Development of Liposome Formulations of Ethambutol, Rifampicin, and Isoniazid for Pulmonary Treatment of Tuberculosis

by

Theresa M. Wiens

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Experimental Medicine Program, Department of Medicine

We accept this thesis as conforming

To the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

June 2003

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Department of Medicine, Experimental Medicine Program

The University of British Columbia
Vancouver, Canada

Date Sept 30/03

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ABSTRACT

Current tuberculosis treatment consists of rifampicin, isoniazid, ethambutol, and pyrazinamide taken orally over a 6 to 12 month period. Although successful when followed correctly, the drugs are relatively toxic, and drug resistance develops when treatment is not followed to completion. Simplification of this protocol, resulting in decreased duration and/or dosage, has been identified as a goal for new TB therapies by the Global Alliance for TB Drug Development and would be a significant improvement in tuberculosis therapy, leading to increased compliance and disease eradication.

We have developed liposomal formulations of three first-line anti-tuberculosis drugs, isoniazid, rifampicin, and ethambutol, which are suitable for pulmonary delivery, with the goal of improving the pharmacokinetics of each drug such that concentrations will be maintained at the disease site for prolonged periods. All three drugs have been formulated using a remote loading method into sphingomyelin/cholesterol (SM/CHOL) (63/37, mol/mol) liposomes. Although isoniazid and rifampicin have been formulated into liposomes previously, this lipid composition and loading technique has not been utilized for these drugs. In addition, no ethambutol formulation or a formulation combining two drugs, such as the isoniazid and ethambutol formulation developed in this thesis, has been described previously.

Each formulation has been optimized in terms of drug/lipid ratio, buffering capacity, and loading kinetics and is capable of encapsulating 76-100% of available drug. Characterization of these formulations in vitro showed prolonged release
kinetics, suggesting that drug pharmacokinetics can be altered \textit{in vivo}. Stability studies also indicate that each formulation is stable over 2 months at 5°C.

Our hypothesis is that liposome encapsulation of isoniazid, ethambutol, and rifampicin by remote loading will maintain efficacy \textit{in vitro} against Mycobacteria. \textit{In vitro} susceptibility testing against \textit{M. bovis} Bacille Calmette-Guérin (BCG), as a model organism for \textit{Mycobacterium tuberculosis}, showed equivalent activity of each liposome formulation in comparison to the free drug in culture and in a THP-1 human macrophage infection model. Fluorescent microscopy also showed that the liposomes are taken up by infected macrophages, although they do not localize to the same compartment as \textit{M. bovis} BCG.

Our opinion is that significant improvements in the therapeutic profile of first-line agents can be achieved by optimizing drug delivery to the disease site. The results presented in this thesis suggest that all four liposome formulations have the potential to be administered by pulmonary delivery, providing an alternative approach to the treatment of tuberculosis. The potential for altered pharmacokinetics \textit{in vivo} will also permit decreased dosing and frequency of administration, resulting in improved compliance and ultimately decreasing the development of resistance, increasing eradication and decreasing relapse rates.
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABELCET™</td>
<td>liposomal amphotericin B</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>Ambisome™</td>
<td>liposomal amphotericin B</td>
</tr>
<tr>
<td>API</td>
<td>active pharmaceutical ingredient</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>BCG</td>
<td>M. bovis Bacille Calmette-Guérin</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CFU</td>
<td>colony-forming units</td>
</tr>
<tr>
<td>CHOL</td>
<td>cholesterol</td>
</tr>
<tr>
<td>CHS</td>
<td>cholesterol sulfate</td>
</tr>
<tr>
<td>CL</td>
<td>clearance rate</td>
</tr>
<tr>
<td>Cmax</td>
<td>concentration maximum in serum</td>
</tr>
<tr>
<td>CMI</td>
<td>cell-mediated immunity</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>DaunoXome™</td>
<td>liposomal daunorubicin</td>
</tr>
<tr>
<td>DLPC</td>
<td>1,2-dilauroyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DMPG</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine</td>
</tr>
<tr>
<td>DOTS</td>
<td>directly observed therapy (short-course)</td>
</tr>
<tr>
<td>DOXIL™</td>
<td>liposomal doxorubicin</td>
</tr>
<tr>
<td>DTH</td>
<td>delayed-type hypersensitivity</td>
</tr>
<tr>
<td>ELSID</td>
<td>evaporative light scattering detector</td>
</tr>
<tr>
<td>EMB</td>
<td>ethambutol</td>
</tr>
<tr>
<td>ETH</td>
<td>ethionamide</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GI</td>
<td>gastro-intestinal</td>
</tr>
<tr>
<td>GSD</td>
<td>geometric standard deviation</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks balanced salt solution</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-gamma</td>
</tr>
<tr>
<td>IL-2</td>
<td>interleukin-2</td>
</tr>
<tr>
<td>INH</td>
<td>isoniazid</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>MAC</td>
<td>Mycobacterium avium-intracellulare complex</td>
</tr>
<tr>
<td>MDR</td>
<td>multi-drug resistance</td>
</tr>
<tr>
<td>MIC</td>
<td>minimal inhibitory concentration</td>
</tr>
<tr>
<td>MMAD</td>
<td>mass median aerodynamic diameter</td>
</tr>
<tr>
<td>Myocet™</td>
<td>liposomal doxorubicin</td>
</tr>
<tr>
<td>OADC</td>
<td>oleic acid-albumin-dextrose-catalase</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PA</td>
<td>phosphatidic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PAS</td>
<td>para-amino salicylic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PEG</td>
<td>poly-ethylene glycol</td>
</tr>
<tr>
<td>pKa</td>
<td>acid dissociation constant</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly (DL-lactide-co-glycolide)</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol-myristate acetate</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonucleocytes</td>
</tr>
<tr>
<td>PVA</td>
<td>polyvinyl alcohol</td>
</tr>
<tr>
<td>PZA</td>
<td>pyrazinamide</td>
</tr>
<tr>
<td>Rd</td>
<td>rhodamine</td>
</tr>
<tr>
<td>RES</td>
<td>reticuloendothelial system</td>
</tr>
<tr>
<td>RMP</td>
<td>rifampicin</td>
</tr>
<tr>
<td>sd</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium-dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SM</td>
<td>sphingomyelin</td>
</tr>
<tr>
<td>STR</td>
<td>streptomycin</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt;</td>
<td>time corresponding to concentration maximum</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>half-life in serum</td>
</tr>
<tr>
<td>TB</td>
<td>tuberculosis</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>THP-1</td>
<td>human leukemic cell line</td>
</tr>
<tr>
<td>V&lt;sub&gt;D&lt;/sub&gt;</td>
<td>volume of distribution</td>
</tr>
<tr>
<td>WHO</td>
<td>world health organization</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Yossef Av-Gay for his support and guidance during this thesis project. I would also like to thank the members of my thesis supervisory committee, Dr. Thomas Redelmeier, and Dr. Rick Stokes, for their direction and support throughout my project. I am grateful to Northern Lipids Inc., for their collaboration on this project, and the scientists at NLI who provided and offered their expertise. I have appreciated the opportunity for dialogue with colleagues and associates in the laboratory of the Infectious Diseases Department. This thesis was also partially supported by a B.C. Science Council GREAT Scholarship. Finally, I wish to acknowledge the support provided by my family during the completion of this project.
1.0 INTRODUCTION

1.1 Mycobacterium tuberculosis

1.1.1 Impact

The causative agent of tuberculosis (TB), *M. tuberculosis*, is the most common cause of death (infectious or parasitic) in the world, killing 2-3 million people per year.\(^\text{15}\) It is estimated that one third of the world’s population is infected with *M. tuberculosis* and one third of AIDS patients die of TB.\(^\text{23}\) TB infection is most prevalent in underdeveloped countries in Africa, Southeast Asia, and the former Soviet Union states.\(^\text{34}\) However, with the spread of HIV/AIDS, tuberculosis is also becoming increasingly problematic in developed countries. In addition, the emergence of multi-drug resistant strains is increasing. Together, these factors prompted the World Health Organization to declare tuberculosis a global emergency in 1993.\(^\text{120}\)

1.1.2 Pathogenesis

1.1.2.1 Primary Infection

*M. tuberculosis* can establish infection within the lung after inhalation of as few as 1-3 bacilli.\(^\text{24}\) However, the mucociliary elevator eliminates most bacteria before reaching the alveolar space. Bacilli that pass this defence mechanism are engulfed by alveolar macrophages, the first-line of cellular defence in the lung.\(^\text{118,112}\) At this stage, bacilli may be inactivated by the macrophage and the protective immune response limits tissue damage and spread.\(^\text{124,39,112}\) Nevertheless, some *M. tuberculosis* bacteria are able to resist killing by macrophages and survive within these cells.
1.1.2.2 Survival Within Macrophages

Initial phagocytosis of the bacilli may occur by either complement-mediated means or independent of complement.\textsuperscript{6,18,112} This process is mediated by various receptors, including: the Fc receptor; complement receptors (CR1, CR3, and CR4); CD14; Toll-like receptors (TLR); scavenger receptors; and the mannose receptor.\textsuperscript{6,18,112} Upon infection, \textit{M. tuberculosis} prevents phagolysosome fusion within the macrophage. Maturation arrest is characterised by events such as the exclusion of the ATPase,\textsuperscript{41,107,108} preventing acidification of the vacuole, and the retention of the GTPase, Rab 5.\textsuperscript{39} Rab 7 and the lysosome-associated membrane glycoprotein 1 (LAMP-1), markers of late endosomal vacuoles, are also excluded.\textsuperscript{39,18,35} TACO (tryptophan-aspartate rich coat protein, or coronin 1) is also localized to the endosome and may play a role in maturation arrest.\textsuperscript{39} These events provide a more favourable environment for bacterial survival.\textsuperscript{91,111}

Although \textit{M. tuberculosis} has adapted to exploit host macrophages in this way, it may still interact with the phagosome, releasing components that modify the host immune system.\textsuperscript{9} Therefore, although the bacilli remain in a separate compartment, \textit{M. tuberculosis} still has the ability to exchange materials with other compartments and has access to the sorting and recycling pathway.\textsuperscript{9,91,107}

1.1.2.3 Development of Latent Tuberculosis

Interaction of the \textit{M. tuberculosis} bacilli with alveolar macrophages prompt the release of cytokines and chemokines, including IFN-\(\gamma\) and TNF, signalling infection.\textsuperscript{96,112} Replication within these cells, as well as resident lung macrophages, results in migration of dendritic cells and monocyte-derived macrophages to the infection site, and to the
lymph nodes where T cells are sensitized to Mycobacterial antigens. Following T cell activation, there is a migration of macrophages, T cells, and B cells, which form a granuloma surrounding the bacteria and serves to limit spread as well as providing an area for T cell, cytokine and macrophage interaction. The granuloma also contains dendritic cells, fibroblasts, and endothelial cells. At this stage, primary TB infection may be resolved or contained within the granuloma. Immunocompetent individuals may remain persistently infected and are therefore carriers of latent TB, but are asymptomatic and are not infectious at this stage.

During the latent stage of infection a small population of bacteria, thought to be less than 100,000 bacilli, remain in a non-replicating, or dormant, form. These bacteria typically reside walled-off within the granuloma, an aggregate of macrophages, giant cells and epithelioid cells surrounding necrotic tissue. This reduced metabolic activity allows survival under the nutrient and oxygen deprived environment of the granuloma, and confers resistance to conventional anti-TB drugs. The bacteria are not culturable at this stage but remain able to initiate a clinical disease state. M. tuberculosis may resume growth when the conditions become favourable, such as having increased oxygen availability. Eradication of this disease state is difficult due to insensitivity of the latent bacterial population to antibiotics.

1.1.2.4 Reactivation of TB

People with low resistance, such as the young, elderly, and those with HIV/AIDS, are susceptible to the development of active TB upon primary infection, as well as reactivation of the disease. Although there is only a 2-23% lifetime risk of reactivation TB, this number increases to a 10% annual development risk for immunocompromised
individuals.\textsuperscript{124} These numbers represent a large proportion of active and infectious cases.\textsuperscript{120} Although the mechanism of reactivation remains to be elucidated, it typically occurs upon immunosupression, when the host is no longer able to mount an immune response capable of restraining the bacteria. At this stage of disease liquefaction of the granuloma can occur, providing an oxygen-rich environment for bacterial growth.\textsuperscript{124} Bacteria can spread through the lymph, blood or bronchioles and seed the formation of other granulomas.\textsuperscript{18} Ten-percent of cases will develop extrapulmonary TB.\textsuperscript{15}

1.2 Tuberculosis Therapy

Several characteristics of the \textit{M. tuberculosis} bacilli make infections difficult to treat, including the high lipid content and complexity of the cell envelope,\textsuperscript{8,10} its slow replication time,\textsuperscript{8,10} and the fact that it is a facultative intracellular parasite, initially infecting and surviving within the phagosome of the alveolar macrophage.\textsuperscript{8,10} Therefore, anti-TB agents should remain active at the phagosomal pH.\textsuperscript{10} Granulomas, the site of advanced TB infection, also present increased eradication difficulty since they are not vascularized and therefore antibacterial agents do not readily diffuse into the interior space that harbours the bacteria. In addition, \textit{M. tuberculosis} may exist in a state of latency, rendering agents that target actively growing bacteria ineffective.

1.2.1 Current Drug Therapy

The discovery of the first anti-TB drug, streptomycin, in 1944, was followed by the development of all major drugs used today, culminating with pyrazinamide in 1970.\textsuperscript{29} No major discoveries have been made since then and rifampicin, developed in 1965, remains the most potent antibiotic that is currently prescribed as part of the recommended treatment regimen. Although rare, chromosomal mutations within the \textit{M. tuberculosis}
genome have made it possible to acquire resistance to every anti-TB drug available and the risk of resistance increases upon inadequate treatment. Of new cases, it is reported that 10.7% are resistant to at least one drug, and 1% are resistant to multiple drugs. As a result, current treatment involves the use of combinatorial drug therapy, relying on three or four first-line antibiotics to combat the development of resistance and also to enhance efficacy. In addition, due to the chronic nature of the infection, prolonged therapy over 6 to 12 months is required.

1.2.1.1 First-Line Drugs

First-line anti-TB agents include streptomycin (STR), isoniazid (INH), rifampicin (RMP), ethambutol (EMB) and pyrazinamide (PZA) (Table 1). Nine-month treatment regimens that include isoniazid and rifampicin, with streptomycin or ethambutol in the beginning, are estimated to cure 95% of patients. However, adding pyrazinamide in the first two months can reduce treatment length to 6 months and maintains the 95% cure rate. Currently, the recommended regimen therefore consists of isoniazid and rifampicin, with the addition of either ethambutol or pyrazinamide. These drugs must be administered concurrently, in doses given daily or 2-3 times weekly to maintain efficacious concentrations, over a period of 6 to 9 months.

