area of the ETL. Ribosomes and lipid bodies are clearly observed in Figure 4.2 A. The lipid bodies are present likely due to the initial incubation on 7H10 agar containing OADC. In both cases, the CL is observed, which appears to be thin, broken and slightly electron translucent in certain areas.

Figure 4.3 displays H37Ra *M. tb* treated with 16mg/L CMS (A, B) and 16mg/L CST (C, D). Firstly, accumulations of membrane-like structures within the cytoplasm were apparent, in addition to mesosomes.

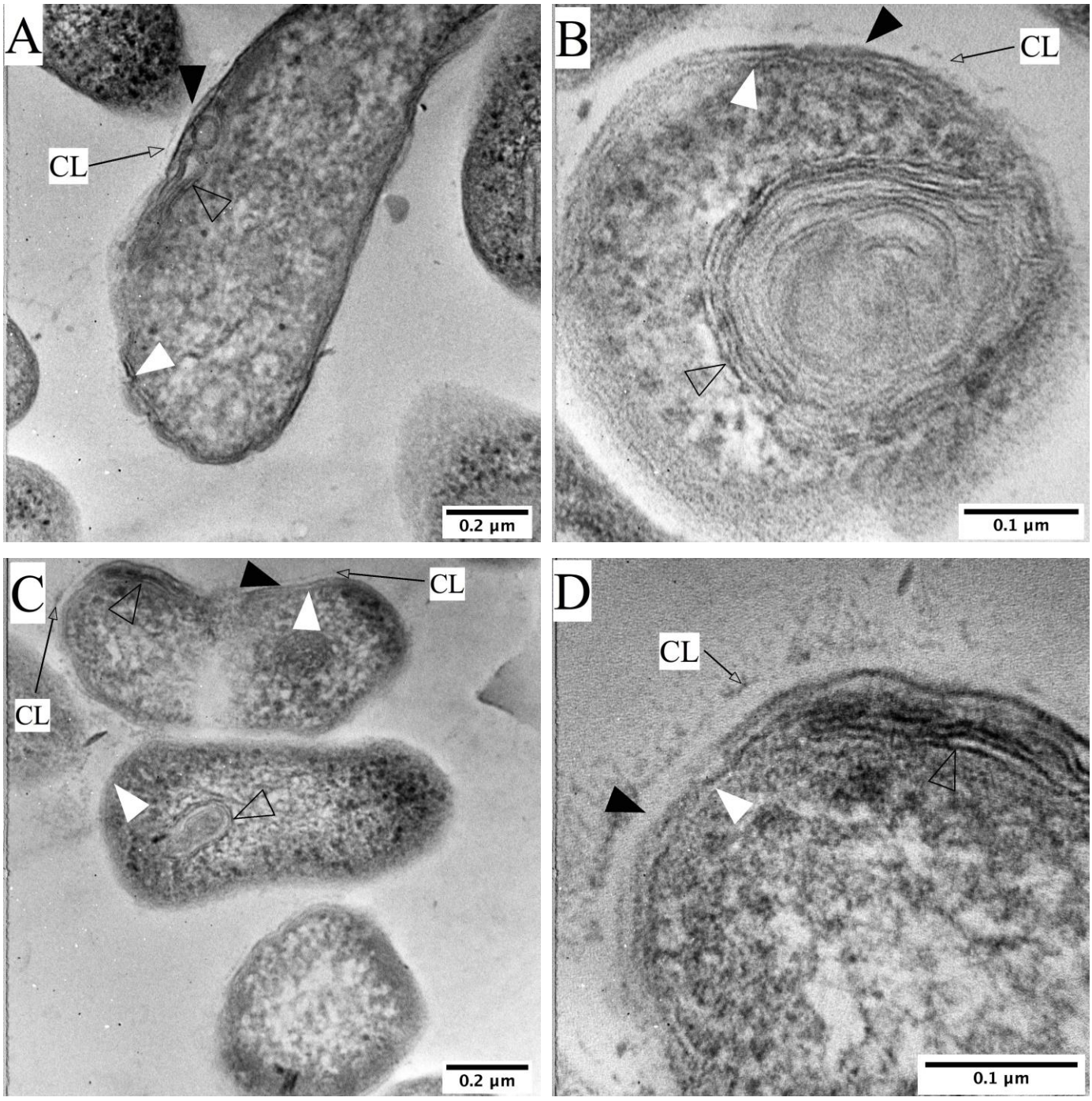


Figure 4.3: Ultrastructure of H37Ra *M. tb* treated with (A, B) CMS 16 mg/L and (C, D) CST 16 mg/L. White arrows display CM, black arrows display MM. The CL is also displayed. Arrows with a black outline display membrane injuries and accumulation of membrane-like structures.

