

Chapter 4: Conclusion

Tuberculosis can be classified as a new disease since HIV/TB co-infection became such a prominent problem in especially third world countries [1]. South Africa currently has the highest incidence of TB per 100 000 (358 per 100 000) people in the world. In 2007 alone 112 000 people died of TB in South Africa, of which 94 000 (72%) were co-infected with HIV [1]. Sputum smear microscopy for detection of acid fast bacilli is still the primary tool for diagnosis of TB especially in resource poor countries [1]. This detection method has low sensitivity particularly in an HIV burdened population [22]. A need for a more rapid diagnostic test is highly desirable [1]. Studies from a gold mine community in South Africa showed that due to the diagnosis delay and inappropriate therapy, drug resistance and disease transmission increased in the HIV co-infected population [9]. Standard first line chemotherapy is not effective for individuals that have MDR-TB and there is practically no cure for XDR-TB. The failure to complete lengthy drug regimens and the pathogens becoming resistant to especially the two first line drugs INH and RIF through mutations are increasingly detected in persons who have been previously treated for TB [16, 18-20]. The approach to control this disease now is to discover new chemotherapies effective against *M.tb*, as well as to enhance the potential of existing drugs to treat MDR-TB [21].

As discussed in chapter 1, effective chemotherapies do exist for TB treatment for more than 50 years but due to the persistence of the bacteria in the human host for several months despite treatment, curing patients becomes quite a challenge. Failure to complete therapy due to non compliance is associated with increased infectious time, relapse and drug resistance [19]. Due to the treatment period for drug susceptible TB of up to 9 months, toxic side effects of the combination therapy asks for special consideration, such that the decision for treatment cannot be taken lightly.

Another drawback of the combination therapy against TB is the low solubility of the drugs, their short half life and rapid clearance from the biological system [16]. One approach in development of more efficient anti-TB drugs is to nanoencapsulate the drugs and to design systems capable of targeting the drug to the site of infection at relatively low drug concentrations in the blood to prevent systemic toxic side effects. There are different approaches that could be followed in order to focus the drug towards its target. The way our group decided on was to investigate targeted nanotechnology based drug delivery systems with slow release capabilities. The long term aim is to reduce the concentrations and frequency of dosing, hoping also for a reduced treatment period earned from more efficient and compliant treatment.

In developing countries patient non-compliance turned out to be a major cause of treatment failure and the development of drug resistance [16]. To address this particular issue a DOTs (Directly Observed Treatment) programme was introduced by the WHO, where a trained person directly observes the medication being swallowed daily by the TB patient [10]. This programme is human resource intensive. The cost of this service could be reduced by developing a nano-encapsulated anti-TB drug delivery vehicle, which can be taken weekly instead of daily due to the slow release properties of the nanoparticles.

In the TB strategic plan for 2007 to 2011, as set out by the South African Department of Health, the role that the scientific community should play is in the development of new drugs, diagnostics and vaccines for the prevention and control of TB. The CSIR (Council for Scientific and Industrial Research) embarked on a national health strategy since 2008, which includes research towards the development of novel solutions and pharmaceutical products that could treat diseases such as malaria, HIV/AIDS, and tuberculosis. The CSIR annual report of 2008/2009 announced our group's contribution to this strategy in focusing on slow-release nanomedicines against tuberculosis. Instead of new therapeutic compounds, our group focused on improved uptake and delivery of existing drugs by exploring the practical feasibility

of encapsulating drugs into PLGA nanoparticles. In addition, this study focused on MA as targeting ligand to be included in the nanoparticle together with the anti-TB drugs. The product's toxicity to and efficiency of uptake in host macrophages was investigated, to prepare for animal studies and eventual clinical trials in humans.