To ensure compliance with the above treatment regimen, DOTS (directly observed treatment, short-course) is commonly employed, however, 100% compliance is not achieved in patients enrolled in DOTS. Still, this program is considered successful and the WHO is moving to expand the program world-wide as it has been proven to result in net savings due to decreased relapse and resistance rates, as well as prompt cure.
<table>
<thead>
<tr>
<th>Agent/Class</th>
<th>Specific Agent in Class</th>
<th>Mechanism of Action</th>
<th>Daily Dose</th>
<th>MIC (µg/ml)</th>
<th>Activity</th>
<th>Peak Serum Level (µg/ml)/Dose Administered</th>
<th>Tmax (hr)</th>
<th>t1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid</td>
<td>NA</td>
<td>Blocks mycolic acid synthesis by inhibiting InhA and/or KasA</td>
<td>5 mg/kg</td>
<td>0.01-0.025</td>
<td>Bactericidal</td>
<td>3.5 @ 300 mg</td>
<td>1-2</td>
<td>0.5-2</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>NA</td>
<td>Inhibits fatty-acid biosynthesis by targeting FASl</td>
<td>15-30 mg/kg</td>
<td>6.25-50</td>
<td>Bacteriostatic</td>
<td>30-40 @ 1.5 g</td>
<td>1-2</td>
<td>9-10</td>
</tr>
<tr>
<td>Rifamycins</td>
<td>Rifampicin</td>
<td>Inhibits DNA-dependent RNA-polymerase; suppresses initiation of chain formation</td>
<td>10 mg/kg</td>
<td>0.005-0.02</td>
<td>Bactericidal</td>
<td>4-32 @ 600 mg</td>
<td>1-4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Rifabutin</td>
<td></td>
<td>5-10 mg/kg</td>
<td>0.04-0.08</td>
<td>Bactericidal</td>
<td>0.49 @ 300 mg</td>
<td>2-3</td>
<td>12-18</td>
</tr>
<tr>
<td></td>
<td>Rifapentine</td>
<td></td>
<td>600 mg twice/week</td>
<td>&lt;0.125</td>
<td>Bactericidal</td>
<td>15 @ 600 mg</td>
<td>5-6</td>
<td>13</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>NA</td>
<td>Inhibits introduction of D-arabinose into LAM and arabinogalactan</td>
<td>15-25 mg/kg</td>
<td>1-5</td>
<td>Bacteriostatic</td>
<td>2-5 @ 25 mg/kg</td>
<td>2-4</td>
<td>3-4</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Streptomycin</td>
<td>Inhibits protein synthesis by binding to the 30S ribosomal subunit</td>
<td>15 mg/kg</td>
<td>0.25-2</td>
<td>Bactericidal</td>
<td>25-50 @ 1 g</td>
<td>0.5-2</td>
<td>2-4</td>
</tr>
</tbody>
</table>

*NA: not applicable

Table 1: First-line anti-tuberculosis drugs 12,22,23,49,61,125
<table>
<thead>
<tr>
<th>Agent/Class</th>
<th>Specific Agent in Class</th>
<th>Mechanism of Action</th>
<th>Daily Dose</th>
<th>MIC (µg/ml)</th>
<th>Activity</th>
<th>Peak Serum Level (µg/ml)/Dose Administered</th>
<th>Tmax (hr)</th>
<th>t(\text{1/2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycosides</td>
<td>Kanamycin</td>
<td>Inhibits protein synthesis by binding to the 30S ribosomal subunit</td>
<td>15 mg/kg</td>
<td>1.5-3</td>
<td>Bactericidal</td>
<td>22 @ 7.5 mg/kg</td>
<td>0.5-2</td>
<td>2-4</td>
</tr>
<tr>
<td></td>
<td>Amikacin</td>
<td></td>
<td>15 mg/kg</td>
<td>0.5-1</td>
<td>Bactericidal</td>
<td>55 @ 15 mg/kg</td>
<td>0.5-2</td>
<td>2-4</td>
</tr>
<tr>
<td>Ethionamide</td>
<td>NA</td>
<td>Inhibits mycolic acid synthesis</td>
<td>0.5-1 g</td>
<td>2.5-10</td>
<td>Bacteriostatic</td>
<td>20 @ 1 g</td>
<td>1.25</td>
<td>2-3</td>
</tr>
<tr>
<td>Capreomycin</td>
<td>NA</td>
<td>Inhibits protein synthesis</td>
<td>15 mg/kg</td>
<td>1.25-2.5</td>
<td>Bacteriostatic</td>
<td>20-47 @ 1 g</td>
<td>1-2</td>
<td>4-6</td>
</tr>
<tr>
<td>Quinolones</td>
<td>Ciprofloxacin</td>
<td>Inhibits DNA gyrase subunit A</td>
<td>1-1.5 g</td>
<td>0.12-2</td>
<td>Bactericidal</td>
<td>3.4-5.4 @ 1 g</td>
<td>0.5-2</td>
<td>3-5</td>
</tr>
<tr>
<td></td>
<td>Ofloxacin</td>
<td></td>
<td>400-800 mg</td>
<td>0.12-2</td>
<td>Bactericidal</td>
<td>2.9-5.6 @ 400 mg</td>
<td>0.5-2</td>
<td>4-8</td>
</tr>
<tr>
<td></td>
<td>Levofloxacin</td>
<td></td>
<td>500-1000 mg</td>
<td>0.12-2</td>
<td>Bactericidal</td>
<td>5.7 @ 500 mg</td>
<td>0.5-2</td>
<td>6-8</td>
</tr>
<tr>
<td>PAS (p-aminosalicylic acid)</td>
<td>NA</td>
<td>May competitively inhibit conversion of aminobenzoic acid to dihydrofolic acid and/or inhibit iron uptake</td>
<td>150 mg/kg</td>
<td>1-10</td>
<td>Bacteriostatic</td>
<td>9-35 @ 4 g</td>
<td>0.8-8</td>
<td>1</td>
</tr>
<tr>
<td>Cycloserine</td>
<td>NA</td>
<td>Inhibits cell wall synthesis</td>
<td>0.5-1 g</td>
<td>6.25-25</td>
<td>Bactericidal</td>
<td>10 @ 250 mg</td>
<td>2-4</td>
<td>10</td>
</tr>
</tbody>
</table>

*NA: not applicable

Table 2: Second-line anti-TB drugs\(^{21}\)
1.2.1.2 Second-Line Drugs

Second-line drugs have also been developed but are more expensive, show decreased efficacy, and increased toxicity in comparison to first-line drugs. This group is used when first-line drugs are ineffective due to developed resistance or toxicity, such as hepatotoxicity resulting from INH treatment. Second-line antibiotics include ethionamide (ETH), cycloserine, p-aminosalicylic acid (PAS), capreomycin, amikacin, kanamycin, quinolones (such as ciprofloxacin), and clofazime (Table 2).

1.2.2 Drugs Formulated in this Thesis

1.2.2.1 Isoniazid

Isoniazid was initially synthesized by Meyer and Mally in 1912, but was first put into clinical use in 1952. It is a small molecular weight (137.15 g/mol) synthetic pro-drug, which is activated in vivo by the enzyme KatG (Figure 1). It is soluble and stable in aqueous solutions up to 125 mg/ml but is rapidly degraded when exposed to serum or plasma at room temperature. The key structural components responsible for the activity of isoniazid are the pyridine nucleus and the carboxylic acid hydrazide.

Isoniazid is specific for Mycobacteria, with a MIC of 0.01-0.25 μg/ml against M. tuberculosis. It is bactericidal to actively growing bacteria. Isoniazid inhibits mycolic acid synthesis, which results in increased permeability of the cell wall to hydrophilic solutes and irreversible activity after 12 hours. Drug resistance primarily develops by mutations in the katG gene, leading to a loss of catalase-peroxidase activity and therefore loss of drug activation, but may also occur by mutations in InhA, and perhaps KasA, which are both drug targets involved in fatty acid synthesis. Mutations in AhpC may also play a role in drug resistance.
Figure 1: Chemical structures of (A) isoniazid; (B) rifampicin; and (C) ethambutol.
The volume of distribution (V_D) of isoniazid, a measure of the apparent volume into which a drug has been dissolved, is 0.6 to 2 L/kg. This suggests that isoniazid is widely distributed and therefore diluted within the body, indicating that localized delivery may increase the effectiveness of isoniazid. The t_max, or the time required to reach the peak drug concentration, occurs within 0.5–2 hours after oral dosing, where a concentration range of 3-6 μg /ml is achieved after administration of 900 mg. The pharmacokinetic parameter, together with the time at which the drug is reduced to 50% within the body (t_1/2), which ranges from 1-4 hours, indicate that the drug persists for a relatively short time in vivo, accounting for the requirement of frequent dosing intervals.

Isoniazid is typically administered orally at 10-20 mg/kg, and is given in combination with two other antibiotics over 6-months. There are relatively few major side-effects associated with isoniazid treatment, however, hepatitis may occur, especially in patients with liver problems or increasing age, limiting its usefulness in the elderly.

1.2.2.2 Rifampicin

Rifampicin was the first drug discovered in the rifamycin class. It was isolated from Amycolatopsis mediterranei and further developed in the 1960s. It has a broad range of activity against both gram-positive and gram-negative organisms, including M. tuberculosis for which it is a first-line drug. Rifampicin is bactericidal, with a MIC of 0.025-0.2 μg/ml. It is thought that rifampicin has a rapid onset of activity (within 12 hours), which may act against slow growing bacteria during bursts of metabolism. Rifampicin is also able to concentrate within macrophages and has been found in polymorphonucleocytes (PMNs) at concentrations 5-fold greater than in surrounding tissue.
The extensive ring structure of rifampicin is the active moiety, binding to the $\beta$-subunit of the procaryotic DNA-dependent RNA polymerase and preventing initiation of chain formation (Figure 1).\textsuperscript{70} As for other anti-tuberculosis agents, resistance develops and therefore necessitates the use of combinational therapy. Resistance to rifampicin results upon development of a mutation in the $\beta$-subunit of the RNA polymerase (RpoB), preventing rifampicin binding.\textsuperscript{125}

Rifampicin distributes to most tissues, including cerebrospinal fluid (CSF), with a volume of distribution of 1 L/kg.\textsuperscript{47} It has a relatively short elimination half-life, 3 hours,\textsuperscript{90} necessitating daily dosing to maintain therapeutic drug levels. Like isoniazid, it is thought that the most important pharmacokinetic parameter is the Cmax:MIC ratio, where drug efficacy increases when concentrations greater than the MIC are maintained for longer periods to kill actively growing bacteria and those undergoing bursts of replication.\textsuperscript{84}

Rifampicin is administered orally, at a dose of 600-1200 mg/day, or 10-20 mg/kg i.v.\textsuperscript{45} It may trigger some mild side effects, including GI distress, headache, dizziness, fever, cutaneous reactions, hepatotoxicity,\textsuperscript{45} and colouring of the saliva, urine and tears. Rifampicin is a potent inducer of drug metabolism and therefore drug interactions are prevalent. This characteristic is particularly important to consider when treating HIV patients taking anti-retroviral therapy.

1.2.2.3 Ethambutol

Ethambutol (2,2'-ethylenediimine-di-1-butanol) is a small molecular weight (277.2 g/mol) lipophilic amine that was developed in 1961 (Figure 1).\textsuperscript{61} It requires two centres, which are thought to act as chelating agents, for its activity.\textsuperscript{61} Ethambutol is only
active against *Mycobacteria*, inhibiting synthesis of mycobacterial metabolites by EmbB, which encodes arabinoyl transferases.\textsuperscript{125} The result is the inhibition of cell wall synthesis.\textsuperscript{61} It is bacteriostatic, with a MIC of 1-5 μg/ml.\textsuperscript{61} Ethambutol is only efficacious against actively growing bacteria and requires 1-2 days in culture to become effective,\textsuperscript{61} but this activity is reversible. Since ethambutol causes the breakdown of the cell wall, it allows other drugs to pass through the bacterial cell wall, accounting for its synergistic activity with other antibiotics.\textsuperscript{61}

Ethambutol widely distributes in all body fluids and tissues.\textsuperscript{45} It does not bind highly to plasma proteins. It is rapidly absorbed (75 to 80%) and reaches a peak serum level \(C_{\text{max}}\) of 2 μg/ml to 5 μg/ml in 2 to 4 hours after administration of a 35 mg/kg dose.\textsuperscript{45} The half-life of ethambutol is 3-4 hours in normal patients and is undetectable 24 hours after administration\textsuperscript{85} and repeat administration is required to ensure that ethambutol is maintained at efficacious levels since it is relatively slow to act in comparison to isoniazid or rifampicin.

As with the other first-line tuberculosis drugs, ethambutol must be given in combination since random spontaneous genetic mutation confers resistance to *M. tuberculosis* rapidly. In the case of ethambutol, resistance involves both the overexpression of EmbB, the target of the drug, and mutations within the gene for this enzyme.\textsuperscript{125} The drug is typically administered at 15 mg/kg/day, for a minimum of 6 to 9 months.\textsuperscript{45} Side effects include optical toxicity and regular visual testing should be carried out during treatment.\textsuperscript{45}
1.2.3 Development of New Therapies

Although tuberculosis is a curable disease, cases of drug resistant infections continue to rise and 1/3 of the world's population continues to be infected with latent tuberculosis. These facts make the development of new tuberculosis therapies a necessity. The Global Alliance for Tuberculosis Drug Development has identified three objectives to ensure improvement of TB therapy. These include: decreasing the duration and dose of drugs required (the aim being sterilization in 2 months); developing new drugs against resistant strains (i.e. novel compounds with a unique mechanism of action); and developing drugs active against latent bacteria (required to completely eliminate tuberculosis).

The development of new therapies is a long process, and the most significant contribution so far has been the development of long-acting rifamycins, including rifapentine, which was approved by the FDA in 1998, and rifabutin, which shows decreased drug interactions. The fluoroquinolones also show promise as potential new anti-TB agents, including gatifloxacin, moxifloxacin, and sparfloxicin, which have been investigated for use against MDR-TB since they concentrate in respiratory tract fluid, bronchial tissue, and alveolar macrophages. These agents may eventually be preferred over currently employed second-line agents. In addition, two novel classes of drugs show promise. The oxazolidinones, including linezolid, are currently used as broad-spectrum antibiotics for acute bacterial infections. The nitroimidazopyrans, including the lead compound PA-824, have a novel mechanism of action and activity against non-replicating bacilli, suggesting potential sterilizing ability.
As mentioned, the need to decrease treatment length and reduce the frequency of administration would significantly improve TB therapy, the rationale being that more people would complete their treatment, resulting in less resistance development and decreased relapse rates. However, the development of new classes of antibiotics is a long and costly process. It would therefore be beneficial to develop new delivery systems using available antibiotics that have proven activity against *M. tuberculosis*.