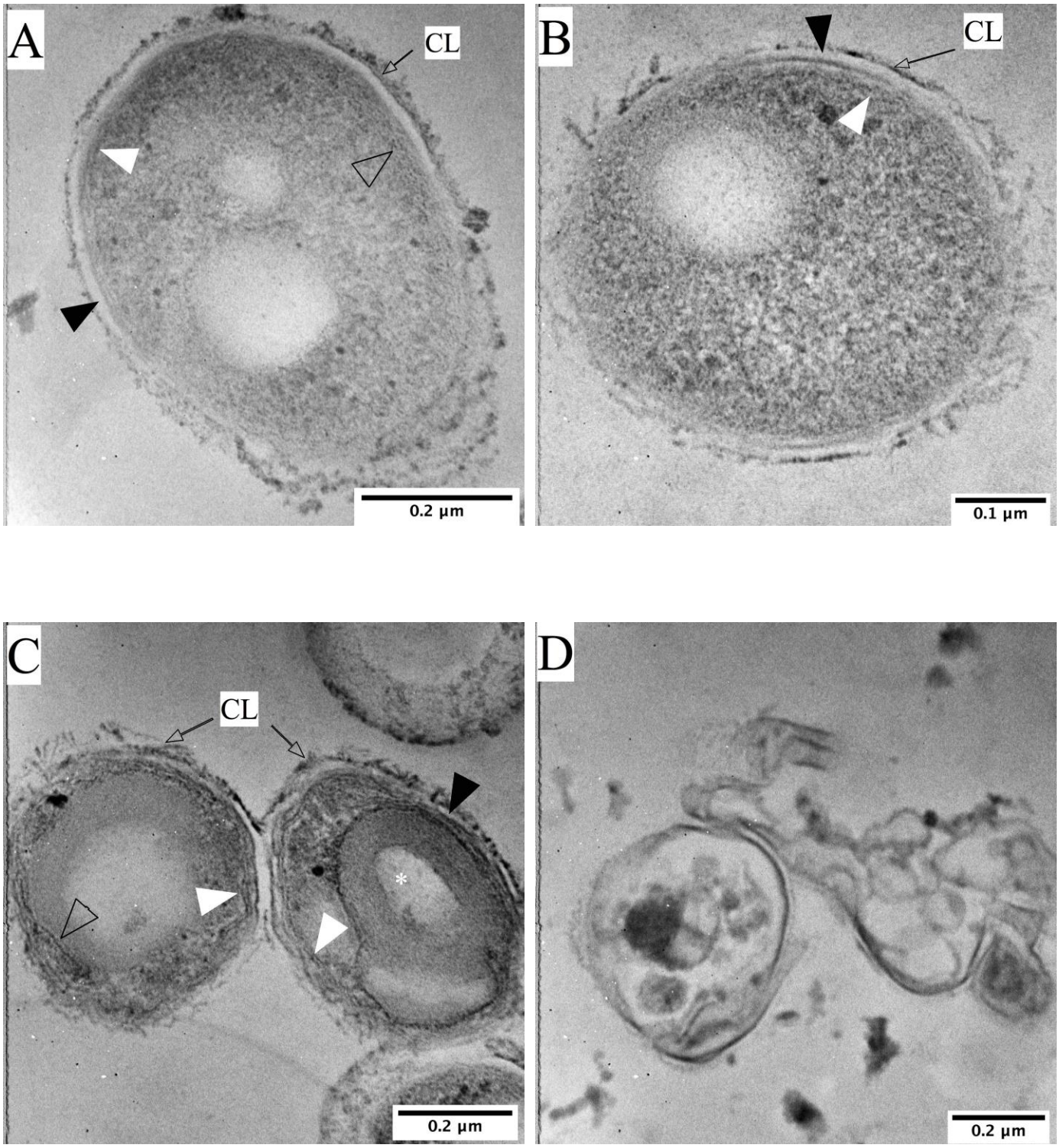


Figure 4.4: Ultrastructure of H37Ra *M. tb* treated with (A, B) CMS 64 mg/L and (C, D) CST 64 mg/L. White arrows display CM, black arrows display MM. The CL is also displayed. Arrows with black outline display membrane injuries and accumulation of membrane-like structures. (C) displays a multilayered membrane structure (*) different to other structures observed.

Along the CM, accumulation of other membrane-like structures were observed in response to a damaged CM. Different staining profiles of the CM were observed, compared to the control. Clearly the CM was observed to be damaged and deformed. For CMS/CST (16mg/L) (Figure 4.3), the CL did not appear to be much different from that of the control (Figure 4.2), but on closer observation, damage could be observed (Figure 4.3 B, D). More obvious in Figure 4.3 was the 'patchy appearance' of the CL, but this was not as apparent for CMS. Likely due to CMS being converted to colistin over a 24 hour period. No apparent damage was determined for the EDL or the ETL, although, in certain instances, the EDL seemed to become more electron dense. Figure 4.4 displays H37Ra *M. tb* treated with 64mg/L CMS (A, B) and 64mg/L CST (C, D). For CMS (64mg/L), the most apparent ultrastructure effect was that of the CL becoming more electron dense, thick and patchy, similar to CST (Figure 4.4 C). In general, for both CMS and CST (64mg/L), *M. tb* was clearly more damaged when compared to 16mg/L and the control.

Accumulation of membrane-like structures were also more abundant, i.e., along the CM and within the cytoplasm of the cells, even though this might not be apparent in Figure 4.4. Concerning CST (64mg/L)(Figure 4.4 C), an additional large multilayered membrane structure was observed. This structure was different to other membrane structures observed. It appeared to have an electron translucent centre and was hypothesised to be a remnant of a lipid body. Interesting, very few lipid bodies were observed when compared to the control from the initial treatment (16mg/L). In addition for CST (64 mg/L)(Figure 4.4 D), a large number of lysed cells were apparent. This was in a larger amount than that of CMS (64mg/L).

Figure 4.5 displays H37Ra *M. tb* treated with 256mg/L CMS (A, B) and 256mg/L CST (C, D). For CMS (Figure 4.5 A), similar effects as CST (64mg/L)(Figure 4.4 C) were observed. The presence of the multilayered membrane structures were observed that were hypothesised to be a remnants of lipid bodies due to an electron translucent centre. Thickening of the CL is very prominent with a 'bud' clearly being displayed. These buds were also observed in certain instances for CST (64mg/L)(not shown). For CMS (Figure 4.5 B) a larger amount of lysed cells were present. For CST (Figure 4.5 C, D), most cells were found to be lysed. Only remnants of membrane-like structures appeared to remain.

The following deductions can be made; (1) there is an increase in CM-like structures as well as mesosomes with an increase in CMS/CST concentrations. (2) An increase of membrane-like structures along the CM are observed with an increase in concentration of CMS/CST. (3) With an increase in concentration, the CL became more electron dense and thicker with a patchy appearance.