Nanotechnology can be used to overcome the main technological obstacles of therapeutic agents. By making use of polymeric nanoparticles such as PLGA based nanoparticles, the efficacy of the drugs could be enhanced by targeting the molecules to the site of infection and thereby reducing the dose needed for treatment [16]. Because the degradability of the nanoparticles could be controlled, the advantage of slow release of the therapeutics could be achieved [37]. As described in chapter 1, there is a wide array of nano-carrier vehicles to choose from. PLGA was the polymer of choice mainly because of its non-immunogenic properties, biodegradability and having the capacity to encapsulate hydrophobic and hydrophilic agents [34, 54, 55]. An additional advantage that PLGA is reported to have over other drug delivery vehicles is that the particles are selectively taken up into macrophages and dendritic cells [61], the main target for infection of *M. tuberculosis* [32, 51], [45, 172]. The results shown in literature, as mentioned above for PLGA polymers as carrier vehicle, support sustained release and the possibility of enhanced uptake in the macrophages without the use of targeting ligands. Several literature references show research done on PLGA nanocarriers in the field of TB. One such group is lead by G.K. Khuller, who showed that PLGA drug carrier microparticles released antitubercular INH and RIF over several days [205]. The frequency of dose administration could also be reduced by making use of carrier systems. In a guinea pig model it was shown that the treatment frequency could be reduced 9-fold by encapsulating RIF or INH into PLGA nanoparticles due to the sustained release of the drugs from the carrier vehicle [206]. Another laboratory showed that PLGA particles containing RIF and mannitol by using a four-fluid nozzle spray drying technique, led to improved uptake by alveolar macrophages in rats [69].

The discovery of the structural relationship between cholesterol and MA in our group [121] provided an excellent opportunity for specific drug targeting. The initial observation was made in an ELISA assay to detect antibodies to MA in human patient sera. Cross-reactivity between cholesterol and MA by antibodies from tuberculosis patients was so strong that the cholesterol could equally well suit as a substitute for MA as antigen in the ELISA, without affecting the outcome significantly [118]. Thereafter the cholesterol nature of MA was more directly demonstrated by showing the strong interaction between MA and Amphotericin B - an antifungal macrolide agent known for its binding to cholesterol [162] - on a wave-guide evanescent field biosensor system. The structural specificity of this attraction was demonstrated by showing that the methylester of MA was unable to bind Amphotericin B [121]. The same principle was confirmed with a surface plasmon resonance biosensor in a follow-up study [117], in which it was shown what the effect of chemical modification of MA would be on the manifestation of its cholesterol nature, i.e. its propensity for binding to Amphotericin B. Mycolic acids were shown to attract cholesterol from liposomes in an evanescent field biosensor [121]. In another study by a member of our group, evidence of a more circumstantial nature was found: Cholesterol was shown to hinder the efficacy of INH to eradicate *M.tb* in radiometric culture experiments [208]. One important target of INH is the enzyme called InhA, which catalyzes the final stage of the mycolic acid biosynthesis pathway. This enzyme is known to be related to the steroid dehydrogenase family of enzymes, suggesting the possibility that cholesterol could compete with the synthesized MA for binding to the substrate binding site of the enzyme, thereby competing with INH for binding into the active centre. Competitive inhibition between MA and cholesterol for binding to an enzyme's active site would further support the notion of a structural mimicry between MA and cholesterol.

Mycobacterial MAs are immunogenic. Stimulation of human CD4⁺, CD8⁺ T-lymphocytes to proliferate is done by MA upon CD1b presentation [116, 209]. Anti-MA antibodies generally occur in human TB positive patient serum [119, 137]. In one targeting scenario, MA incorporated into nanoparticles may interact with the anti-

mycolic acid antibodies that are anticipated to be present in higher concentrations at the infected areas. In this way, targeting may be achieved by an accumulation of the nanoparticles in immune complexes at the site of infection.

Furthermore, free MA is likely to play a role in the drug tolerant persistence of *M.tb* infection in the extracellular caseous lesions of a tubercle [141, 142]. Free MA has been reported to being pumped out of the bacilli for the specific purpose to form a biofilm. It cannot be excluded that mycolic acids simultaneously fulfils the role of attracting cholesterol to the biofilm as a carbon source for the slow growing, persistent mycobacteria. Cholesterol has been shown to be present in foamy macrophages surrounding the granulomas [14, 126] and can accumulate around the bacteria [148]. In this way, it is anticipated that foci of high cholesterol concentration is associated with late stage persistent TB infection in the lungs and other affected organs. Therefore, in another targeting scenario, the MA incorporated into nanoparticles could also serve as a ligand for cholesterol-rich areas, due to the cholesterol nature of MA and the fact that MA is attracted to cholesterol [121].