This thesis focuses on the development of liposomal formulations of isoniazid, rifampicin, and ethambutol. These three drugs were chosen because they are currently used in first-line therapy and have short half-lives *in vivo* that would be improved by liposome encapsulation. In addition, they were considered to have good potential for liposome encapsulation using remote loading. Formulation of each drug would therefore improve the delivery of these drugs to the disease site and prolong their release. These characteristics will allow simplification of the current treatment regimen.

### 1.3 Liposomes

Liposomes, vesicles formed upon hydration of phospholipids, are capable of entrapping water-soluble and lipophilic compounds, and have thus been exploited as drug delivery vehicles for a variety of agents (Figure 2). They are biodegradable, biocompatible, and relatively non-toxic due to the fact that they are natural components of the cell.\(^{109}\) Although studies involving administration of lipid emulsions in the same lipid range as human studies (7-22 mg/kg) have shown impaired macrophage function in mice, the effects, resulting in increases in liver and spleen weight, do not typically result in an immune response and are reversible in animal studies.\(^{109}\) In addition, administration
Figure 2: Structure and formation of a liposome
of liposome encapsulated drugs, such as amphotericin B and doxorubicin, show decreased toxicity in comparison to the free drug form.\textsuperscript{109}

Liposomes, identified as particulate material by the immune system, are naturally cleared by the reticuloendothelial system (RES).\textsuperscript{3} Liposomes are phagocytosed primarily by the liver at low doses (approximately 0.1-1 \textmu m moles/mouse), followed by clearance by the spleen, lymph nodes and bone marrow as doses increase.\textsuperscript{3} Therefore, they have the potential to increase efficacy of treatment for intracellular pathogens like TB by modifying cellular uptake or intracellular distribution of drug.\textsuperscript{94}

1.3.1 Targeting and Delivery

Liposomes can be delivered by passive targeting, which is dependent on the distribution of unmodified liposomes, or by active targeting, by enhancing liposome interaction with specific cell types by the addition of ligands.\textsuperscript{99} Increased targeting may also be accomplished by altering the route of administration. Although liposomal formulations are typically delivered i.v., administration routes such as topical or pulmonary are also possible. Since tuberculosis primarily infects the lung, passive targeting of liposomes by pulmonary delivery is the ultimate goal for formulations developed in this study.

1.3.2 Drug Loading

Two general modes of drug encapsulation are available for liposome formulations. The first, passive encapsulation, involves drug trapping during the formation of the liposomes,\textsuperscript{78} while the second, active loading, involves drug loading into pre-formed liposomes.\textsuperscript{63} This thesis will utilize the active loading method, which results
in higher concentration drug formulations than passively entrapped formulations. This is an important component since drug delivery to the lung is restricted by the volume that can be administered, and therefore the drug concentration must be maximized to achieve efficacious levels.

1.3.3 Drug Release

The pharmacokinetics and efficacy of a formulation are dependent on drug release. Therefore, it is important to optimize this parameter in order to achieve the desired release properties and efficacy in vivo. As long as drug remains associated with the liposome, it takes on the properties of the carrier. For the formulations developed in this thesis, the desired therapeutic effect will only occur when drug is released and its concentration is maintained at levels greater than the MIC. Drug release typically occurs upon interactions with proteins, such as high-density lipoproteins (HDL), low-density lipoproteins (LDL), and to a lesser extent complement factors, which result in pore formation. Release from remote loaded formulations may also occur upon partial lysis due to osmotic stress, or diffusion across the membrane.

1.3.4 Factors Affecting Drug Loading and Release

Before administration of any drug, a formulation must be developed that is appropriate for the desired route and target so that improvements in the pharmacokinetics and pharmacodynamics are achieved. This development process involves consideration of both drug loading and release, which are dependent on the properties of the drug and liposome formulation.

The physiochemical properties of the drug influence the extent of drug incorporation, as well as the orientation within the liposome itself. Highly polar, water-
soluble drugs tend to be entrapped in the aqueous compartment, while lipophilic (hydrophobic) drugs may intercalate within the lipid bilayer. Amphipathic drugs can also partition across the membrane-aqueous interface. Either very hydrophobic or very hydrophilic drugs tend to be retained to a greater extent in the liposome, whereas agents with intermediate partition coefficients may diffuse more readily.

The properties of the liposomal carrier are also important. Lipid composition dictates the rigidity of the membrane, as well as the charge. These properties in turn affect the extent of drug-lipid interaction and therefore encapsulation, as well as the rate of release. Vesicle size plays a role in the kinetics of cell uptake, which may also determine the kinetics of drug release. Additional factors affect drug uptake during remote loading. These include: the size and stability of the pH gradient, buffer composition (buffering capacity), loading kinetics, lipid composition, and drug/lipid ratio. Each factor was considered when developing the formulations in this thesis in order to achieve sufficient drug delivery to the disease site by pulmonary administration and to prolong drug release at concentrations high enough to kill the bacteria.

1.3.5 Rationale for the Use of Liposome Encapsulated Antibiotics

As mentioned previously, current tuberculosis treatment relies upon three or four first-line antibiotics that are administered concurrently over a 6 – 12 month period. The long length of exposure to anti-tuberculosis drugs is required in part due to the inability of antibiotics to effectively permeate macrophages or sites of necrosis and disease. Therefore, although blood levels of drug may reach the MIC, the intracellular bacteria are not exposed to sufficiently high concentrations for periods approaching the time required to divide. This problem may be circumvented by increasing oral dosages of existing
first-line antibiotics, but would likely result in serious side effects, while not improving compliance or the time to achieve an effective response.

There is considerable interest in developing novel approaches to treat tuberculosis that would lead to enhanced (more rapid) killing of the bacteria, especially for those individuals who exhibit chronic disease or who are infected with antibiotic resistant strains. Our working hypothesis is that significant improvements in the therapeutic profile of first-line agents can be achieved by optimizing drug delivery to the disease site.

We propose that these objectives may be achieved by administering a pulmonary formulation of liposome encapsulated first-line agents to treat tuberculosis. The therapeutic properties of many active pharmaceutical ingredients (API) can be improved by incorporation into liposomal systems. Improved characteristics may derive from the effective solubilization of "lipid-like" drugs, altered drug pharmacokinetics (by sustaining drug release from the carrier), biodistribution (by enhanced delivery to disease sites), protection from toxic effects, and protection of the active drug species from metabolism. Several liposome formulations, including: the anticancer agents doxorubicin (Myocet™, DOXIL™) and daunorubicin (DaunoXome™); the anti-fungal agent amphotericin B (ABELCET™, AmBisome™); and a benzoporphyrin (Visudyne™); have recently been introduced into the US, European and Japanese markets, and a relatively large number of second generation products are currently undergoing clinical investigations. The proven safety and efficacy of lipid-based carriers make them attractive candidates for the formulation of pharmaceuticals.
1.3.6 Previous Liposome Encapsulated Anti-Mycobacterial Formulations

1.3.6.1 In vitro Studies against MAC Infections

Several *in vitro* studies have been carried out to characterize the activity of antimycobacterial agents against *M. avium-intracellulare* complex (MAC). Such studies have typically focused on the encapsulation of fluoroquinolones, which are effective against MAC and have also been used to treat MDR tuberculosis. These studies have shown increased efficacy of ofloxacin, clarithromycin, ciprofloxacin, and azithromycin in comparison to equivalent concentrations of free drug, and have also noted that potency increased with increasing negative charge on the vesicle carriers. Encapsulated aminoglycosides, specifically streptomycin and amikacin have also shown significantly increased activity against MAC.

1.3.6.2 In vivo Studies against MAC Infections

In animal studies, encapsulated streptomycin showed equivalent efficacy to 5-100 times the concentration of free streptomycin against MAC infected beige mice, while liposomal rifabutin showed increased efficacy after 2-3 weekly injections. The most extensive research has been carried out using amikacin, an aminoglycoside related to kanamycin, which is used as a second-line anti-tuberculosis drug. Upon i.v. injection, encapsulated amikacin shows increased maintenance of drug for up to 9 days in the lung and up to 28 days in the spleen. Encapsulated amikacin is also 4-10 fold more efficacious than free amikacin. Further investigation in the rhesus monkey showed favorable pharmacokinetics and in administration to a patient with MDR TB, serum levels were detected at 100 mg/L after 46 hours and the elimination half-life was extended to 180 hours.
1.3.6.3 *In vivo* Studies against *M. tuberculosis*

Further analysis has been undertaken by several groups to determine the efficacy and pharmacokinetics of encapsulated anti-tuberculosis drugs *in vivo*. Investigations characterizing the activity of encapsulated streptomycin against tuberculosis after i.v. administration showed increased efficacy in the spleen and liver, but not the lung.\(^{69,113}\) Favorable pharmacokinetic parameters were also measured for these formulations, characterized by 6-9 fold greater area under the curve (AUC), which suggests higher drug bioavailability, and three-fold greater \(C_{max}\),\(^{59}\) ensuring that the MIC is surpassed and indicating the potential for increased activity.

Encapsulation of rifampicin and isoniazid, the two most important first-line drugs, has been the focus of the most intensive research. Typically, with varying lipid formulations, several research groups have found that both drugs are more efficacious than free drug following i.v. administration to mice. Deol et al. showed that liposome formulations of both rifampicin and isoniazid showed significant improvement in efficacy in comparison to the free drugs as measured by reduced bacterial load, organomegaly, and histopathology.\(^{26}\) The same group, when testing formulations with polyethylene glycol (PEG), added to increase circulation time, found a 40% improvement in organ accumulation, resulting in equivalent efficacy of a 4 mg/kg dose of encapsulated isoniazid in comparison to a 12 mg/kg dose of free drug.\(^{26}\) However, efficacy in the lung was the lowest of all organs. Agarwal et al. also found that incorporation of tufstin, a peptide known to increase uptake by macrophages, improved efficacy in comparison to their previous liposome preparations.\(^2\)
Finally, pharmacokinetic studies have shown that liposomal formulations are retained in tuberculous mice for longer periods than free drugs. Specifically, Labana et al. found that liposomal rifampicin and isoniazid could be detected in plasma for up to 5 days and in organs for up to 7 days, in comparison to the free drugs, which were only detectable for 24 hours. Even at 1/3 of the therapeutic dose, liposome encapsulated drugs showed similar release patterns. Tmax and T1/2 values were greater for encapsulated formulations and Cmax levels, although lower than for free drugs, were sustained for longer periods at concentrations greater than the MIC.

1.3.7 Microparticles - Alternative Drug Delivery Systems

More recently, scientists have been investigating polymeric drug delivery systems, including polymeric microparticles, which entrap drug within pores and then release it upon hydrolysis of the particles in vivo. Sreenivasa et al. found that coated ethylcellulose beads, with 52% drug loading, exhibited slow release. Most notably, a co-polymer of lactic acid and glycolic acid, poly (DL-lactide-co-glycolide) (PLGA), has been studied by several groups for the development of drug delivery systems for tuberculosis therapy. Dutt and Khuller designed particles capable of releasing isoniazid for up to 2 days in the plasma and organs, while hard microparticles showed INH release for up to 2 days in the lung and liver, and up to 27 days in the spleen. In addition, the same group observed drug release over 6-7 weeks when particles were coated with PEG. Results by Barrow et al. suggest that small microparticles are similar in efficacy to free drug, but when combined with larger particles, efficacy is significantly increased, releasing drug over 21-26 days at concentrations greater than the MIC (0.06-0.25μg/ml). Finally, Saurez et al.
administered PLGA-rifampicin microparticles by insufflation 24 hrs prior to infection, as well as 10 days later by nebulization. After 28 days, the bacterial load in the lungs and spleen was significantly reduced.\textsuperscript{98} They also showed that 20 \( \mu \)g of rifampicin entrapped in PLGA particles, versus 6 \( \mu \)g of free rifampicin, remained in the airways for 72 hours.\textsuperscript{98}

1.4 Pulmonary Delivery

Currently, anti-tuberculosis drugs are administered orally, or i.v. However, pulmonary delivery is the intended route of administration for the drug formulations designed during this project. This mode of delivery will allow local delivery of the antibiotics directly to the lung, the location of alveolar macrophages and sites of granuloma formation. The lung anatomy makes this route of administration plausible, and has been exploited in the past for treatment of other pulmonary disorders, such as asthma, bronchitis, cystic fibrosis and adult respiratory distress syndrome.\textsuperscript{94,97,122}

1.4.1 Lung Anatomy

Upon inhalation, air enters the lung after passing through the ciliated trachea and the bronchi, which branch 22 times (forming the bronchial network) before becoming terminal bronchiole.\textsuperscript{82} At this point are the alveoli, tiny air sacs that are surrounded by capillary networks. Typically, an adult has approximately 600 million alveoli, with a total epithelial surface area of approximately 100 m\(^2\).\textsuperscript{82} These air sacs, the site of oxygen exchange, are very permeable since they are made up of a single layer of cells, in contrast to the pulmonary epithelium, which is a largely impermeable, tightly packed cell layer covered with cilia and mucous.\textsuperscript{82} In fact, absorption is four-fold higher in the alveoli than in the bronchioles. The anatomy of the respiratory system therefore provides a favourable environment for drug deposition in the alveolar space, as well as providing
systemic delivery since large molecules may pass into the bloodstream in this area. Pulmonary administration may therefore be suitable for the treatment of pulmonary TB infections as well as miliary TB.

1.4.2 Pulmonary Drug Delivery

Since pulmonary administration enables localized delivery, it has the advantage of requiring lower dosing and enhancing the therapeutic ratio, as well as ultimately leading to decreased toxicity (in comparison to systemic delivery), lower costs, and enhanced targeting. Bioavailability is particularly high in the lung because of its large surface area and low metabolic activity. However, efficacy is ultimately determined by the amount of drug reaching the lung, a factor dependent on particle and drug delivery device technology. Less than 30% of the administered dose typically reaches the lower airways, so it is crucial to develop a formulation that has optimal characteristics such as particle size, charge, shape and density.

Aerosol size is reported as the median mass aerodynamic diameter (MMAD), which takes into account the size, shape and density of a particle. The optimal aerosol droplet size range for lower lung delivery is less than 7 μm, which is governed by three main mechanisms of deposition within the lung. The first two mechanisms, sedimentation and impaction, are important for larger particles and often result in deposition within the mouth or trachea. The third mechanism, brownian diffusion, is dependent on random movement upon collision with a gas molecule. This process usually governs deposition of particles in the alveoli and is inversely proportional to the diameter of particles less than 0.5 μm.
Factors such as the lipophilicity, hygroscopic character and release rate can also be optimized to favour slow clearance and absorption rates. In addition, the anatomy of the respiratory system and breathing pattern of the patient comes into play. The ideal situation would be an aerosol containing particles with an MMAD of <7 µm applied slowly and steadily with a period of breath-holding.

1.4.3 Delivery Devices

The device chosen for administration also plays a role in delivery efficiency. Three general categories of delivery devices are available for pulmonary administration. Personal meter-dosed inhalers are the most common but tend to leave a large proportion of the dose in the mouth of the patient. Dry-powder inhalers are breath-activated and turn milled powders into smaller aggregates capable of being respired upon airflow. Finally, nebulized inhalers, the most popular being airjet (air powered) or ultrasonic (frequency dependent), can produce smaller droplets than the other systems. It is important to remember that the most important characteristic of any system is output, distribution and intra and inter-reproducibility.