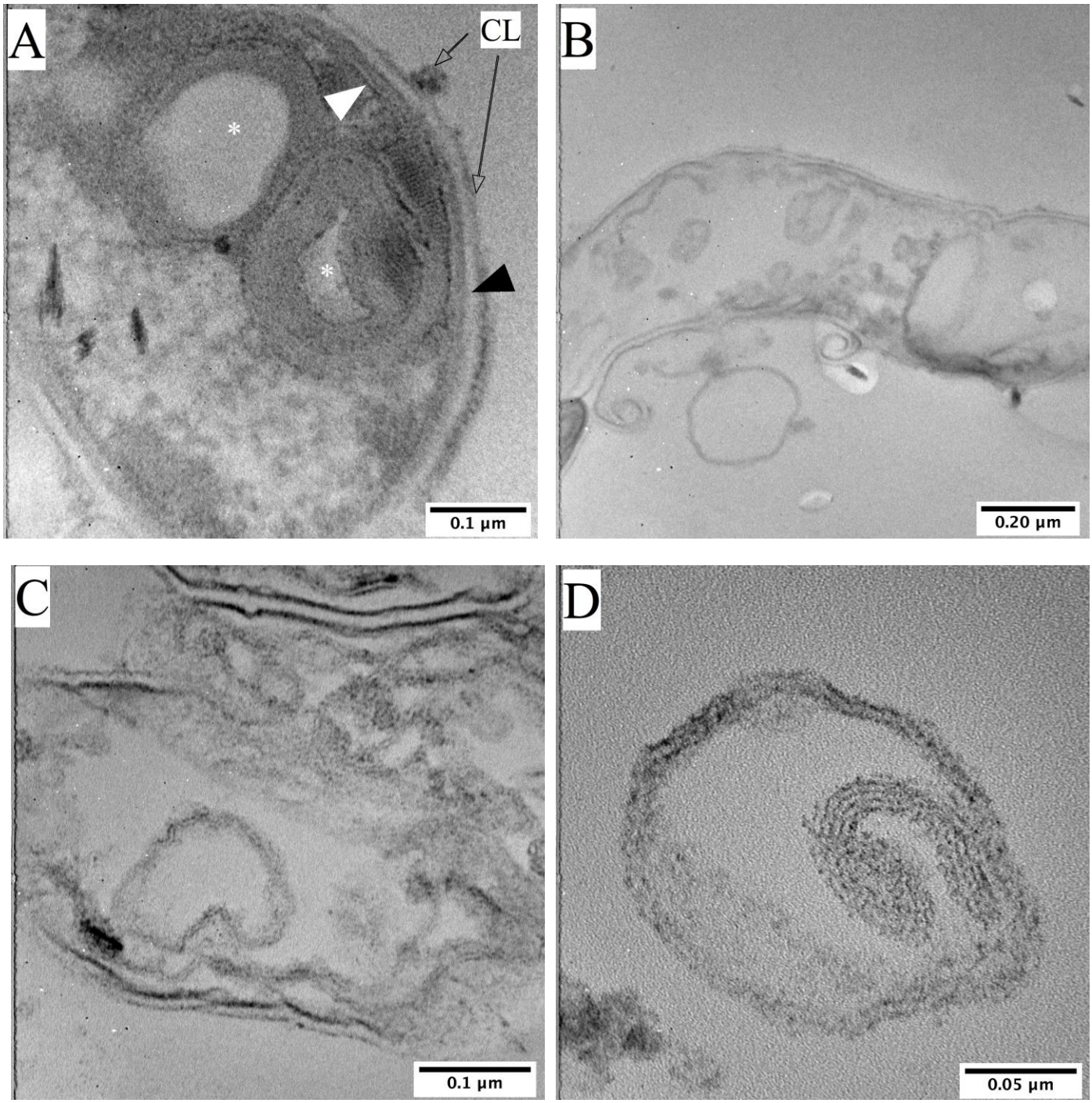


Figure 4.5: Ultrastructure of H37Ra *M. tb* treated with (A, B) CMS 256 mg/L and (C, D) CST 256 mg/L. White arrows display CM, black arrows display MM and CL is also displayed. (A) displays a multilayered membrane structure (*) different to other structures observed. (B-D) displays lysed *M. tb*.

At higher concentrations of CST (64mg/L) and CMS (256mg/L), the CL appeared to have 'buds'. (4) A multilayered membrane structure hypothesised to be a remnant of a lipid body appeared at higher concentrations, i.e., CST (64mg/L) and CMS (256mg/L). (5) At higher concentrations, lysed cells became abundant. Similar results, but not entirely identical were described for bactericidal concentrations of colistin used against *M. aurum*, i.e., CM injuries, patchy appearance of the CL and some cell lysis [David and Rastogi 1985].

For CMS and CST the same effects on the ultrastructure of *M. tb* were observed. The only difference was for CST; some of the effects were more prominent at lower concentrations. This makes sense, as determined in Chapter 3, CMS is in the process of conversion to colistin over a 24 hour period. This also proves that the first 24 hours of colistin formation from CMS is very important for reduction of viable cells, explaining the difference in reduction of viable cells observed for CMS and CST (Chapter 2). Since the same effects were observed, colistin (A and B) are responsible for disruption of the ultrastructure.

Strangely, different results were obtained here for H37Ra *M. tb* compared to David *et al* [1986]. This is likely because the authors were more concerned if the lytic cycle of Mycobacteriophage D29 in *M. tb* (H37Ra) would be affected. The authors did confirm an interaction of colistin with the CM.

It is likely that colistin formed from CMS would first interact with the CL of *M. tb*. This was apparent by the changes in CL observed during this investigation. This was also observed and suggested for *M. aurum* [David and Rastogi 1985]. Concerning section 1.2.3, the CL contains a variety of different types of lipids and polysaccharides [Daffé 2015]. It is likely that colistin from CMS will interact with these via hydrophobic interactions, causing a perturbation in the cell and even extracting these lipids and polysaccahrides. Interestingly, thickening of the CL in *M. tb* has been observed previously in low oxygen tension, i.e., similar to that found in granulomas [Cunningham and Spreadbury 1998]. The authors determined that thickening of the CL was due to a 16-kDa small heat shock protein or α-crystallin homolog. The 16-kDa protein was highly associated with the cell envelope, fibrous peptidoglycan-like structures, and intracellular and peripheral clusters. This thickening was linked to assisting the bacilli survive in hypoxic environments, providing a coat to offer protection in toxic surroundings. Thus, it is highly likely that the same 16-kDa protein is trying to protect *M. tb*, by thickening of the CL when in the presence of colistin. The patchy appearance determined (Figure 4.3 D, Figure 4.4 A, B, C and Figure 4.5 A) suggests that this protective coat is disrupted by colistin.

Once colistin from CMS has interacted with the CL, it is taken up by the self-promoted uptake mechanism as described by Hancock and Chapple [1999]. Colistin from CMS then can interact with the

CM, forming micelles/water channels. It is likely that other components such as complex lipids and MA might be extracted during this process. This is evident by the presence CM injuries observed throughout the various concentrations tested (Figure 4.3-4.5). This has been confirmed for *M. tb* treated with colistin [David *et al* 1986]. All these interactions elicit a response within *M. tb*. This response is an attempt for *M. tb* cells to repair their damaged membranes. This is evident by the production of multilayered membrane-like structures observed within the cell as well as thickening of the CL, to prevent cell lysis. When the concentration of colistin overcomes the repair mechanisms, cell lysis occurs. These multilayered membrane structures are hypothesised to be remnants of lipid bodies present with *M. tb*. The multilayered structures are likely formed to help protect the damaged membranes of *M. tb*. It is also likely that colistin enters the cytoplasm, interacting negatively charged components such as DNA or RNA [Hancock and Chapple 1999].