Cholesterol is also immunogenic, but only at high concentrations, when their homogenous existence with phospholipids is no longer possible and cholesterol separates out to form islands of high cholesterol concentration in liposomes or membranes. Such foci of high cholesterol concentration in natural eukaryotic cell membranes are known as lipid rafts. At lower than 40% cholesterol concentration in the liposomes, antibodies tend not to locate the cholesterol as it is hidden in the phospholipids [166, 167, 210]. Monoclonal IgG and IgM antibodies to cholesterol have been developed by immunizing animals with high cholesterol concentrations in liposomes [166, 167]. Such antibodies have the ability to recognize and bind to areas of high cholesterol concentration, such as the lipid rafts of immune cells and even lipoproteins [166]. Rather than binding to single cholesterol molecules, these antibodies tend to bind to cholesterol clusters [170]. Antibodies to cholesterol have been reported to exist in all human beings, but their role in the body has not been

unequivocally determined to date. Proposals of the auto-antibodies to cholesterol playing a role in activation of complement and/ or the phagocytic system in order to remove dead cells and viruses to assist in pathological conditions seems very likely [166]. Antibodies to cholesterol become elevated during HIV-progression to AIDS, but decrease when the patient is put on anti-retrovirals. As with TB, HIV also relies on cholesterol for infection of host cells. Measles and influenza viruses are other examples where cholesterol plays a role in the progression of disease: they rely on lipid rafts for budding [210]. It could well be that the dependence on cholesterol for pathogenicity increases the immunogenicity of host cholesterol. In all these cases, targeting of nano-encapsulated drugs to cholesterol-rich foci may be beneficial for treatment. MA may therefore be a useful targeting agent in more applications than TB.

From the discussions above the specific role that anti-cholesterol and anti-MA antibodies play in TB infected individuals may be contemplated. It was reported that anti-cholesterol antibodies bind to human lipoproteins with a possible role in opsonisation and removal by scavenger macrophages [211]. From the observation that persons infected with TB have hypocholesterolemia [212], it may be concurred that anti-cholesterol antibodies may aid in the uptake of cholesterol containing lipoproteins in macrophages. A question that could be investigated in the future is whether the anti-MA antibodies assist in this process. This could be a possible mechanism in which the *M.tb* bacteria use the host in order to accumulate cholesterol in the macrophages that it infected. Thus, in order to understand the strategy of MA targeting, it may be necessary to take a look into how the pathogen uses the lipid metabolism to create a cholesterol rich environment that enables the survival of *M. tb* in the persistent state.

Cholesterol was recently shown to play a very important role in *M.tb* infection as it can be utilised as a carbon and energy source for the mycobacteria. The bacteria need to adapt to their nutrient environment during the cycle of infection, persistence and

spread. In nutrient poor conditions the mycobacteria are able to convert their nutrient dependence towards the use of mainly cholesterol [125, 148, 155]. Another benefit to the pathogen that is gained from cholesterol, is its incorporation into the free lipid zone of the cell wall of *M.tb*, to affect cell wall permeability. In this way, uptake of antagonist compounds such as RIF is inhibited [148]. This may especially be important to gain a competitive advantage over other antibiotic-producing microorganisms that may compete for infecting the ailing host.

Lipids from the host play an important role in mycobacterial pathogenesis. In one study it was shown that when the *M.tb* infection reached the chronic phase, the bacteria produce intracellular lipophilic inclusions [85]. Bacteria in general and *M.tb* in particular, make use of the complexity of the host lipidome in order to escape the immune system [213]. In the case of *M.tb* a number of genes code for production of enzymes involved in lipogenesis and lipolysis [149]. The lipids of the host are not only used as a source of carbon but are also manipulated to enable the survival and replication of the bacteria. Inhibition of phagosome lysosome formation is an important mechanism that *M.tb* uses to be able to survive within the macrophages. Various mechanisms have been identified that contribute to the inhibition of phagolysosome formation that have been described in a recent review of Van der Meer-Jansen *et al.* [213]. The feature that interested our group was where host cholesterol plays a role in the virulence of the bacteria.