1.5 Pulmonary Delivery of Liposome Encapsulated Drugs

1.5.1 Drawbacks to Pulmonary Administration of Free Drugs

The drugs of interest to this thesis, isoniazid, rifampicin, and ethambutol, are orally active, exhibiting high volumes of distribution (Vₐ ∼ 0.5 – 2 L/kg), and clearance rates (CL > 0.2 L/hr/kg). These properties are suitable for orally administered drugs that target lung tissue, but are less optimal for drugs administered directly to the lung by aerosolization or dry powder inhalation. Under these conditions, small molecules with high clearance rates are released from the lung with a t₁/₂ in the serum from 1 to 3 hours,
as observed for rifampicin, isoniazid, and ethambutol, which have $t_{1/2}$ values of 2.4, 1, and 2.5 hours respectively.\textsuperscript{85,86,87} These considerations limit the utility of direct administration to the lung for tuberculosis treatment since drug concentrations would not be maintained at therapeutically efficacious levels.

1.5.2 Previous Pulmonary Liposome Formulations

Pulmonary delivery and liposome encapsulation would combine the benefits of both systems, altering pharmacokinetics and providing sustained release at the target site not achievable by orally administered drugs. Toxicity would also be limited by encapsulation and minimized systemic exposure. Targeting using pulmonary administration prevents a dilution effect, increasing the dose encountered by the bacilli.

Pulmonary delivery of several liposome encapsulated drug formulations has been investigated previously, including: amphotericin B for the treatment of lung aspergillosis;\textsuperscript{90} surfactant for the treatment of respiratory distress syndrome;\textsuperscript{57} IL-2 for the treatment of lung cancer;\textsuperscript{57} beclomethasone for the treatment of asthma;\textsuperscript{93} tobramycin for the treatment of \textit{P. aeruginosa} in cystic-fibrosis patients;\textsuperscript{94} and ciprofloxacin for the treatment of various intracellular pathogens including \textit{Francisella tularensis}.\textsuperscript{19,38} Direct lung administration of encapsulated cytosine arabinoside, a DNA-synthesis inhibitor, showed 12-fold prolonged maintenance in comparison to free drug.\textsuperscript{57} Extrapulmonary drug was also significantly lower than after i.v. administration.\textsuperscript{57}

1.5.3 Effect of Device on Liposome Delivery

As in every pulmonary delivery formulation, the device and the conditions are important when considering administration of liposomes.\textsuperscript{74} Specifically, the presence of liposomes affects the efficiency and the aerodynamic properties of the aerosol\textsuperscript{113} and it is
important to note that the aerosol droplet size, rather than the vesicle size, determines the site of lung deposition. Shear rates of the device may affect drug encapsulation since any disruption of the liposome bilayer could result in drug release, ultimately affecting the pharmacokinetics and disposition of the drug.

Previous studies aimed at pulmonary delivery of liposomes have typically looked at jet nebulizers. Waldrep et al. found that there was no significant difference in the delivery of beclomethasone-DLPC liposomes, in regards to encapsulation and MMAD and GSD, when testing 18 different jet nebulizers. Finlay and Wong (when testing five jet nebulizers and one ultrasonic nebulizer for the delivery of ciprofloxacin-PC/CHOL liposomes) found that the LC STAR (a jet nebulizer) and the Sonix 2000 (an ultrasonic nebulizer) significantly outperformed the other nebulizers tested in regards to delivery of drug to the lung and drug encapsulation after nebulization. However, it is important to note that the type of nebulizer used should be determined for each particular formulation.

1.6 Summary of Project

This project was designed to develop liposome formulations of three first-line drugs used in primary therapy of patients with tuberculosis. Our approach utilized remote loading of rifampicin, isoniazid, ethambutol and combined isoniazid/ethambutol into SM/CHOL (63/37, mol/mol) liposomes to maximize the drug/lipid ratio and potency. The use of remote loading and SM/CHOL should also maximize the sustained release of the antibiotics, thereby maintaining drug concentrations above the MIC at the site of infection for prolonged periods.

The efficacy of each formulation was compared to equivalent concentrations of free drugs in vitro against extracellular M. bovis BCG, as a model organism for M.
tuberculosis. Extracellular assays included using the disc diffusion assay and the MIC macrobroth dilution assay. The intracellular efficacy was also tested using a THP-1 infection model, again using M. bovis BCG. The efficacy was compared to free drug over a 48 hour and 1 week period at concentrations corresponding to peak serum levels in humans, and over 48 hours at levels below the MIC of each drug. The localization of liposomes in infected cells was also visualized by fluorescence microscopy.

It is the intent to deliver these formulations by pulmonary administration in order to target infection within the lung. Hopefully, by coupling the optimal characteristics of liposome encapsulation with pulmonary delivery, the approach to TB therapy can be improved, leading to less frequent administration, lower development of resistant strains and ultimately improved compliance.

**Hypothesis:** Liposome encapsulation of isoniazid, ethambutol, and rifampicin by remote loading will maintain efficacy in vitro against Mycobacteria.

**Objectives:**

1. Develop liposome formulations of three first-line anti-TB agents, isoniazid, rifampicin, and ethambutol, by remote loading under optimized conditions.

2. Characterize the formulations in terms of in vitro drug release and stability.

3. Determine in vitro efficacy against extracellular bacteria.

4. Determine in vitro efficacy against intracellular bacteria.

5. Characterize intracellular localization of bacteria and liposomes.
2.0 MATERIALS AND METHODS

2.1 Sources of Materials

All lipids were supplied by Northern Lipids Inc. (Vancouver, Canada). *M. bovis* BCG Pasteur (ATCC 35374) was purchased from the American Type Culture Collection (Rockville, MD). The strain used in this thesis contained a plasmid expressing green fluorescent protein (GFP) as described in Cowley et al.\textsuperscript{20} *M. smegmatis* mc\textsuperscript{2}155 was provided by Dr. W. R. Jacobs (Albert Einstein College of Medicine, Bronx, NY). Isoniazid (cat#I3377), rifampicin (cat#R3504), ethambutol (cat#E3640), RPMI-1640, Hank’s balanced salt solution (HBSS) and mouse serum were purchased from Sigma (St. Louis, MO). The THP-1 cell line was purchased from the American Type Culture Collection (Rockville, MD). Middlebrook 7H9 and 7H10 media (Difco) were purchased from VWR Scientific (Mississauga, ON). Fetal calf serum (FCS) was purchased from Invitrogen (Burlington, MO). Other tissue culture reagents were purchased from Stem Cell Technologies Inc. (Vancouver, Canada). All other reagents were of analytical grade.

2.2 Drug and Lipid Solubilization

Isoniazid and ethambutol are soluble in aqueous solutions and were dissolved in 115 mM Na\textsubscript{2}SO\textsubscript{4}/50 mM Na\textsubscript{2}HPO\textsubscript{4} buffer for the purposes of these experiments. Rifampicin is not aqueous soluble and is more soluble in basic solutions. Therefore, absolute ethanol was routinely used for solubilization, with the addition of 1 M NaOH to pH 10.

Sphingomyelin (Lipoid, Germany) and cholesterol (Lipoid, Germany) were solubilized together in t-butanol/absolute ethanol (1/1, v/v) (Fisher, Edmonton, Canada) in a 60°C waterbath for approximately 30 minutes, with intermittent vortexing.
2.3 Preparation of Liposomes

Liposome formulations of the candidate drugs were prepared essentially as described in Jia et al., 2001\textsuperscript{49} (Figure 3). Liposomes were prepared from solubilized SM/CHOL (63/37, mol/mol) by dilution in 5 parts 0.5 M sulfuric acid (Fisher, Edmonton, Canada) by injection through a 22-gauge needle (BD) attached to a 3 ml syringe (BD), quickly followed by vortexing. Vesicles were size reduced through two stacked 80 nm filters (Nucleopore, USA) and one drain disc (Nucleopore, USA) using a LIPEX\textsuperscript{TM} Extruder (Northern Lipids Inc., Vancouver, Canada) to a range of 110-140 nm.

2.4 Drug Loading

Remote loaded vesicles were prepared based on the methods of Madden et al.\textsuperscript{63} and Jia et al.\textsuperscript{49} For isoniazid loading, size reduced vesicles were diluted 1/10 in 115 mM Na\textsubscript{2}SO\textsubscript{4}/50 mM Na\textsubscript{2}HPO\textsubscript{4} followed by pH adjustment to pH 4.5 with 1 M NaOH. Isoniazid, solubilized in the same buffer, was added at a drug/lipid ratio of 1:15 (mol/mol), unless otherwise specified, after pH adjustment. Drug was loaded into the liposomes by incubating the solution in a 60°C water-bath for 30 minutes. The same procedure was followed for the loading of ethambutol into vesicles except that the ethambutol was added to the liposomes at a drug/lipid ratio of 1:3 (mol/mol) (unless otherwise specified) after adjustment to pH 7.5 with 1 M NaOH. The same protocol was followed for rifampicin except for the following changes: loading was carried out for one hour at 37 °C at a drug/lipid ratio of 1:15 (mol/mol) (unless otherwise specified) after exterior pH adjustment to pH 7.5. The same empty vesicles were used to load both isoniazid and ethambutol at the same time, at a drug/lipid ratio of 1:15 for each drug, after exterior buffer pH adjustment to pH 6.0, followed by a 30-minute incubation at
1. Solubilization and Dilution

2. Extrusion

3. Remote Drug Loading

4. Exterior Drug and Solvent Removal/Concentration

Figure 3: Formulation development scheme.
60 °C. During optimization of these formulations, varying drug/lipid ratios and loading kinetics were employed as specified in the results section. However, if no changes are noted, the procedures listed above were followed.

2.5 Removal of Unencapsulated Drug and Solvent

Loaded liposome formulations were concentrated to approximately 12 ml in the first step, followed by diafiltration against ten volumes of 115 mM Na₂SO₄/50 mM Na₂HPO₄ at the drug loading pH. Diafiltration was carried out using a MidGee ultrafiltration column (UFP-30-C-H24LA, 30000 MWCO, 42cm², 0.5mm lumen diameter) (Amersham Biosciences, Piscataway, NJ), to remove unencapsulated drug and solvent, which was confirmed as described below. In the last step, formulations were concentrated to achieve approximately 100 mg/ml total lipid (15 fold total concentration) on the same column to maximize total drug concentration achieved for in vitro testing.

2.6 Drug Quantification and Encapsulation Determination

Both isoniazid and rifampicin were quantified using a spectrophotometric (Schimadzu UV 160IPC, Mandel Scientific, Guelph, ON) assay by measuring absorbance against a free drug standard curve, at 263 nm and 479 nm respectively, in 70% ethanol. Vesicles were disrupted by vortexing in 70% ethanol, with heating to 60°C, to release encapsulated drug. Ethambutol was quantified using the Pierce modified Lowry protein assay (MJS Biolynx, Brockville, ON), against an ethambutol standard curve. Experiments showed that this assay is sensitive to ethambutol in the same range as bovine serum albumin (BSA), following a quadratic curve relationship in the range of 0-1.5 mg/ml drug. Total drug was quantified after solubilization of the vesicles in 10% SDS/16% ethanol with heating at 60°C.
Encapsulation of each drug was determined by separation of the liposome associated drug from free drug by centrifugation at 13000 rpm for 10 minutes through microcon-30 filtration devices (Millipore, Billerica, MA), where the percentage encapsulation is determined by: \[1-(\text{drug concentration in eluant/drug concentration loaded})\]*100.

2.7 Lipid Detection

Sphingomyelin or cholesterol was quantified separately using a Waters HPLC. Sphingomyelin was quantified using an Evaporative Light Scattering Detector (ELSD MK III) after separation using a polyvinyl alcohol (PVA) Silica (4/6 x 250mm) column and chloroform/isopropanol/water mobile phase. Cholesterol was quantified by a Waters LC spectrophotometer at 207 nm after separation on a Spherisorb ODS1 (4.6 x 250 mm) column using methanol as the mobile phase. Either lipid was quantified for a given sample, allowing for calculation of the second lipid based on proportional recoveries during processing.

2.8 In vitro Release Assay

The in vitro release of each drug was quantified at 37°C (VWR Scientific Products Incubator, Model 1545, VWR, Edmonton, AB) in the presence or absence of serum (Sigma, St. Louis, MO) and ammonium sulfate (Fisher, Nepean, ON). The specified formulation was diluted two-fold in either PBS pH 7.4 or mouse serum and subsequently incubated at 37°C for 24 hours with aliquots removed at the indicated timepoints. Ammonium sulfate was added to appropriate samples at a final concentration of 10 mM and diluted two-fold in PBS before incubation as for other samples. All samples were further diluted five-fold in PBS and encapsulation was determined at each
time point as described above. Since serum proteins interfere with the Lowry assay, total ethambutol concentrations for serum containing samples were based on total drug measured in equivalent PBS diluted samples. Controls containing known drug concentrations with serum added, as well as with serum alone, were processed in the same manner as experimental samples to ensure that background detection of serum proteins in the eluant did not interfere with the levels of drug assayed in these release studies.

2.9 Vesicle Size Analysis

Vesicle size was determined using a Nicomp 300 ZS (Particle Sizing Systems, Santa Barbara, CA) by diluting to 0.5 mg/ml total lipid in 0.9% saline. Vesicle size determination using this machine is based on quasi-elastic light scattering (QELS). In this method a laser beam is diffracted at varying scattering intensities by vesicles in suspension that is dependent on particle size. This method enables calculation of a diffusion coefficient over time, which is converted to particle size by taking into account the temperature and viscosity of the sample.

2.10 pH Gradient Determination

All formulations were diluted ten times in 115 mM Na₂SO₄/50 mM Na₂HPO₄ at the appropriate exterior pH as indicated for drug loading. ¹⁴C-methylamine (NEN, Boston, MA) was added at 0.1 μCi/ml and the ethambutol, isoniazid and combined isoniazid/ethambutol formulations were incubated at 60°C for 30 minutes, while the rifampicin formulation was incubated at 37°C for 1 hour. After incubation an aliquot was centrifuged at 13000 rpm for 10 minutes through microcon-30 (Millipore, Billerica, MA) filtration devices to separate exterior methylamine. Samples then underwent
scintillation counting for total and exterior methylamine, where the pH gradient is
determined by the equation \( \log \left( \frac{[\text{MeAm}^+]_{\text{in}}}{[\text{MeAm}^+]_{\text{out}}} \right) = \Delta \text{pH} \). This technique utilizes
methylamine as a probe since it is able to cross the membrane in the uncharged form and
becomes protonated once inside the vesicle, rapidly equilibrating across the membrane.
The methylamine concentration gradient therefore reflects the pH gradient.