These observations can explain the enhanced bactericidal activity of CMS + INH observed in Chapter 2 as well as the antagonistic nature of CMS + RIF. INH enters *M. tb* via passive diffusion (section 1.2.5.1) [Bardou *et al* 1998], which is not affected by the MA layer [Jackson *et al* 1999]. Since at physiological pH INH is non-charged [Pinheiro *et al* 2014b] and slightly hydrophilic, the hydrophobic and multilayered structure of the *M. tb* cell wall sufficiently prevents the passive diffusion of INH into the cell. Disruption of the cell wall, i.e., CL and the CM can facilitate an influx of INH into the cell. In addition to this and described by Hancock and Chapple [1999], other small molecules can be taken up by bacteria during self-promoted uptake caused by polymyxins because when colistin interacts with the CM, micelle-like aggregates form that act similar to ‘water channels’ allowing for the transport of hydrophilic compounds, i.e., INH from the surrounding environment. It is thus likely that disruption of the CL and CM together with the self-promoted uptake of colistin allow for an increased uptake of INH into the cytoplasm of *M. tb*. As for RIF [Pinheiro *et al* 2013; Pinheiro *et al* 2014b], due to the hydrophobic and lipophilic nature, RIF can easily cross the hydrophobic membranes of *M. tb*. This is further evident by the MIC for INH and RIF against H37Ra *M.* tb, i.e., 0.064 vs 0.0032mg/L. Thus, increasing the permeability of the mycobacterial membrane will not increase the uptake of a hydrophobic molecule like RIF. This might also explain the antagonism observed; by disrupting the hydrophobic nature of the mycobacterial membrane (CL and CM), RIF cannot effectively enter the cytoplasm of the cell because key hydrophobic components that allow for RIFs uptake have been extracted/disrupted.

4.4 Conclusion:

H7 states: CMS has an observable damaging effect on the CL and CM of *M. tb* that is similar to that of CST. This was clearly observable and thus H7 is accepted.

Considering the evidence obtained from the TEM investigations, it is hypothesised that colistin, formed from CMS interacts with the CL and is then taken up by the cell via the self-promoted uptake mechanism as described by Hancock and Chapple [1999] where colistin forms micelle-like aggregates spanning the CM ('water channels'). During this process, it is also likely that other components such as complex lipids and MA are extracted along the way, making the cell wall less hydrophobic. It is also possible colistin enters the cytoplasm, disrupting DNA and RNA [Hancock and Chapple 1999]. After uptake, there is also response from within the cell to repair this damage. This has been previously observed for *M. tb* and colistin [David *et al* 1986]. The response is a repair mechanism causing the formation of the many membrane-like structures and thickening of the CL, in an attempt to prevent the cell from lysing. The likely source of these membrane-like structures are from the available lipid bodies within *M. tb*. At high concentrations, i.e., > 16mg/L, the amount of colistin damage is irreparable, causing cell lysis. This hypothesis can also explain the enhanced bactericidal activity when using CMS with INH. INH is hydrophilic and can be excluded by the hydrophobic membranes of *M. tb*, not being affected by MA. Disruption of the hydrophobic barrier can allow for an influx of INH, leading to enhanced bactericidal activity. Another possibly is that during the self-promoted uptake, INH has an enhanced uptake from the environment by the cells, especially via the micelle-like 'water channels' that form, leading to an influx within the cell and enhanced bactericidal killing [Hancock and Chapple 1999]. Since RIF is hydrophobic in nature [Pinheiro *et al* 2013; Pinheiro *et al* 2014b] and can easily transition through the hydrophobic mycobacterial cell wall, disruption of the hydrophobic nature would rather exclude RIF, explaining the likely antagonistic nature observed with CMS.

Chapter 5: Concluding discussion

Currently, TB is one of the world's most deadly infectious diseases. During 2013, there were an estimated 480 000 cases of MDR *M. tb*, with an estimated 210 000 deaths [World Health Organisation 2014]. Currently in the late phase of clinical development are ten new or re-purposed anti-TB drugs, with two new drugs being approved for MDR *M. tb* treatment under specific conditions [World Health Organisation 2014]. Investigating the use of 'off the shelf' antimicrobials, that have anti-TB potential and alternative delivery routes, i.e., inhalation, could eventually lead to improved treatment success. One such class of 'off the shelf' antimicrobial is colistin (polymyxin E). The last decade has seen an increase in pulmonary administration of antibiotics for the treatment of respiratory infections. Use of pulmonary administration allows for high local drug concentrations at the site of infection. In addition, other advantages of pulmonary administration include rapid onset, minimising systemic exposure and adverse effects [Yapa *et al* 2013].

PST and CST are described as having amino acids (D and L) arranged to form a cyclic heptapeptide ring, with a tripeptide side chain covalently bound to a fatty acid moiety [Nation and Li 2010]. While PST is administered parenterally as such, CST is administered parenterally in the form of CMS, in which methanesulfonate moieties are attached through covalent bonds to the primary amines present on the amino acids [Nation and Li 2010]. In contrast to PST and CST, CMS is negatively charged at physiological pH, and is readily converted to colistin in microbiological broth [Bergen *et al* 2006], and *in vivo* [Li *et al* 2003; Li *et al* 2005].

Broad antimycobacterial activity for CST has been known since 1986, but the drug has never been used systemically for TB. This is due to high MIC and systemic toxicity concerns. Many publications exist that investigate the effects of polymyxins on Mycobacteria [David *et al* 1986; David and Rastogi 1985; Korycka-Machała *et al* 2001; Malaviya *et al* 2008; Mogi *et al* 2009; Rastogi *et al* 1986b; Rastogi *et al* 1987; Rastogi *et al* 1988]. The most significant publication determined that the MIC and MBC values for CST against *M. tb* were 5mg/L and 50mg/L respectively, as determined by broth dilution and by out-plating appropriate dilutions onto Löwenstein-Jensen media [Rastogi *et al* 1986b]. Considering the fact that the authors [Rastogi *et al* 1986b] focused on CST and its relation to parenteral administration and its systemic toxicity concerns, investigating the less toxic CMS, that can be administered via inhalation, minimising systemic exposure, is a research topic that will add much value.