Cholesterol could influence the interaction between the pathogen and its host in the membrane-trafficking pathways. Host cholesterol mediates the binding of TACO, an actin binding protein [214], on live mycobacteria-containing phagosomes in order to prevent lysosomal degradation of the mycobacteria [81, 96, 154]. TACO is not only retained by cholesterol from the host but is also actively retained on the phagosomes by the mycobacterial protein, coronin interacting protein (CIP)50 or later identified as lipoamide dehydrogenase C (LpdC) [215]. LpdC is the E3 component of the pyruvate dehydrogenase complex. Cholesterol depletion of the phagosome membrane inhibited

the TACO – LpdC interaction showing the cholesterol dependence of the association [215]. TACO is responsible for activating the Ca^{2+} dependent phosphatase calcineurin. This leads to the inhibition of lysosomal delivery [90, 213, 216]. In previous studies it was shown that upon cholesterol depletion of pre-existing phagosomes, the close apposition between the phagosome membrane and the mycobacterial surface is loosened and fusion with lysosomes occur [96]. Thus from the observations from literature it can be expected that cholesterol will not only concentrate around the granulomas of the infected macrophages but also within the macrophages on the phagosome membrane of active *M.tb* [14, 80, 81, 96, 126, 151]. Cholesterol was also shown to accumulate in the free lipid zone of the cell walls of the bacteria and that the accumulation affected cell wall permeability [148]. Therefore there is an accumulation of cholesterol from the outside of the granuloma to the inside of the macrophage, up to the bacterial cell wall.

In this study it was shown that no individual synthetic MA subclass was responsible for the cholesterol nature of the MA. All the antigenic synthetic MA subclasses contribute to the cholesterol nature, whether tested separately or as a mixture as it exists in the natural MA. The importance of antigenic configuration of MA, stabilized through hydrogen bonding at the carboxylic acid moiety of the MA, was indicated by the effect that carboxylic acid modification had on the biological activity of the MA molecule. Furthermore a structure-function relationship between the stereochemistry and the antigenicity/cholesterol nature of the synthetic mycolic acids were observed. Recent ELISA evidence from monoclonal antibody scFv fragments generated from a chicken antibody gene library against MA's indicated that one antibody cross-reacted with cholesterol and the other not, indicating a mixture of anti-cholesterol and anti-MA antibodies in human patient serum or a single antibody with cross-reactive specificity [217].

In this thesis, the structure of synthetic mycolic acids in relation to their antigenicity in tuberculosis was determined. The attraction of MA to cholesterol might be due to their hydrophobic nature (Van der Waals forces) or through a more specific hydrogen

bonding by the structural features of both molecules [121]. Keto MA exhibited a W-shaped configuration with exceptional rigidity in Langmuir monolayers, whereas methoxy- and alpha-MA exhibited a more flexible conformation towards variation of experimental parameters [102, 103]. Therefore the packing of MA appears to be influenced by the orientation of the functional groups. MA appears to assume different conformations for interaction with antibodies in sera. The results obtained showed that the methoxy MA of *M.tb* had the strongest functional antigenicity. This property is so specific, that even the stereochemistry on the functional groups on the mero chain influenced the binding activity. However, the functional groups or their chemical modifications on the distal position did not hold the key to differentiate between antigenicity or the cholesterol nature of MA. TB negative serum also bound best to that synthetic MA homologue that was recognised best by TB positive patient serum. If the antibody cross-reactivity to cholesterol [118] is taken into consideration, the argument can be made that the methoxy MA also has the highest cholesterol cross-reactivity. This may imply that methoxy MA may also be the strongest cholesterol attractor. It is likely that a surface created by packed mycolic acids is likely to be the structure that is recognized by antibodies, similar to the case of monoclonal antibody recognition of cholesterol [170]. MA subclasses are present in mixtures in *M.tb* and it may well be that different types and variations of MA subclasses form part of an overall structure, such that the natural mycolic acid antigen(s) may never be re-made synthetically by the use of a single species of pure synthetic mycolic acid homologue.

The contribution that our research is able to make is to specifically target PLGA nano-encapsulated anti-TB drugs to TB infected macrophages. The uptake of drugs by infected macrophages would not only be enhanced by the synthetic PLGA polymer carrier vehicle but also by the presence of MA that may facilitate opsonisation of the nanoparticles for macrophage uptake mainly at the site of MA antigen production. In addition, the cholesterol nature of MA and its ability to bind cholesterol could also cause a physical attraction to the cholesterol rich sites of persistent *M.tb* infection.