2.11 Stability Studies

Vesicle size, drug encapsulation, and pH gradients were determined as described
above at various timepoints after incubation at 5°C, ambient temperature, and 37°C over a
period of 8-12 weeks.

2.12 In vitro Efficacy Characterization

2.12.1 Disc Diffusion Assay

Bacterial lawns of BCG containing green fluorescent protein (GFP) were
prepared by swabbing a mid-log phase culture, grown in Middlebrook 7H9 media (Difco,
Sparks, MD) + 50 μg/ml hygromycin (Calbiochem, La Jolla, CA) + 0.5% Tween-80
(BDH, Toronto, ON) to 0.4 to 0.6 units at OD580nm, onto Middlebrook 7H10 (Difco,
Sparks, MD) + 0.5% glycerol (Fisher, Nepean, ON) + 10% OADC (Difco, Sparks, MD)
+ 75 μg/ml hygromycin agar plates. Ten μl of a two-fold serial dilution of free drug or
liposome encapsulated drug was added to filter discs (S+S Biopath Inc, VWR,
Edmonton, AB) and dried before placement on the bacterial lawn. Initial stock
concentrations of drugs were: 1 mg/ml of ethambutol, 200 μg/ml of isoniazid, and 100
μg/ml of rifampicin diluted in PBS pH 7.4 for both the free and liposome drug
formulations. Testing of combined isoniazid and ethambutol was based on 200 μg/ml of
isoniazid, with the appropriate concentration of ethambutol added to correspond to the
EMB:INH ratio of the liposome formulation. Plates were incubated for 18 days at 37°C after which time the zone of inhibition around each disc was measured. Experiments utilizing *M. smegmatis* mc²155 were incubated at 37°C for three days. Five two-fold serial dilutions of drugs were added to the discs with the highest concentrations being 10 μg, 20 μg, 55 μg and 0.5 μg for the ethambutol, isoniazid, rifampicin and isoniazid (with the appropriate ratio of EMB added) for the combined formulation respectively. Activity of empty liposomes was established by using the same dilution as for loaded vesicles.

2.12.2 MIC Assay

MIC determination was carried out for each formulation, in comparison to free drug, against *M. bovis* BCG. A two-fold dilution series in 1 ml of 7H9 broth + 10% OADC were prepared from stock antibiotic solutions at 1.6 μg/ml of isoniazid, 0.32 μg/ml of rifampicin and 8 μg/ml of ethambutol. A stock solution of 8 μg/ml of ethambutol was also prepared and combined with INH at the same ratio as the combined liposome formulation. All dilutions were inoculated with 10 μl of mid-log phase bacterial culture diluted to 0.22 OD units measured at A₅₈₀nm. Dilutions of empty liposome controls were also set-up with an equivalent concentration of lipid to the respective liposome formulations. All cultures were incubated at 37°C in a shaking incubator over three weeks. MIC values were determined by reading A₆₀₅nm at two and three weeks in Costar 96 well plates (Corning, New York, USA) using a BioRad Model 550 plate reader (BioRad, Hercules, CA). BCG cultures were sealed with mylar plate sealers (Dynex Technologies, Chantilly, VA) before removing from biosafety hood.

The same general procedure was followed for *M. smegmatis* mc² 155 except that cultures were incubated for 3 days at 37°C, after inoculation with 10 μl of culture at 0.2
to 0.5 OD_{580nm} units. Stock antibiotic solutions were prepared at 64 μg/ml of isoniazid, 256 μg/ml of rifampicin and 5 μg/ml of ethambutol. A 5 μg/ml ethambutol stock was prepared as described above for combined EMB/INH testing.

2.13 Intracellular Efficacy Assay

2.13.1 Tissue Culture

THP-1 monocyte cells were cultured in RPMI-1640 media (Sigma, St. Louis, MO) with 10% fetal calf serum (FCS) (Invitrogen, Burlington, MO)/1% L-glutamine/1% penicillin/streptomycin (StemCell Technologies, Vancouver, BC). Before infection cells were differentiated with the addition of phorbol myristate acetate (PMA) (Sigma, St. Louis, MO) at 20 ng/ml and plated at 0.75×10^6 cells per well in 24 well culture plates (Corning, New York, USA), followed by overnight incubation at 37°C and 5% CO₂. Cells were subsequently washed three times with Hanks buffered salt solution (HBSS) (Sigma, St. Louis, MO) and 1 ml fresh RPMI/1% L-glutamine/10% FCS (no antibiotics) was added. Cells were left to rest at 37°C/5% CO₂ in fresh media for 1 hour before infection for the 48-hour peak serum experiments and for 48 hours for the other experiments.

2.13.2 Infection

After resting, cells were infected in the presence of 10% human serum, to opsonize bacteria, at a ratio of 50:1 (M. bovis BCG:THP-1 cells) for 48 hour experiments and at a ratio of 5:1 for 1 week experiments. Cell viability was observed for an uninfected well by noting the extent of cell adherence. Bacteria was cultured in 7H9 media + 50 μg/ml hygromycin, protected from light, with shaking at 37°C (Lab-line ORBIT Environ Shaker, H.Hermann Technologies, BC). Before infection the A_{580nm} was
determined to ensure that the culture was in mid-log phase before use (0.4 to 0.8 absorbance units at 580 nm). The required volume of culture was then spun down at 14000 rpm (EBA 12, Hettich, Zentrifugen, Rose Scientific, Edmonton, AB) for 5 min and resuspended in RPMI-1640/1% L-glutamine. After removal of media, bacteria were added to the THP-1 cell monolayer in a total volume of 1 ml RPMI-1640/10% human serum/1% L-glutamine per well. The infection mixture was incubated for 3 hours at 37°C/5% CO₂.

2.13.3 Antibiotic Treatment

After infection, cells were washed 3 times with HBSS to remove uningested bacteria and dead cells. One well of infected cells was lysed in 0.1 ml of 1% Triton X-100 (Sigma, St. Louis, MO) in PBS for 10 minutes. Cells were then diluted in 7H9 media and plated to determine colony-forming units (CFU) at time zero. Liposomal or free antibiotic solutions, empty liposomes, or control media solutions were then added at appropriate concentrations in 1 ml RPMI-1640/1% L-glutamine/10% FCS and incubated for 48 hours at 37°C/5% CO₂.

For 48 hour and 1 week serum level experiments, liposomal and free drugs were added at the same concentrations, where concentrations added corresponded to maximum serum concentration levels. Ethambutol was added at 6 and 12 μg/ml, isoniazid at 2.5 and 5 μg/ml, and rifampicin at 5 and 10 μg/ml. Previous experiments have been conducted at similar drug concentrations. For the 48 hour sub-MIC experiments, ethambutol was added at 0.5, 1, 2, and 5 μg/ml, isoniazid was added at 0.05, 0.1, 0.5, and 1 μg/ml, and rifampicin was added at 0.25, 0.5, 1, and 2 μg/ml. To test the combined formulation, isoniazid was added as above, with ethambutol added at the appropriate
concentration to reflect the INH:EMB ratio of the combined liposome formulation. After a 48 hour incubation, treatments were washed off and cells were either lysed and plated or fresh media without antibiotics was added and incubated for another 5 days at 37°C/5% CO₂, as conducted by Majumdar et. al.⁶⁴

2.13.4 Cell Lysis and CFU Determination

Following incubation, adherent cells were lysed as above. Plates were viewed under the microscope to confirm efficient removal. Cells were then sonicated (Fisher 60 Sonic Dismembrator, Fisher Scientific, Edmonton, AB) twice at 5 Hz for 10 seconds in a waterbath to disrupt aggregates and ensure lysis of macrophages. Serial dilutions were prepared in 7H9 media and the appropriate dilutions plated onto 7H10 + 10% OADC + 50 μg/ml hygromycin. Viable bacteria were counted after a 18-22 day incubation at 37°C to calculate CFU.

2.14 Fluorescence Microscopy

Fluorescence microscopy was used to determine the localization of liposomes and bacteria after addition to the THP-1 cell monolayer in 24 well plates. The THP-1 infection model used to determine CFU after drug treatment was followed in this set of experiments except that the cells were not lysed for plating. Instead, cells were initially plated on coverslips in each well. Following treatment timepoints of 3 hrs, 24 hrs, 48 hrs, and 1 week, the cells were washed three times with HBSS, followed by incubation with 0.3 ml of 2% paraformaldehyde (Fisher Scientific, Edmonton, AB) (prepared in PBS to pH 7-7.4) at 37°C for 20-30 minutes. After incubation, cells were washed two times with HBSS for 5 minutes each. This was followed by a quick wash with distilled water, after which time the coverslips were removed from the wells and placed cell layer down onto a
drop of fluorosave (Calbiochem-Novabiochem Corp, La Jolla, CA) on a glass slide. Slides were then viewed using a fluorescent microscope (Zeiss Axioplan 2 Imaging, Zeiss, Thornwood, NY), with the filters set for GFP and Texas Red to observe the M. bovis BCG and liposomes respectively. Northern Eclipse 6.0 Software (Empix Imaging Inc., Missisauga, ON) was then used to merge the images to observe any co-localization and to edit the image.

Empty and rifampicin loaded liposomes were prepared as previously described except that 0.5 mol percent Lissamine-rhodamine labelled phosphatidylethanolamine (Molecular Probes, Toronto, ON) was incorporated into the liposomes during lipid solubilization. These liposomes did not undergo concentration but were dialysed overnight at ambient temperature, against 20 volumes of 115 mM Na₂SO₄/50 mM Na₂HPO₄ pH 7.5 to remove solvent, with one buffer change after 4 hours. Presence of the fluorescent tag was confirmed by fluorescent microscopy and its stability was measured over a one week period by quantitating the relative fluorescence of a sample using a fluorescent microplate reader (Spectramax Gemini XS, Molecular Devices, Sunnyvale, CA). Liposomes were also passed over 1 ml Sephadex G-50 (Sigma, St. Louis, MO) spin columns, prepared in 1 ml BD syringes (VWR, Edmonton, AB) in exterior buffer. Two hundred microlitres of sample was added to the top of the columns and spun at 2000 rpm for 3 minutes. Samples recovered should contain only fluorescent lipid associated with the liposomes and remove any tag that may have been cleaved off under the acidic conditions of vesicle preparation.
2.15 Statistical Analysis

GraphPad Prism 2.0 Executable (Graphpad Software Inc., San Diego, CA) was used for statistical analysis by the two-tailed student’s paired t-test.
3.0 FORMULATION DEVELOPMENT

3.1 Introduction

Liposomes are particularly desirable as drug delivery systems as they are biodegradable, biocompatible, and are relatively non-toxic.\textsuperscript{102} They also have the ability to sustain release of the drug, altering the pharmacokinetics and pharmacodynamics.\textsuperscript{10,51} In this project, we have chosen to focus on the development of liposomes that are not formulated to specifically interact with a certain cell type, but that will be passively targeted to the site of infection by pulmonary administration. The aim is therefore to deliver antibiotics to macrophages and sustain release at concentrations greater than the MIC within infected cells. Altering treatment in this manner will enable increased dosing intervals, leading to increased patient compliance.

3.1.1 Drug Loading

Two general modes of drug encapsulation, passive or active, may be considered when developing a liposome formulation. Passive encapsulation is achieved by co-dispersion of the drug and lipid and entrapment occurs due to hydrophilic, hydrophobic, and electrostatic interactions.\textsuperscript{78} For example, one method of passive encapsulation is to hydrate a drug/lipid film in buffer. Typically, liposome drug formulations of water-soluble drugs, like ethambutol and isoniazid, prepared by this mode result in encapsulation of 1-59%.\textsuperscript{78} However, up to 100% encapsulation of lipid-soluble drugs can be achieved. Rifampicin, which is partially lipid soluble, has been previously encapsulated up to 86% using passive trapping techniques.\textsuperscript{76}

For this project we have chosen an active loading, or remote loading, method. This entrapment procedure can be used for drugs that have ionophilic or cationic
characteristics since they can be charged and move into the liposome in response to an ionic gradient. It is therefore unrestricted in regards to drug class and is generally useful. Specifically, in this project weak bases are loaded into the interior compartment of pre-formed vesicles in response to a pH gradient, where the interior is acidic (Figure 4). When the exterior pH is slightly lower than the drug pKa, the neutral form may pass through the bilayer, where it is subsequently protonated inside. Therefore, the range of the pH gradient is the driving force of drug loading and may result in 100-fold greater encapsulation than passive trapping techniques. For example, a 3-unit pH gradient can generate a 1000X drug concentration gradient.

3.1.2 Liposome Characteristics Affecting Entrapment

Four main characteristics: lipid composition, charge, vesicle size, and surface moieties, dictate the ability of vesicles to entrap drug, as well as the clearance and distribution of drug within the body. Upon administration, lipid dose and the route of administration also play a role. Although remote and passive loading allow variation of all of these parameters, remote loading leads to much higher drug/lipid ratios than passive trapping methods. Although efficacy and toxicity ultimately govern the drug to lipid ratio, high ratios are preferred by the pharmaceutical industry and optimization includes determining the highest drug/lipid ratio possible.

3.1.2.1 Lipid Composition and Charge

The first characteristic, lipid composition, affects drug incorporation, drug release, and effector activity. Encapsulation of charged drugs may be improved by incorporation of oppositely charged lipids into the bilayer to provide points of ionic
Figure 4: Scheme of remote loading of weak acids in response to a pH gradient. The internal pH is lower than the exterior pH.
interaction. Effector activity may also be enhanced by the addition of negatively charged lipids to enhance uptake of liposomes by certain cell types, as has been demonstrated for macrophages.\textsuperscript{108} Finally, drug release can be changed by lipid choice as it may alter the structural integrity of the membrane,\textsuperscript{81} perhaps increasing resistance to protein absorption or enzyme attack. Therefore, it is important to consider the desired speed of release, as well as the site of action, when developing a liposome formulation. For this thesis, a composition of sphingomyelin/cholesterol (63/37, mol/mol) was chosen since this combination is highly ordered, preventing rapid drug release.

3.1.2.2 Vesicle Size

Vesicle size determines internal volume, and is proportional to the amount of drug that can be entrapped within the interior. Vesicles in the range of 100-150 nm, as used in this thesis, have approximately 1-3 $\mu$L/mol phospholipid trapped volume.\textsuperscript{78} Size may also play a role in cell interaction \textit{in vivo},\textsuperscript{73} where decreasing vesicle size results in lower concentrations being taken up by the liver.\textsuperscript{78} Finally, vesicle size is important in the production of formulations since smaller vesicles (generally 170 nm or smaller) are easier to filter sterilize through 0.22$\mu$m, a necessity for \textit{in vivo} studies and eventual use as a pharmaceutical product.