CST is highly synergistic with RIF against highly-resistant gram-negative MDR organisms [Lee *et al* 2013; Tascini *et al* 2013; Landman *et al* 2008] and CMS is being used as last-line therapies to

treat highly-resistant gram-negative MDR bacterial infections. Treatment occurs often in critical care units [Evans *et al* 1999; Falagas and Kasiakou 2005; Li *et al* 2005; Li *et al* 2006; Zavascki *et al* 2007]. Investigating CMS with INH, RIF or both, against MDR *M. tb* could provide a synergistic interaction, allowing for reduction of resistant INH and RIF MICs back to clinically relevant concentrations, i.e., overcoming the resistance imposed by mutations in *rpoB* and *katG*. With respect to sensitive *M. tb* (H37Ra), synergistic interactions of CMS, INH and RIF could allow for bactericidal concentrations to be achieved at lower concentrations. Unfortunately H37Rv was not included in this study mainly due to unavailability. However, recently, an independent research group using H37Rv and CST reported very similar data as in this thesis [Bax *et al* 2015], supporting our conclusions.

Understanding the chemistry of polymyxins allowed for *in vitro* conditions using Sauton media to be used that minimised the loss of the peptide during investigations. MABA was used to determine the MIC (≥ 90% prevention in Alamar Blue® reduction) of CMS, CST and PST against H37Ra and MDR *M. tb*. MICs were determined to be 16, 16 and 8mg/L for CMS, CST and PST against both H37Ra and MDR *M. tb*. Next, using exactly the same *in vitro* conditions and determining cell viability, the MBC (≥ 99% reduction in viable cells) was determined for H37Ra and MDR *M. tb*. H37Ra *M. tb* had an MBC of 256, 64 and 64mg/L for CMS, CST and PST respectively. MDR *M. tb* was determined to have an MBC of 64, 64 and 128mg/L for CMS, CST and PST. However, these values did not differ statistically from each other, i.e., CMS of 64mg/L for H37Ra *M. tb* was statistically the same as MDR *M. tb* CMS MBC of 64mg/L. This led to the conclusion that polymyxins have a similar mechanism of action against H37Ra and MDR *M. tb*. It was noticed that CST caused a greater reduction of viable cells at lower concentrations compared to CMS (Figure 2.6). Thus, the decision for quantification of colistin formed from CMS being undertaken. H37Ra and MDR *M. tb* were most sensitive to PST with regard to MIC, likely due to inhibiting alternative NADH dehydrogenase and even malate: quinone oxidoreductase [Mogi *et al* 2009] leading to the disruption of the hypoxic or aerobic respiratory chain. This does not apply to bactericidal activity as determined in Chpater 2 and perviously observed against *M. smegmatis* [Mogi *et al* 2009]; polymyxin E has a greater bactericidal effect than polymyxin B.

With regard to MIC and MBC, PST and CST fail as possible drug candidates due to the concentrations being unachievable [Nation and Li 2010]. CMS also may appear as unlikely to be used in the clinical setting due to its high MIC, bacteriostatic action and an MBC/MIC ratio greater than 4 [Nation and Li 2010]. However, CMS has the additional ability of being administered via inhalation. Use of CMS DPI using the Turbospin® for inhalation [Schuster *et al* 2013] was hypothesised to allow for further drug development of CMS against *M. tb*. Using sputum as a representation of local drug concentrations after inhalation of CMS DPI, it was shown that 38% of patients had colistin levels ≥

128mg/L while the remaining patients had levels ≤ 128mg/L [Schuster *et al* 2013]. Thus, if CMS was to be used in this manner in the clinical setting, it was highly important to understand how PS would affect it.

Using the same i*n vitro* conditions and 1mg/ml porcine surfactant, it was determined that CMS was antagonised eight fold (16mg/L vs 128mg/L). Antagonism was likely due to interaction with the various polar lipids present in the porcine surfactant and the hydrophobic proteins, in a similar way that interaction would occur with the lipids in the CM and outer membrane of gram-negative organisms [http://curosurf.com]. It is consequential to note that surfactant concentrations used have been suggested to be 100 times higher than that found in epithelial lining fluid [Schwameis *et al* 2013]. This data also only considers CMS being used as a mono therapy, and thus it was important to understand if any synergistic interaction of CMS, INH and RIF exists. PS was not used in any further investigations due to various reasons, but consideration must be taken that antagonism would take place during inhalation of CMS.

Using a modified checkerboard MABA under the same *in vitro* conditions described, indifference was determined for the combinations of CMS, INH and RIF for both H37Ra and MDR *M. tb*. INH and RIF concentrations for MDR *M. tb* were that of clinically relevant values while the INH and RIF MICs used for H37Ra *M. tb* were the same as those determined during the initial MABA investigation. The determined Σ_{FIC} for all combinations were determined to be 0.5-4 (indifference). Even though indifference was determined, for H37Ra *M. tb*, differences in concentrations of CMS, INH and RIF were observed when used in combination, compared to their determined MICs. It was hypothesised that a difference in kinetic killing may be present. No such trend was observed for MDR *M. tb*, rather the only differences observed were for changes in CMS concentrations. The combinations that produced the lowest Σ_{FIC} were selected to be investigated in a time-kill assay.