The objective in this study was to determine the feasibility of encapsulating anti-TB chemotherapies into nanoparticles together with MA that will be taken up by macrophages with little or no toxicity to the host. Successful encapsulation of 14% INH together with MA was achieved with the double emulsion-solvent evaporation freeze drying technique used, resulting in a negative zeta potential that may prevent unwanted agglomeration [32, 33]. The percentages of the INH and MA inside of the nanoparticles were not optimised for the purpose of this study. It was shown that the particles were taken up into macrophages with little toxicity to the cells. The results reported here corroborate those of others who have demonstrated the uptake of various formulations of PLGA drug carriers by macrophage cell lines [32, 186, 199].

Endocytic traffic studies indicated that the endpoint of the MA containing nanoparticles was not the lysosomes, but it is not yet confirmed whether the particles actually end up in the cytoplasm or not. We speculate that the particles may probably escape into the cytoplasm, in accordance with a report that showed that endo-lysosomal escape of PLGA nanoparticles was possible by a mechanism of surface cationization [29]. It would be favourable for our particles to reside in the cytoplasm and not be degraded in the lysosomes, as the nanoparticle should preferably be slowly degraded to result in a sustained therapeutic effect. Additional experiments are warranted to further elucidate particle trafficking. For instance, it would be interesting to learn if the MA present in the cytoplasm of the macrophage could interfere with the LpdC–TACO association. With MA and cholesterol having an affinity to one another, they could compete for binding to the LpdC protein that may affect the way that phagolysosome formation is avoided.

The BACTEC studies that were conducted, aimed at determining the efficiency of the nanoencapsulated INH to kill and suppress the growth of *M.tb* in the infected macrophage. It also determined how this may be influenced by the simultaneous inclusion of MA in the nanocapsules. Targeting efficiency of the MA was not tested, as the required physiological conditions were not observed *in vitro*. These include the

presence of antibodies to MA and cholesterol in the patient, as well as the establishment of cholesterol-rich foci around tubercles in the persistent phase of tuberculosis. Experiments to determine the efficiency of the MA as targeting ligand fell beyond the scope of this thesis at this stage. The experimental work was done to illustrate the plausibility to encapsulate the MA into the nanoparticles without negatively affecting uptake or toxicity of the particles. It prepares the way for an investigation to determine whether targeting of the anti-TB drug containing nanoparticles to the sites of infection can be effected with MA inclusion.

In this study the cholesteroid nature of MA was confirmed. It was demonstrated how this related to the fine structure of the MA and how dependent the cholesteroid nature of MA was to structural changes of natural mixed MA. This was done by measuring its recognition by Amphotericin B after chemical modification. In particular, it demonstrated that MA could be made fluorescent by esterification to fluorescein via the MA carboxylic acid, without significantly changing its cholesteroid nature. This prepares the way to use fluorescein labelled MA in extracellular and intracellular trafficking studies in future. In addition, the structure of synthetic mycolic acids in relation to their antigenicity in tuberculosis was determined showing that methoxy MA associated the most with antibodies in both TB positive and TB negative patient serum. MA containing nanoparticles were synthesized and assembled successfully and were shown *in vitro* to be taken up in macrophage cell lines, without the MA hindering the uptake of the particles. The toxicity of the MA containing nanoparticles was only slightly worse than that of the nanoparticles alone. This investigation into the feasibility of forming MA containing nanoparticles therefore paves the way for testing MA as a ligand to target anti-TB drugs to mycobacterially infected macrophages in human TB patients.

This MA project forms part of a collaborative programme between the CSIR and the University of Pretoria. Animal studies investigating the immunogenicity of MA and the nanoparticle carrier compared to other carrier vehicles for MA is underway. The

contribution that this programme makes in the larger health strategy of the CSIR is in the investigation of MA and its ability to be used as a ligand for targeted drug delivery. Continuation of this project aims to address the issue of persistence of TB infection that is the major cause of long term therapy. When properly understood, the ideal of shortening the anti-TB therapy regime may eventually be realised. It is not excluded that MA may play a crucial role in the persistence of TB, the understanding of which may provide new direction towards efficient TB therapy by making use of targeted drug delivery.