3.1.2.3 Surface Moieties

As mentioned previously, the addition of surface moieties, such as antibodies, will increase directed targeting to the site of interest \textit{in vivo}. However, the formulations in this study were designed to passively target the disease site. Local delivery will be accomplished by pulmonary administration.
3.1.3 Formulation Development and Optimization Rationale

The above characteristics were considered during the development of the formulations in this thesis, for potential application to in vivo efficacy, toxicity, and pharmacodynamics and pharmacokinetics. However, each characteristic was not varied specifically since direct applicability to in vivo studies is beyond the scope of this investigation. Instead, the development of the liposomal drug formulations for this thesis was directed at optimizing drug encapsulation to achieve higher drug potency.

For remote drug loading, the procedure chosen for this series of investigations, factors that influence formulation potency include: the size and stability of the pH gradient, buffer composition (buffering capacity), loading kinetics, lipid composition, and drug/lipid ratio. Studies involving each of these characteristics were carried out during formulation development and will be discussed further in this chapter. Characterization of the release pattern of the drug in vitro will also be illustrated and related to potential in vivo effects. Finally, stability studies were conducted on the formulations presented here in order to evaluate their potential success as pharmaceutical products.

3.2 Results

3.2.1 Formulation Development and Optimization

3.2.1.1 Remote Loading

As mentioned, remote loading was chosen in order to maximize drug potency for all formulations. Traditional remote loading procedures require the use of a citrate buffer, with an interior pH of 4, and an exterior pH of 7-8, to provide the pH gradient. This procedure was successfully utilized in an experiment with ethambutol, resulting in
90% encapsulation. However, attempts to encapsulate rifampicin using this approach did not result in significant encapsulation (0-66%), and in many cases a drug precipitate was observed. In the case of isoniazid, the citrate buffering system will not work since the pKa of the drug is approximately 5.5, only 1.5 units above the interior pH, resulting in a gradient insufficient to allow substantial drug loading.

Utilizing the remote loading procedure with sulfuric acid maximizes the pH gradient and enables loading of drugs with lower pKas such as isoniazid. For example, a gradient of 4.5 units may be established using an exterior pH of only 4.5 units, still below the pKa of isoniazid. For rifampicin and ethambutol, which both possess basic pKas, a gradient of 7 units can be established. These conditions were found to be optimal for rifampicin. This system was also chosen for ethambutol since sulfuric acid provides greater buffering capacity, and therefore a larger residual pH gradient than citrate, characteristics linked to trapping efficiency. It has been previously shown that residual gradients less than 2 units result in rapid drug release. In our hands, increasing the buffering capacity of sulfuric acid by increasing the molarity from 0.5 M to 0.75 M or 1 M had no effect on the percent encapsulation of ethambutol.

3.2.1.2 Drug/Lipid Ratio

To further optimize the encapsulation of each drug, the effect of varying drug/lipid ratio on encapsulation was determined. As expected, the percentage of entrapped drug, in relation to the amount added, increases with increasing lipid concentration (Table 3). This is a common trend since higher concentrations of lipid in relation to drug provides increased points for interaction and increased vesicle numbers and therefore a greater volume of aqueous space for entrapment. For isoniazid, 95%
<table>
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<th>Drug/Lipid Ratio (mol/mol)</th>
<th>Encapsulation* (%)</th>
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<td></td>
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ND: not determined; *% reported as mean % encapsulation ± SEM when n>2 and mean % encapsulation ± sd when n=2

Table 3: Isoniazid, rifampicin, and ethambutol loading in response to varying drug/lipid ratios. Isoniazid and ethambutol were loaded at 60°C for 30 minutes and rifampicin was loaded at 37°C for one hour. (n) represents the number of replicate experiments. The 30 minute time-point at 60°C for ethambutol corresponds to the data reported in Table 4.
encapsulation was achieved at a drug/lipid ratio of 1:15, whereas 94% encapsulation was observed for the same drug/lipid ratio for rifampicin (Table 3). Ethambutol was encapsulated at higher drug/lipid ratios than the other drugs, being 74% entrapped at a drug/lipid ratio of 1:1, and 83% encapsulated at 1:3, the drug/lipid ratio chosen for this thesis (Table 3). For all three drugs, these levels were considered to be adequate, leaving minimal unencapsulated drug to be removed, and were chosen for further development.

As observed for the three drugs investigated, the degree of loading is not entirely dependent on the drug/lipid ratio or the established pH gradient. For formulations of a given pH gradient and lipid composition, the maximal loading capacity of drugs may be different. These differences may be in part be explained by the membrane-water partition coefficient of the drug, where increasing coefficients lead to increased drug loading. The membrane water coefficient is dependent on the properties of the drug (such as lipophilicity), and is a measure of the partitioning of the amine between the bilayer and the aqueous space. Drug partitioned in the membrane does not contribute to the concentration gradient and therefore higher drug concentrations can be loaded. This effect may in part explain loading of ethambutol at a drug/lipid ratio of 1:3 and isoniazid at a ratio of 1:15, where ethambutol may be more lipophilic than isoniazid. A study characterizing the position of isoniazid in the membrane of DMPC and DMPG vesicles found that isoniazid is located in the aqueous interior space. Ethambutol also possesses two amine groups that may be protonated and therefore drive drug loading to a greater extent than the single amine group of isoniazid.

As for isoniazid, the position of rifampicin in the bilayer has been characterized. However, in the case of rifampicin, it was found to be located partially in the bilayer and
partially in the aqueous space. This may account for slightly greater encapsulation at 1:15 than observed for isoniazid. Zwitterionic drug loading may also be dependent on the basic pKa relative to intraliposomal pH. If the former is high, and the latter low, the amine group should be nearly 100% protonated on the exterior and the drug will therefore act as a weak base. This should be true for rifampicin, where the pKa of the amine is 8.8.

3.2.1.3 Combined Formulation Optimization

Encapsulation of two drugs within the same liposome formulation is more difficult, as the buffering capacity is smaller in relation to each drug individually. In this study, isoniazid and ethambutol were chosen since they were relatively easy to solubilize and substantial encapsulation was achieved in separate formulations. However, these two drugs also vary considerably in regards to their pKas, where ethambutol has two pKas above 8.5, and isoniazid has a pKa of approximately 5.5. Preliminary experiments attempting to encapsulate each drug at the ratios established for the individual formulations were unsuccessful over an exterior pH range of 3.5-7.5. Under these conditions isoniazid did not remain associated with liposomes at pH values greater than 5.5. However, control experiments revealed that the pH was not responsible for this trend since isoniazid alone remained associated with liposomes in the absence of ethambutol at high pH. Therefore, a set of experiments was designed to characterize encapsulation at pH 4.5, 6.0, and 7.5, while varying the drug/lipid ratio of ethambutol (Figure 5).

At an exterior pH of 4.5, the encapsulation was initially high for isoniazid, but decreased as the amount of ethambutol increased, while the opposite pattern was
Figure 5: Encapsulation of combined isoniazid and ethambutol in response to varying drug/lipid ratio at varying exterior pH (n=1). (A) pH 4.5; (B) pH 6.0; (C) pH 7.5. Ethambutol is represented by the triangle, isoniazid by the square. Circle indicates the point of optimal encapsulation. X-axis represents the drug/lipid ratio of ethambutol to total lipid. Isoniazid was maintained at a drug/lipid ratio of 1:15. Loading was carried out at 60°C for 30 minutes.
observed for ethambutol (Figure 5). A similar trend was observed at pH 7.5 (Figure 5). However, if the exterior pH was adjusted to pH 6, a compromise between optimal loading conditions for the individual drugs, greater than 80% encapsulation was achieved at a 1:15 drug/lipid ratio for each drug (Figure 5). This formulation was used in subsequent experiments.

3.2.1.4 Loading Kinetics

Loading kinetics may alter the final encapsulation reached since the rate and extent of uptake are dependent on temperature. An experiment was carried out for each drug formulation to evaluate the conditions for optimal loading. As such, encapsulation was measured over 2 hours at ambient temperature, 37°C, and 60°C (Table 4). In the case of rifampicin, it was found that the drug itself was unstable at 60°C, as observed by a change in colour from orange to dark brown. This may be expected from reports of limited stability in vitro. The optimal loading conditions were determined to be one hour at 37°C, where encapsulation peaked at 93% (Table 4). For ethambutol, optimal loading occurred within 15 minutes at 60°C and isoniazid was loaded at greater than 90% at both 37°C and 60°C (Table 4). Drug loading typically requires elevated temperatures to relax the rigidity of the membrane, allowing increased drug movement through the bilayer. The same optimal loading kinetics were also observed at other drug/lipid ratios.

3.2.1.5 Formulation Optimization Summary

After optimization of the properties discussed (Table 5), all three drugs were formulated in a 63/37 molar ratio of sphingomyelin/cholesterol (SM/CHOL), both neutral lipids. The optimized liposome formulations developed in this study show encapsulation of 76-92% for ethambutol, 88-100% for isoniazid, greater than 90% for rifampicin, and
<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time(min)</th>
<th>Encapsulation (%)* (n)</th>
<th>Rifampicin (n)</th>
<th>Isoniazid (n)</th>
<th>Ethambutol (n)</th>
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<td>92 (1)</td>
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</table>

*reported as mean % encapsulation ±SEM when n>2 and mean % encapsulation ± sd when n=2.

Table 4: Loading kinetics of rifampicin, isoniazid, and ethambutol at varying time and temperature. The drug/lipid ratios used were 1:15 for rifampicin and isoniazid, and 1:3 for ethambutol. (n) represents the number of replicate experiments. The 30 minute time-point at 60°C for ethambutol corresponds to the data reported in Table 3.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug-Lipid Ratio (mol/mol)</th>
<th>% Encapsulation</th>
<th>Maximum Achievable Drug (mg/ml)</th>
<th>Interior pH:</th>
<th>Exterior pH:</th>
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<tr>
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</table>

*Based on 100 mg/ml of total lipid

Table 5: Summary of physical characteristics of liposome-encapsulated first-line anti-tuberculosis drugs
78-100% for both ethambutol and isoniazid in the combined formulation (Table 5), when formulated in vesicles ranging from 125 to 145 nm.

3.2.2. In vitro Drug Release

The ability of liposomes to release drug in vitro was characterized over 24 hours at 37°C, in the presence or absence of 50% mouse serum (v/v), as a source of proteins such as HDL, LDL, and apoprotein, or 10 mM ammonium sulfate, as a means of dissipating the pH gradient (Figure 6). As shown in figure 6, >90% of all encapsulated antibiotics remained associated with the liposomes upon dilution, in the absence of serum. This is expected since there is no agent present that would act to dissipate the transmembrane pH gradient and the membrane potential is maintained. Therefore, the drug remains associated with the vesicles. This suggests that the lipid composition chosen remains tightly packed at 37°C, maintaining the residual pH gradient and preventing ion transport, in this case drug, across the bilayer in the absence of an agent that interacts with the lipid bilayer.

In the presence of serum, where lipid-protein interactions can occur between the bilayer and serum proteins, approximately 25%-30% drug release was measured for rifampicin, isoniazid, and the combined formulation, while ethambutol showed <25% release (Figure 6). Release may therefore be dependent on protein-lipid interactions, a proven mechanism of drug release from liposome formulations. Leakage due to lysis upon changes in the osmotic environment, amplified in the presence of serum, may also occur. The presence of serum proteins was used to give an indication of the effect of in vivo conditions where liposomes may encounter proteins systemically, in the tissues,
Figure 6: *In vitro* drug release at 37°C. All *in vitro* release experiments were conducted at 37°C over 24 hours. Each formulation was incubated alone (square), with 50% serum (triangle), or with 10 mM ammonium sulfate (circle). (a) *In vitro* release of ethambutol. (b) *In vitro* release of isoniazid. (c) *In vitro* release of rifampicin. (d) *In vitro* release of combined isoniazid (closed symbols) and ethambutol (open symbols). Results represent mean % release ±SEM (n=3). SEM is smaller than symbol where no error bars are visible.
or as a component of surfactant in the lung. These studies illustrate the potential for these systems to sustain release in the lung compartment, following pulmonary administration.

Finally, addition of ammonia, which collapses the pH gradient, leads to spontaneous release (90-100% within 4 hours) of isoniazid and rifampicin, while the combined formulation reaches approximately 75-80% release in the same time period (Figure 6). Ethambutol, however, only showed 25% release over 24 hours (Figure 6). Ammonia addition is equivalent to passively trapping drugs at neutral pH where no gradient is established to maintain drugs within the vesicles.

3.2.3 Formulation Stability

There are two categories of stability that should be considered when developing a formulation: physical, pertaining to the size and drug retention properties of the liposomes, and chemical, referring to drug degradation and lipid oxidation and hydrolysis.

The physical stability of these formulations was characterized over a period of up to 4 months, where the vesicle size and drug retention (measured in percentage encapsulation) were quantified at 5°C, ambient temperature, and 37°C (Table 6). The residual pH gradient was also measured as an indication of potential drug release at some timepoints but is not represented in Table 6.

The ethambutol formulation appears to be stable over 4 months at 5°C since there is no change in size, encapsulation, or pH gradient (Table 6). However, at higher temperatures instability is observed by 4 months, as indicated by 55% and 100% drug release at ambient temperature and 37°C respectively (Table 6). The combined
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</table>

*ND=not determined
**NA=could not be determined accurately by assay due to drug precipitate

Table 6: Physical stability of liposome formulations at 5°C, ambient temperature, and 37°C (n=1).
formulation, which was incubated at 5°C, shows the same trend as the ethambutol formulation (Table 6). The isoniazid formulation appears to be slightly less stable than the ethambutol formulation, showing 12% release by the fourth month at 5°C although no vesicle size change is noted (Table 6). However, at ambient temperature and 37°C, the drug encapsulation is below 90% within one month (Table 6). Although no change in vesicle size is observed at ambient temperature, the vesicles appear to have a tendency to fuse within 2 months at 37°C, increasing to 162 nm (Table 6). The rifampicin formulation appears to be the least stable, releasing 25% of drug within 3 months at 5°C, and greater than 30% within one month at higher temperatures (Table 6). The vesicle size only appears to be stable at 5°C (Table 6).