A time-kill assay was conducted, once again under the same *in vitro* conditions and viable cells were monitored over six days. A bactericidal effect was observed for CMS (2mg/L) + INH (0.064mg/L) against H37Ra *M. tb*, i.e., a reduction of 3.16 $\log_{10}$ CFU/ml, which was statistically different to the rest of the combinations. Unfortunately no statistical difference in any of the CMS combinations were observed for MDR *M. tb*, i.e., approximately 1.0 $\log_{10}$ CFU/ml reduction was determined for all cases. Thus, the resistance effects of the *rpoB* and *katG* mutations could not be overcome. In support of these observations, Bax *et al* [2015] determined that using CST with INH or amikacin, resulted in bactericidal effects against H37Rv *M. tb*, similar to that reported throughout this thesis. Thus, even though H37Ra and H37Rv have differences, similar results have been observed independently. The authors also proved that addition of CST prevented the emergence of INH or

amikacin resistance. Reported here and by Bax [*et al* 2015], is that colistin displays antagonistic behaviour with RIF against *M. tb*. Strangely, CST is highly synergistic with RIF against gram-negative organisms [Landman *et al* 2008; Lee *et al* 2013; Tascini 2013]. The paradoxical effect of colistin with RIF on *M. tb* compared to the same combination used on gram-negative organism can be explained by a number of factors. Firstly, the *in vitro* test conditions used differ, i.e., not always at physiological pH and generally cation-adjusted Mueller-Hinton broth is used. Secondly, *M. tb* membranes are vastly different to that of membranes of gram-negative organisms. Polymyxins in general may have a higher affinity for membranes of gram-negative organisms than RIF, whereas in the *in vitro* conditions used here, CMS has a higher affinity for RIF, rather than the membranes of *M. tb* (section 2.3.4). Thirdly, membranes of gram-negative organisms are likely effective permeability barriers to RIF and once disrupted, allow for an influx of RIF, whereas this is the opposite for *M. tb.*

It is very important to note that the enhanced bactericidal activity of CMS (2mg/L) + INH (0.064mg/L) against H37Ra *M. tb* is only concerning an *in vitro* environment. Since it is known that CMS is likely to be antagonised during inhalation by PS, it could also be antagonised by other possible factors such as sputum or caseum; thus, the importance of *in vivo* investigations. Furthermore, investigating if this bactericidal activity is observed in *M. tb* infected macrophages are of importance.

With regard to *M. tb* populations with high vs low metabolism (active vs dormant), bactericidal action was only determined for high and not for low metabolic activity. This phenotype of *M. tb* with low metabolic activity is resistant to the bactericidal action of INH and RIF [Wayne and Hayes 1996], i.e., INH and RIF are only effective against actively dividing *M. tb* since their drug targets are readily available (section 1.2.4). The mechanism of action for colistin has more of an indirect approach, i.e., acting on the cellular membrane. It would be expected that regardless of metabolic state, no phenotypic resistance to colistin should be observed. However, Bax *et al* [2015] did observe differences in colistin bactericidal killing for high vs low metabolically active *M. tb* populations; less killing was observed for the low metabolically active group. Thus one could expect lower inhibition/killing for dormant *M. tb.* However, using better *In vitro* and *in vivo M. tb* persister models need to be undertaken to confirm this. It is suggested here that the effect of colistin on high vs low metabolically sensitive and MDR *M. tb* populations would be the same as they share very similar MIC and MBC.

Quantification of colistin formed from CMS under the *in vitro* conditions described over six days was conducted using SPE column derivatisation with FMOC-Cl and UPLC with UV-VIS detection at 265nm. Difference in amount of colistin A and colistin B were determined for CST and CMS, but the overall concentrations of colistin formed did not differ, i.e., 16mg/L. This explained the reasons for similar CMS and CST MICs determined. It was further suggested that CMS is in the

process of conversion to colistin within the first 24 hours of incubation and is likely to contain a mixture of -3 and +3 CMS and colistin species. Difference in reduction of viable *M. tb* was attributed to the conversion of CMS within the first 24 hours of incubation, oscillating between +3 and -3 species. It was concluded that investigating this conversion at the MIC over 24 hours and the effect it would have on the ultrastructure of *M. tb* would be a great advantage as it would also assist in explaining the enhanced bactericidal effect observed with lower CMS concentrations used with INH.

H37Ra and MDR *M. tb* had very similar MICs for CMS and CST, thus H37Ra *M. tb* ultrastructure was investigated, since the similar mechanism of action applies to MDR *M. tb*. MICs for H37Ra *M. tb* were determined at a higher inoculum, i.e., 1 x 10^7CFU/ml using previously described MABA methods [Carroll *et al* 2009]. Using chemical fixation and room temperature dehydration, ultrastructure investigations of the effect CMS has on *M. tb* were conducted. This method was adopted from previously described reports by Rivas-Santiago *et al* [2013a; 2013b]. The following was observed; (1) increase in cytoplasmic membrane-like structures; (2) increase in membrane-like structures along the CM. These are likely due to CM damage (1 and 2); (3) multilayered membrane structures hypothesised to be remnants of lipid bodies within the cell. This was further hypothesised to be a repair mechanism response. (4) Thickening of the CL, becoming more electron dense, patchy and diffuse with 'buds' in certain instances. Similar observations have previously been made for *M. aurum* [David and Rastogi 1985]. This was hypothesised to be due to a 16-kDa protein [Cunningham and Spreadbury 1998] that provides a protective coat for *M. tb* in toxic environments such as granulomas. The patchy and diffuse appearance of the CL was likely due to extraction of lipids and polysaccharides by colistin formed from CMS. At higher concentrations of colistin (> 16mg/L), the repair mechanism cannot overcome the interactions of colistin with the CL and CM and cell lysis occurs. This data can help explain; (1) the observed bactericidal of CMS and INH, and (2) the antagonistic activity of CMS and RIF.

It is hypothesised that the Fenton reaction does not play a role as a mechanism of action against *M. tb*. Gram-negative organisms are sensitive to the Fenton reaction (section 1.3.3.1). Cell death is achieved via oxidative damage producing very low MICs [Sampson *et al* 2012]. *M. tb* has been shown to be very sensitive to the Fenton reaction when vitamin C is used, producing low MICs [Vilchèze *et al* 2013]. Thus, if the Fenton reaction mediated by polymyxins played a role in killing *M. tb*, lower MICs would be expected, i.e., < 1 mM [Vilchèze *et al* 2013]. This is because the Fenton reaction mediated by polymyxins is different to that of vitamin C. Interestingly, when gram-negative organisms are treated with vitamin C, higher MICs are obtained, thus, gram-negative organisms are not sensitive to the Fenton reaction mediated by vitamin C, i.e., > 16 mM in a similar way that *M. tb* is not sensitive to the

Fenton reaction mediated by polymyxins. The difference in how the Fenton reaction is initiated by polymyxins vs vitamin C could explain the observation. For the Fenton reaction to take place by polymyxins, it is likely that an envelope stress response occurs causing aberrant electron transport [Sampson *et al* 2012]. This causes a production of O_2^- (superoxide), converted to H_2O_2, then interaction with Fe^{2+} forms hydroxyl radicals ($OH^\cdot$) causing damage to DNA, lipids etc., [Sampson *et al* 2012]. For the Fenton reaction to take place by vitamin C, Fe^{3+} is firstly reduced to Fe^{2+}, which can then react with O_2, to form O_2^-, O_2^- via dismutation forms H_2O_2. H_2O_2 then reacts with Fe^{2+} to form $OH^\cdot$ which can lead to further membrane disruption, phospholipid alteration, fatty acid oxidation and DNA damage [Vilchèze *et al* 2013]. However, the difference in Fenton reaction sensitivity formed via polymyxins vs vitamin C is just a hypothesis for *M. tb* and confirmation via an *in vitro* study is required.