The chemical stability of these formulations was not addressed in this study, but the isoniazid formulation turned yellow after 4 months at ambient temperature and 2 months at 37°C, indicating some instability. Instability was also evident in the rifampicin formulation as it changed colour from red/brown to dark brown at elevated temperatures within one month. Lipid degradation was not measured but analysis of liposome formulations of the same composition, prepared in 1 M sulphuric acid, showed no degradation over two months at ambient temperature.\textsuperscript{78}

3.3 Discussion

Rifampicin, isoniazid, and ethambutol were chosen based on their use in first-line therapy, as well as their potential for liposome encapsulation using remote loading. Each drug also has a relatively short half-life \textit{in vivo}, which would be favourably increased upon liposome encapsulation.
All three drugs tested in these studies were formulated in a 63/37 molar ratio of sphingomyelin/cholesterol (SM/CHOL), both neutral lipids. Cholesterol is known to decrease acyl chain mobility, lateral chain packing density, and membrane free volume by packing within spaces of the bilayer,\textsuperscript{100} which decreases sites for protein absorption.\textsuperscript{13,102} It therefore increases the order of the bilayer and acts as a barrier to the transport of drug from liposomes.\textsuperscript{21} Sphingomyelin also provides rigidity and prevents fusion.\textsuperscript{21} Together, these characteristics optimize stability by minimizing interaction with proteins, thereby preventing rapid destabilization and breakdown of the liposome \textit{in vivo} and prolonging drug release.\textsuperscript{63} In general, neutral lipids also tend to interact less with proteins than charged lipids due to the absence of charge interactions.\textsuperscript{103}

The primary goal during initial optimization of liposome formulations is drug encapsulation. It is a critical step when developing formulations since it leads to greater drug potency and high deliverable concentrations with minimal use of excipient. This is of particular importance for pulmonary delivery, since small volumes reach the lung, the site of primary tuberculosis infection. The formulations of rifampicin and isoniazid presented here show better encapsulation and drug/lipid ratios in comparison to previously reported formulations,\textsuperscript{1,11,12,25,42} where encapsulation reached 45% and 86% for isoniazid and rifampicin respectively, while our formulations encapsulated at least 76% of available drug and typically exceeded 90% encapsulation (Table 5). This demonstrates that concentration gradients can be generated that exceed the concentrations achieved by passive loading. No previous formulations of ethambutol have been described to our knowledge.
It is also interesting that the same type of liposome formulation can be altered to allow loading of two drugs at approximately 80% encapsulation of each drug (Table 5). The pH in this case was adjusted to pH 6, a compromise between the optimal exterior pH for each separate drug. Increasing or decreasing this pH resulted in lower encapsulation of either drug (Figure 5). The drug/lipid ratio of ethambutol was also decreased to 1:15, equal to that of isoniazid. At all exterior pH values that were used, a greater ratio of ethambutol than isoniazid resulted in negligible encapsulation of isoniazid.

Combining two drugs into one formulation may increase efficacy simply by achieving delivery of two drugs to the same macrophage simultaneously. Alternatively, two liposome formulations, each containing a different drug, may be combined. Combining drugs in either way may simplify administration enough to improve compliance to tuberculosis therapy. Isoniazid and ethambutol were chosen for developing a combined formulation since they are both aqueous soluble and therefore easier to work with. However, a beneficial combination that may merit further investigation would be isoniazid and rifampicin, the two most common and potent drugs used to treat tuberculosis.

The proportion of loading achieved in our formulations also corresponds to the MIC values for each drug. For example, the MIC of ethambutol is 5-10 times greater than that of isoniazid, which corresponds to the ten-fold higher maximum achievable drug concentration for the ethambutol formulation. The same trend holds true for the combined formulation. In all cases, at a lipid concentration of approximately 100 mg/ml, drug concentrations at least 1000x greater than the MIC of each drug was achieved (Table 5).
Prolonged drug release is a critical factor linked to in vivo efficacy, since it prevents toxicity in surrounding tissue and increases drug concentrations at the disease site. Release from liposomes typically results upon interaction with proteins and enzymes, and may result from degradation in the lysosome by lysozymes that consequently release drug into the cytoplasm. Release may also occur under hyperosmotic conditions when liposomes undergo partial lysis to release osmotic pressure, or by drug diffusion through the bilayer. Remote loaded formulations typically release drug more slowly than passively entrapped drugs, since dissipation of the pH gradient is required and was an important factor in choosing this method of loading.

It is encouraging that the in vitro release data shows that all four liposome formulations have the potential to sustain release at 37°C. Only 20-30% of drug is released over 24 hours when incubated with 50% serum (Figure 6). These results compare favourably to previous liposomal rifampicin formulations. A PC/CHOL/dicetylphosphate formulation prepared by Kamath et al. released 98% of the drug within 20 hours in PBS (Kamath), while a PC/CHOL formulation prepared by Deol and Khuller showed 36% release within 4 hours in the presence of 20% serum. Since current aqueous formulations of rifampicin, isoniazid and ethambutol have a maximum t_{1/2} of 3 hours in vivo, these results suggest that liposome encapsulation will favourably alter in vivo pharmacokinetics, sustaining drug release and potentially altering biodistribution following pulmonary administration.

The stability of the formulations appears to be acceptable over 2 months at 5°C for rifampicin, and for longer periods for the other formulations. The ethambutol and
combined formulations in particular show no signs of drug release over four months at 5°C (Table 6). All three formulations show signs of instability at 37°C, as evidenced by drug release within one month (Table 6). However, if stored refrigerated at 5°C, the formulations should be stable for extended periods. Stability may also be improved by lyophilization, which would require further studies to ensure that drug is not released during this process and that vesicle size is maintained upon rehydration. A powder form may also be amenable to inhalation therapy.

The lipid composition was not altered since the sulfuric acid remote-loading procedure would cause degradation of lipids other than SM, which is more resistant to hydrolysing conditions. In addition, the stability of this composition, which minimizes leakage, is desirable to ensure that the formulations resist drug release before reaching the deep lung during pulmonary administration. One consideration, which may be investigated in the future, is the incorporation of negatively charged lipids such as dicetylphosphate, cholesterol sulfate (CHS), or phosphatidic acid (PA). These lipids have been shown to increase uptake by cells such as alveolar macrophages and may be beneficial for increased targeting in vivo.

Although the vesicle size was not varied in these experiments, the range of 120-150 nm was chosen to optimize the internal trap volume space, and therefore drug entrapment, while still resulting in a formulation capable of being filter sterilized. Filter sterilization is important for quality control during tissue culture experiments but is ultimately important for pharmaceutical processing during scale-up, where terminal sterilization is required. These vesicles are also favourable for passive targeting to the RES, since the most effective liposome formulations are medium sized, negatively
charged vesicles. Finally, this size will also minimize vesicle disruption during nebulization since it is significantly smaller than the typical droplet size.

Together, these results suggest that these formulations have the potential to be an alternative therapy for tuberculosis and that they may be suitable for administration by pulmonary delivery.
4.0 \textit{IN VITRO} EFFICACY TESTING

4.1 Introduction

4.1.1 Drug Development

There is a need for new treatments against \textit{M. tuberculosis}. The majority of anti-TB agents still in use today were developed between 1945-1961, and resistance to these antibiotics is becoming an increasing problem. In particular, multi-drug resistant bacteria are often simultaneously resistant to both isoniazid and rifampicin, the main drugs used to fight TB.\textsuperscript{90} Resistance becomes a particular problem when treating chronic TB infections, since drugs have to be taken daily or several times weekly over 6 months to one year and compliance is often lacking as a result.

Recent research has introduced new potential agents with activities significantly higher than rifampin, the most potent TB drug. For example, rifapentine was recently approved by the FDA, making it the first new TB drug in 25 years. It is efficacious when taken once weekly, starting in the second month of an eight-month short-course therapy regimen.\textsuperscript{90} However, rifapentine and other newer drugs belong to the same class as rifampicin (a rifamycin), and cross-resistance has already been observed.\textsuperscript{95}

Since development of new classes of antibiotics with novel targets is a long and costly process, it is beneficial to develop new delivery systems using available antibiotics that have proven activity against \textit{M. tuberculosis}. Therefore, we propose to encapsulate rifampicin, isoniazid, and ethambutol into liposomes to prolong their release \textit{in vitro}, thereby decreasing dosing in comparison to the current treatment regimen. This should increase compliance and decrease resistance, thereby improving the cure rates of TB infected patients.
4.1.2 In vitro Screening

*In vitro* testing is an integral primary screening method to determine if a drug has acceptable efficacy for development continuation. Since the drugs used in this thesis are active against the target, *M. tuberculosis*, *in vitro* efficacy testing was carried out to verify the effectiveness of each drug following encapsulation. *M. bovis* BCG, the vaccine strain used for TB, was used as a model organism since it has a similar genome, generation time, and antibiotic susceptibility pattern to *M. tuberculosis*.

Disc diffusion is a relatively simple method used to determine the ability of a drug to inhibit its target. Filter discs holding specified drug concentrations are placed on a solid agar plate swabbed with the bacteria of interest. Inhibition is then noted by the size of clearing around the disc, corresponding to a gradient of drug concentration. Minimal inhibitory concentration (MIC) testing was utilized to confirm the results of the disc diffusion assay. This method determines the efficacy of a drug against bacteria growing in solution by determination of the minimum concentration of drug that inhibits the growth of the bacteria. The lowest concentration of drug resulting in no noticeable growth is recorded as the MIC. The above methods are useful tools for primary screening and were carried out to compare the activity of encapsulated drug in comparison to unencapsulated drug. However, they do not give any indication of the effectiveness of drugs against the target when it is in its intracellular environment.

To address intracellular efficacy we set-up a tissue culture model using THP-1 cells, a human monocyte cell line that can be differentiated to a macrophage-like cell-line upon addition of phorbol myristate acetate (PMA). These cells were infected with *M.
bovis BCG and subsequently treated with appropriate drug formulations. We were then able to quantitate intracellular efficacy by determining colony-forming units (CFU).

To supplement these findings, fluorescence microscopy was performed with rhodamine labelled liposomes and bacteria expressing green fluorescent protein (GFP). Experiments using these tools were conducted to determine if the liposome and bacteria localized to the same intracellular compartment and to ensure that the liposomes were being taken up under the conditions provided in this assay.

4.2 Results

4.2.1 In vitro Extracellular Efficacy

4.2.1.1 Disc Diffusion Assay

Results using the model organism M. bovis BCG showed no significant differences in the activity of encapsulated rifampicin (p=0.32-0.81), isoniazid (p=0.68-0.72), or ethambutol (p=0.16-0.6) in comparison to the free drugs (Figure 7), as demonstrated by the diameter of bacterial clearing on an agar plate. The activity of the combined isoniazid and ethambutol formulation also showed equivalent efficacy (p=0.74-1) in comparison to the same combination of free drugs on filter discs (Figure 7). Empty liposome controls did not show any inhibitory activity against M. bovis BCG when added to the filter discs. Similar results were also observed when the efficacy of each formulation was tested against the faster growing M. smegmatis mc² 155. This demonstrates that efficacy is maintained when each drug is encapsulated within liposomes.
Figure 7: *In vitro* efficacy against *M. bovis* BCG measured by the disc diffusion assay. Liposome encapsulated antibiotics (closed triangle) were compared to free antibiotics (closed square). Empty vesicle control showed no activity (results not shown). (A) Efficacy of Ethambutol (n=4). (B) Efficacy of Isoniazid (n=3). (C) Efficacy of Rifampicin (n=3). (D) Efficacy of Combined Ethambutol and Isoniazid (n=3). Results are reported as mean diameter ±SEM.
4.2.1.2 MIC Determination

The minimal inhibitory concentrations determined by the macrobroth dilution assay confirmed the results of the disc diffusion assay, showing that the activities of the liposome encapsulated drugs against *M. bovis* BCG were equivalent to free drugs (Table 7). Ethambutol inhibited the bacteria at 2-4 μg/ml after two weeks of growth for each form of the drug (Table 7). Isoniazid showed a two-fold difference in the MIC concentration range, having a free drug MIC of 0.1-0.2 μg/ml and an encapsulated drug MIC of 0.1-0.4 μg/ml (Table 7). The combined formulation showed the same trend as the isoniazid formulation. In this combination the MIC of isoniazid remained the same but that of ethambutol was decreased by approximately ten-fold in combination. The MICs determined for rifampicin in free form and encapsulated form were 0.008-0.015 μg/ml and 0.008 μg/ml respectively (Table 7). The minimal inhibitory concentrations determined from this assay also correspond to known MIC ranges, where the MIC of ethambutol is 1-5 μg/ml,\(^1\) the MIC of isoniazid is 0.01-1 μg/ml,\(^2\) and the MIC of rifampicin is between 0.016 μg/ml-0.25 μg/ml in 7H11 media, depending on the strain used.\(^3\)

As with the disc diffusion assay, the empty control liposome formulations did not show any inhibition of *Mycobacterial* growth in the MIC broth assay. MICs against *M. smegmatis* \(^4\) 155 were also equivalent when comparing free drug and liposome encapsulated drug. Since the media used for both assays contains ammonia at sufficient concentrations to dissipate the pH gradient across the liposome bilayer, these experiments demonstrate that the bioavailability of all three drugs is maintained after release.
<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>Free</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>2-4</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>0.1-0.2</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.008-0.015</td>
</tr>
<tr>
<td>Combined Ethambutol</td>
<td>0.13</td>
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<tr>
<td>Combined Isoniazid</td>
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Table 7: MIC of free versus liposome encapsulated first-line anti-tuberculosis agents against *M. bovis* BCG after 2 weeks of incubation at 37°C. Results reported as range from three experiments.
It is also possible that drug is released upon drying on the filter discs used for the disc diffusion assay.

4.2.2 In vitro Intracellular Efficacy

4.2.2.1 48 Hour Peak Serum Level Experiment

To determine if encapsulation of anti-tuberculosis agents enhances delivery to the macrophage, in vitro efficacy was investigated using a THP-1 macrophage infection model. This assay was set-up to determine activity of the formulations against intracellular bacteria over 48 hours. The rifampicin and combined isoniazid/ethambutol liposomal formulations showed significant activity (p=0.0001-0.035) at all concentrations in comparison to the control (Figure 8). Free rifampicin also showed significant activity (p=0.035), while the combination of the free drugs did not significantly reduce the colony forming units (p=0.27-0.42). Neither the free form (p=0.13-0.15), nor the encapsulated form of ethambutol (p=0.1-0.23), significantly inhibited bacterial growth, while encapsulated isoniazid showed significant activity at 5 µg/ml (p=0.01) and at the lower concentration for free drug (p=0.03). Rifampicin showed the highest activity of all drugs.

The concentrations utilized in these experiments were chosen to mimic drug concentrations achievable in serum. A concentration of half of the T_{max} was also chosen for isoniazid (2.5 µg/ml)\(^8\) and rifampicin (5 µg/ml)\(^7\) to determine if the liposomal drug formulation could improve efficacy at lower drug concentrations. Ethambutol was added at the peak serum concentration and two times this amount (6 and 12 µg/ml)\(^5\) as preliminary experiments suggested that it was less permeable to macrophages, or less active, than the other two drugs. The concentrations used for testing combined EMB and INH was based on the peak serum levels of isoniazid, since the ratio of EMB:INH in the
Figure 8: In vitro efficacy against intracellular M. bovis BCG after 48 hours at peak serum drug levels. Empty control liposome treatment showed no activity in comparison to media control shown (polka-dot). (A) Efficacy of encapsulated ethambutol (checkered) and free ethambutol (horizontal lines). (B) Efficacy of encapsulated isoniazid (checkered) and free isoniazid (horizontal lines). (C) Efficacy of encapsulated rifampicin (checkered) and free rifampicin (horizontal lines). (D) Efficacy of combined isoniazid and ethambutol formulation. Results are reported as mean CFU±SEM. (n=3).
combined liposome formulation approximately corresponded to the peak serum level and half of this level for ethambutol.