Discussing the CMS mechanism of action observed against *M. tb* and the synergistic vs antagonistic properties of INH and RIF with CMS, is best done using a schematic diagram (Figure 5.1). Scenario A describes how INH and RIF penetrate the cell wall of *M. tb*. Hydrophilic INH cannot cross the hydrophobic membranes and thus crosses the membrane using passive diffusion through specialist porins such as OmpATb (1). Since RIF is lipophilic, it sufficiently distributes across the cell wall of *M. tb* and thus easily enters the *M. tb* (2). Scenario B describes the possible antagonistic nature of RIF with CMS. Due to RIF being hydrophobic and with possible negative charges, it will interact with CMS/colistin (1) preventing the polymyxin from disrupting the *M. tb* cell wall, i.e., likely the CL (2) [Pinheiro *et al* 2014a]. This explains why higher concentrations of CMS are required with RIF to produce the same level of killing without RIF. Scenario C shows how colistin formed from CMS disrupts the cell wall of *M. tb* (1-4). Colistin likely interacts with the CL (1) and complex lipids within the MM via hydrophobic and covalent interactions. This displacement of glycoplipids/complex lipids etc., can cause cracks in the membrane (2). Via the self-promoted uptake mechanism [Hancock and Chapple 1999], colistin is taken up by the cell (3) until interaction with the CM occurs. Colistin then has the ability to displace phospholipids within the CM, forming ‘water channels’, due to formation of micelle like channels via positive charges and hydrophobic interactions from colistin. In addition, colistin can likely enter the cytoplasm and interact with DNA or RNA. During the entire process, it is possible to hypothesise that hydrophobic components such as MA in the cell wall can be disrupted and extracted, making the entire membrane less hydrophobic. This all allows for an influx of INH through these ‘cracks’ (5) and the micelle ‘water channels’ within the CM (6). This influx and easy access for INH into cell can explain the bactericidal effect observed when used with colistin. Another explanation

for the antagonistic effect observed with RIF is due to this disruption of the membrane, making it more hydrophilic. The lipophilic RIF cannot distribute within the cell wall of *M. tb* and thus is excluded (7). For RIF, the antagonistic nature described in scenario B and C could be due to a combination of these effects. David *et al* [1986] described that colistin observed to interact with the inner leaflet of the CM. This was determined using Thiéry cytochemical staining and observing an asymmetric staining profile. But, it is likely that formation of micelle 'water channels' in the CM will allow for the stain to cross from the outer leaflet and stain the inner leaflet. In addition to this, from these investigations, it can clearly be seen that CMS would likely act synergistically and even have a greater bactericidal effect with other hydrophilic compounds. For MDR *M. tb* treatment, the following drug combinations are used; PZA, a fluoroquinolone, a parenteral agent (kanamycin, amikacin or capreomycin), ethionamide (or prothionamide), and either cycloserine or PAS [Falzon *et al* 2011]. It would be a good idea to investigate the synergistic effect of PZA with CMS, mainly due to PZA being hydrophilic in nature. Possible synergistic interactions of ethionamide (or prothionamide), and either cycloserine or PAS could also be expected. Synergism in this method might lower CMS concentrations low enough for inhalation to be conducted, as observed for CMS + INH in H37Ra *M. tb*. Greater bactericidal effects may also be observed with a fast clearance of the drug-resistant population of bacteria. This motivates the use of CMS in MDR, and it is of importance to further investigate these interactions. It is not suggested that CMS is used with aminoglycosides since adverse events such as nephrotoxicity and ototoxicity might occur.

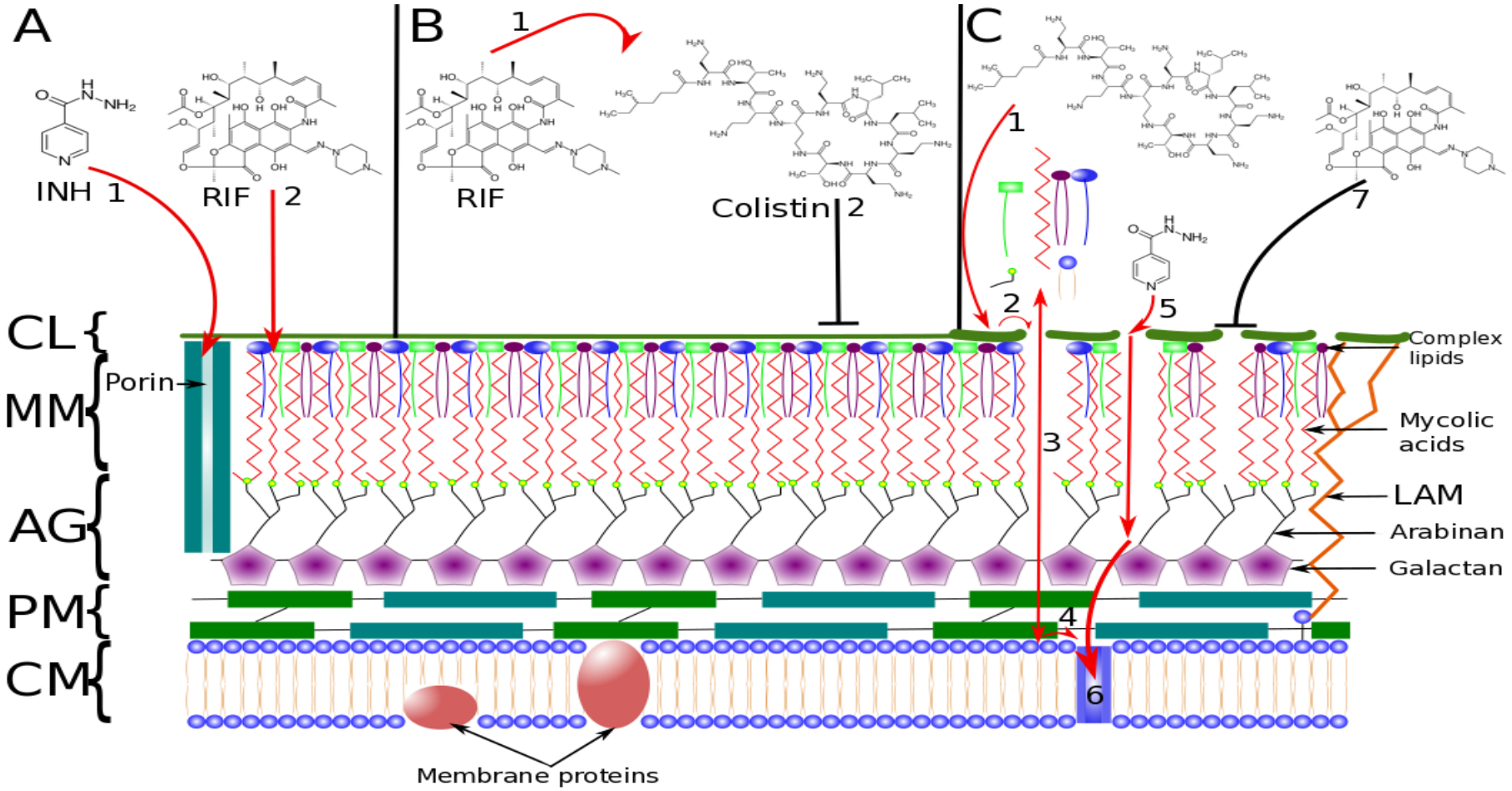


Figure 5.1: Schematic diagram (not to scale) of the effects colistin has on the M. tb membrane. The CM, PM, AG, MM and CL are displayed along with a porin, complex lipids, MA, LAM and AG. (A) INH penetrates the M. tb cell wall via passive diffusion/porins (1). RIF is lipophilic and easily penetrates M. tb cell wall (2). (B) Due to hydrophobic charges and negative charges, RIF interacts with colistin formed from CMS (1), causing antagonism (2). (C) Colistin formed from CMS interacts with CL, causing disruption, resulting in thickening (1) and 'cracks'(2). Due to the self-promoted uptake, colistin crosses the membrane to the CM, likely causing extraction complex lipids, MA and phospholipids causing further 'cracks' (3). Interacting with the CM, extraction of phospholipids likely forms micelle 'water channels' (4). The less hydrophobic membrane allows for influx of hydrophilic INH (5), crossing the CM via the micelle 'water channel' (6). The less hydrophobic membrane prevents lipophilic RIF from crossing the disrupted M. tb membrane (7).

Chapter 6: Future work

The following studies are suggested based on the data obtained in this thesis; (1) investigation of CMS with PZA, ethionamide, cycloserine or PAS against MDR *M. tb*. It is likely to expect synergistic interactions of these drugs due to the effect CMS has on the ultrastructure of *M. tb* and its observed bactericidal effect with INH. (2) Even though Bax *et al* 2015 observed differences of colistin on low vs high metabolic activity, it would still be interesting to investigate with a different model, i.e., investigate the effect CMS would have against *M. tb* in a dormancy model [Deb *et al* 2009]. Interestingly, *M. tb* bacilli that enter a non-replicating persister-like state *in vitro* have been determined to express triacylglycerol synthase associated with being lipid body positive and acid-fast with comparable phenotypes having been found in sputum. It is believed that this *M. tb* phenotype is specific for transmission as it will improve the survival of the bacilli [Garton *et al* 2008]. *M. tb* bacilli were observed to develop a non-replicating persister-like state with a loss of acid-fastness (which returns upon resuscitation) and accumulation of triacylglycerol and waxy ester as well as phenotypic resistance; all the major characteristics of dormancy. Triacylglycerol accumulation has been hypothesised to be used as an energy source by *M. tb* during the disease [Deb *et al* 2009]. It is likely that during this, non-replicating persister-like state that *M. tb* bacilli obtain nutrients, lipids and triacylglycerol from foamy macrophages accumulating lipid droplets in its own cytoplasm. These lipid inclusions are believed to be mainly composed of triacylglycerol and not cholesterol and could also play a role as an energy source during resuscitation [Neyrolles 2014]. It is well known that *M. tb* accumulates storage lipids such as triacylglycerol during dormancy and has been hypothesised to prepare the cells for transmission when in sputum [Daniel *et al* 2011; Garton *et al* 2008]. Thus, since CMS can achieve very high concentrations in sputum, it would be interesting to monitor how CMS changes these bacteria in sputum with a combination of Nile red and Auromine O staining. CMS could likely prevent transmission of this population of bacteria in sputum by disrupting triacylglycerol. (3) Conduct a transmission study using guinea pigs as a model to detect transmission at the AIR facility in Witbank, South Africa. Reduction of transmission is likely, especially if patients use CMS DPI with Turbospin®. It would be important to monitor sputum for a reduction in acid-fast positive to acid-fast negative bacilli. Colistin sputum levels could also be monitored and a possible change in the presence of bacilli in sputum, which could be linked to a reduction in transmission and thus fewer infected guinea pigs. (4) Investigations using CMS with INH in a population of mono-resistant (RIF resistant) *M. tb* bacilli would be beneficial and eventually could be used in a clinical trial for patients being only RIF resistant. (5) It would be interesting to study macrophages infected with *M. tb* to observe if any

killing occurs *in vitro*. Since colistin is antagonised by PS, it would be interesting to determine if colistin would access and inhibit *M. tb* within macrophages. Since Rastogi *et al* [1988] observed that colistin did not increase the β-lactam (amoxicillin, carbenicillin, clavulanic acid) susceptibly of *M. avium in vitro* or intracellularly-multiplying bacilli (in J-774 macrophages), it should be investigated with regard to CMS and INH.

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Appendix A

Appendix B