All four liposome formulations, isoniazid (p=0.11-0.27), rifampicin (p=0.1-0.31), ethambutol (p=0.13-0.16) and the combined isoniazid/ethambutol (p=0.17-0.22) formulations did not show statistically significant differences in efficacy in comparison to free drug after 48 hours of incubation (Figure 8). However, the trends show that isoniazid and ethambutol showed decreased activity (Figure 8A and B) when encapsulated, while rifampicin (Figure 8D) and the combined isoniazid/ethambutol formulation (Figure 8C) showed equal or greater activity than the equivalent concentration of free drug. Empty control vesicles did not show any antibacterial activity.

4.2.2.2 48 Hour Sub-MIC Experiment

Another series of experiments was conducted to determine if the drug concentration levels tested in the previous series of studies led to a saturation effect of drug activity. These experiments were set-up at sub-MIC levels expected against *M. tuberculosis*, where ethambutol, isoniazid, and rifampicin were added at concentrations ranging from 0-5 μg/ml, 0-1 μg/ml, and 0-2 μg/ml respectively. The combined drug treatment was added so that the isoniazid concentration was 0-1 μg/ml, with ethambutol added to achieve the same ratio of INH:EMB found in the combined formulation.

The results after 48 hours of incubation with treatment and immediate cell lysis showed no significant differences at any concentration point between the free and encapsulated forms of isoniazid (p=0.14-0.46) and rifampicin (p=0.15-0.97) (Figure 9). However, the trend suggests that the encapsulated formulation is slightly more
Figure 9: *In vitro* efficacy against intracellular *M. bovis* BCG at sub-MIC concentrations of antibiotic after 48 hours. Empty control liposome treatment showed no activity in comparison to media control shown. (A) Efficacy of encapsulated ethambutol (square) and free ethambutol (triangle). (B) Efficacy of encapsulated isoniazid (square) and free isoniazid (triangle). (C) Efficacy of encapsulated rifampicin (square) and free isoniazid (triangle). (D) Efficacy of combined isoniazid and ethambutol formulation. Efficacy of encapsulated drugs (square) and free drugs (triangle). Results are reported as mean CFU±SEM (n=3).
efficacious then free drug at very low concentrations. That is, in the range of 0-0.25 µg/ml for both isoniazid and rifampicin. However, the same trend is not apparent for the two formulations containing ethambutol. In both the individual ethambutol formulation and combined isoniazid/ethambutol formulation, the trend shows that the liposomal forms of the drug are slightly less efficacious (Figure 9). This trend is not significant for the ethambutol formulations (p=0.12-0.84) except at the 2 µg/ml concentration point (p=0.02) (Figure 9) or at any concentrations of combined INH and EMB (p=0.27-0.66). Therefore, these results suggest that there may be some saturation in drug concentrations, however, they are not significant and therefore would not account for either the lack of differences in efficacy, or decreased efficacy, when comparing liposomal antibiotics to free antibiotics.

4.2.2.3 One Week Peak Serum Level Experiment

Finally, the efficacy of the encapsulated formulations was compared to the free drug over a one-week period to determine if a longer incubation period would permit increased release of encapsulated drug. The increased length of incubation may also have a greater impact on the viability of M. bovis BCG since it will undergo several more rounds of replication during this time. In the analysis of this data, the CFU was compared to the carrier, that is, free drug to media and encapsulated drug to empty liposomes. In each experiment wells containing empty control vesicles showed slightly higher growth than the media controls.
Figure 10: *In vitro* efficacy against intracellular *M. bovis* BCG after 1 week. Efficacy of encapsulated drug (square) and free drug (triangle). Empty control liposome treatments showed increased growth in comparison to media control. (A) Efficacy of ethambutol. (B) Efficacy of isoniazid. (C) Efficacy of rifampicin. (D) Efficacy of combined isoniazid and ethambutol. Results are reported as mean % ±SEM (n=3).
Under these conditions the efficacy of liposomal isoniazid (p=0.19-0.27), rifampicin (p=0.5-0.9) and combined formulations (p=0.39-0.42) were equivalent to the free drugs (Figure 10). However, as in the other intracellular assays, the trend showed that encapsulated ethambutol was less efficacious than free drug, although the result was not significant (p=0.15=0.25) (Figure 10). The data collected for these experiments suggest that sustained release may occur within this period, since the efficacy between the encapsulated and free forms of each drug shows less variation than observed for the 48 hour time point.

4.2.3 Intracellular Localization of Liposomes and Bacteria

Fluorescence microscopy was carried out on *M. bovis* BCG (which expresses GFP) infected THP-1 cells to determine if vesicles reach the same macrophages as the bacteria, or localize within the same vacuoles. Rifampicin loaded vesicles were added to the infected cells and incubated over 3, 24, and 48 hours, as well as 1 week. Empty vesicles and infected cells without liposomes were also visualized as controls.

The bacteria could be visualized under the microscope after the 3 hour infection procedure (Figure 11). Internalization of the liposomes was apparent 3 hours after addition, with liposomes mostly on the edges of the cells. After 24 hours, a more intense fluorescent pattern was seen around the edges of the cell. Fluorescence intensified over 48 hours to one week, with distribution of the rhodamine throughout the cell over the one week period (Figure 11). However, no co-localization of bacteria and liposomes was observed at any time-point (Figure 11), suggesting that they are taken up into different vacuoles within the macrophage.
Figure 11: Localization of rhodamine-phosphatidylethanolamine labelled liposomes and *M. bovis* BCG expressing GFP in differentiated THP-1 cells after indicated treatment periods.
It is unclear if the rhodamine fluorescent tag remains associated with the liposome over the entire incubation period. Experiments conducted to ensure that the fluorescence was not acid sensitive were conducted before beginning this investigation by measuring the absolute fluorescence over one week of storage. The liposomes were also passed over a gel filtration column to ensure that only liposomes containing the tag, and not free rhodamine, were added to the cells. However, rhodamine may still be processed within the cells and distribute to the endosomal processing pathway, a similar processing route that liposomes follow. Further experiments would be required to confirm the distribution of rhodamine in relation to the liposomes.

4.3 Discussion

*In vitro* efficacy testing of antibiotics is typically carried out to determine or verify the activity of drugs against the bacterial target. In this thesis, *in vitro* testing was utilized to establish the efficacy of encapsulated antibiotics in comparison to free anti-tuberculosis agents. The activity of encapsulated drugs *in vitro* is a balance between the drug released from the liposome and the drug that remains associated with the liposome and therefore unavailable to contribute to activity. Sphingomyelin/cholesterol formulations were specifically designed in this thesis to provide stable liposomes that would minimize immediate release in order to provide the opportunity for decreased dosing frequencies for TB treatment. However, the media used for the extracellular efficacy studies contained sufficient concentrations of ammonium sulfate to dissipate the pH gradient. This would allow drug release over the two-week incubation period of the assay since the *in vitro* release kinetics showed >90% release over 24 hours in the presence of ammonia sulfate for every formulation but ethambutol. Even ethambutol
should be released over the two-week period used in this assay. Under these conditions it is evident that the activity of each drug, either in an individual formulation or in a combined formulation, remains equivalent to free drugs solubilized in media (Figure 8).

The intracellular THP-1 infection model more closely represents an infection in vivo. Under these conditions there is no ammonium sulfate present so drug release is presumably the result of liposome-serum interaction or liposome-cell interaction. Liposomes may release drug after disruption of the membrane due to protein insertion or osmotic lysis before entering the cell. However, most liposomes will likely by phagocytosed by macrophages, which may then be degraded by enzymes in the phagosome, allowing drug release.52 Some liposomes may also be taken-up through adsorption, lipid exchange, or fusion.52

Under these conditions drug release did occur, as illustrated by the efficacy of encapsulated drugs, which was generally found to be equivalent to free drugs (Figures 9, 10, 11). Other investigators have found that encapsulated anti-mycobacterial drugs were superior to free drugs against intracellular MAC5,33,44,64 in vitro as well as against BCG or TB in vivo.1,2,25,26,58,59,69,79 However, the formulations used in these studies were typically prepared by passive encapsulation, which results in faster release of drug since there is no pH gradient to preserve encapsulation. Only ciprofloxacin has been prepared by remote loading methods similar to those utilized in this thesis.76 In addition, not all investigators indicated if free drug was removed. Finally, it was also noted that the incorporation of negatively charged lipids increased the efficacy, which may have a significant effect in vitro.76
At concentrations of approximately $\frac{1}{4}$ of the MIC, it appeared that the liposome formulations may be slightly more efficacious, although the results were not statistically significant. This observation may be due to increased penetration of the macrophages by liposomes in comparison to free drug, which may not reach high enough intracellular concentrations when sub-MIC concentrations are added. Results over one week of incubation suggest that efficacy may be increased over longer time periods since the efficacy of the encapsulated drugs more closely mirrored the trends observed for free drugs over one week than after 48 hours (Figure 9 and 11). This may be the result of increased drug release and increased exposure to actively replicating bacteria.

After phagocytosis, *M. tuberculosis*, and similarly *M. bovis* BCG, inhibits fusion of the phagosome with the lysosome. These events prevent acidification and maturation of the vacuole, a more favourable environment for bacterial survival.$^{91,107,111}$ Although *M. tuberculosis* has adapted to exploit host macrophages in this way, it may still interact with the endocytic processing pathway,$^{91,107}$ releasing components that modify the host immune system.$^9$ Therefore, although the bacilli remain in a separate compartment, it still has the ability to exchange materials with other compartments and has access to the sorting and recycling pathway.$^{9,93,107}$

Therefore, although co-localization of the liposomes and bacteria were not visualized by fluorescence microscopy, this does not mean that the drug does not come into contact with the bacteria. The liposomes will be degraded within the phagolysosome and drug will be released and may then be free to interact with the bacteria contained in other compartments. Since efficacy of the encapsulated formulations was observed in the intracellular experiments we know that this must occur.
Of significance *in vivo* is the formation of granulomas, which consist of macrophages and other immune cells that surround the bacteria. The macrophages that were initially infected with *M. tuberculosis* may actually die and newly recruited circulating macrophages may remain on the periphery. In this situation, drug may be present in dying macrophages and be released upon death, or liposomes already released from macrophages may diffuse through the granuloma.

The *in vitro* efficacy results compiled for this thesis show that encapsulated drugs are efficacious against both extracellular and intracellular *M. bovis* BCG. These experiments, along with the fluorescence microscopy results, confirm that isoniazid, rifampicin and ethambutol are active upon release from the liposomes, and that they are capable of interacting with bacteria within the macrophage after phagocytosis. Together with the characteristics outlined in the previous chapter, this suggests that these antibiotic liposome formulations have the ability to kill *M. tuberculosis* over a prolonged release period.
5.0 DISCUSSION AND FUTURE DIRECTIONS

5.1 Discussion

Our aim is to develop an inhalation therapy for the treatment of tuberculosis. As such, and as the first stage of research, the formulations developed in these studies were designed to optimize drug potency in an attempt to develop the most promising candidates for efficacious delivery of anti-tuberculosis agents directly to the lung. This is of particular importance for pulmonary delivery, since small volumes reach the lung, the site of tuberculosis infection. The prerequisites we have in mind for such a delivery agent include: (i) capability to carry high potency (50 mg/ml active range); (ii) capable of nebulization or other form of administration; (iii) safe (non-irritating, low toxicity); (iv) chemically and physically stable; (v) slow release upon administration (t_{1/2} release from lung of 12-72 hours); (vi) passive targeting to disease locations; (vii) enhanced therapeutic index; and (viii) enhanced response rate.

5.1.1 Drug Potency

When developing a liposome drug formulation, encapsulation optimization is critical to achieving high drug potency. As outlined, studies involving pH gradients, loading kinetics, buffering capacity, and drug/lipid ratio were carried out in order to optimize this factor, resulting in formulations of rifampicin and isoniazid that show up to 11% and 45% higher encapsulation than previously reported formulations\textsuperscript{2,25,26,79,116} respectively. Our formulations also showed prolonged release in comparison to previous formulations. Therefore, our objective of increasing drug encapsulation using the remote loading procedure was satisfied in this thesis and clearly demonstrates that the concentration gradients generated exceed those achieved by passive loading techniques.
previously utilized for rifampicin and isoniazid loading. The development of a combined isoniazid/ethambutol formulation, as well as a single ethambutol formulation has not been described previously.

The liposome formulations presented in this report increase the potency achievable for each antibiotic. Nevertheless, at a lipid concentration of 100 mg/ml, the highest drug concentration achievable is 18 mg/ml for the ethambutol formulation, short of the predicted required potency of 50 mg/ml. However, at a lipid concentration of approximately 100 mg/ml, drug concentrations at least 1000x greater than the MIC of each drug were achieved (Table 6), which should be sufficient to deliver therapeutic levels to the lung.

5.1.2 Suitability for Nebulization

Although no experimentation has yet been conducted on the stability of these formulations to nebulization, their small size (130nm) suggests that the vesicles are well below the size of an aerosolised droplet, which is typically in the range of 1 μm- 7 μm, depending on the device. As expected, increasing vesicle size leads to increased vesicle disruption and drug release, where studies conducted using liposomes in the range of 0.2 μm and 0.4-1 μm showed approximately 8% and 20% disruption of vesicles respectively. Studies using specific aerosolised drug formulations have also shown efficacy, including beclomethasone for the treatment of asthma and ciprofloxacin for the treatment of intracellular pathogens including Francisella tularensis.

5.1.3 Toxicity

The most significant improvement of approved liposome formulations such as Ambisome™ and Myocet™ is their reduced toxicity in comparison to aqueous drug
formulations, which illustrates the potential usefulness of liposomes to decrease the
toxicity of a wide range of drugs. In addition, studies conducted to investigate the
toxicity of chronic pulmonary administration of liposomes has shown no toxicity to
macrophages by this route, as measured by changes in morphology, or phagocytic and
killing functions.\textsuperscript{72} Toxicity studies by Deol and Khuller, using rifampicin and isoniazid-
loaded liposomes, has also shown reduced toxicity in comparison to free isoniazid and
rifampicin.\textsuperscript{25}

5.1.4 Formulation Stability

Our physical stability studies, as measured by vesicle size, encapsulation, and
residual pH gradient, have established that all four formulations are stable over 2 months
at 5°C (Table 4). In fact, the ethambutol and isoniazid/ethambutol formulations show no
signs of variation in any of these parameters over 4 months at 5°C. In the future, studies
should be conducted to determine the chemical stability of encapsulated drugs subjected
to the same conditions.

5.1.5 Prolonged Release

All four formulations, which have the same sphingomyelin/cholesterol (63/37,
mol/mol) lipid composition, were designed to optimize the slow release capabilities of
liposomes. The \textit{in vitro} release kinetics quantitated in this thesis support the potential for
sustained release, since only 20-30\% of any drug was released over a 24 hour period after
dilution and in the presence of mouse serum, achieving superior prolonged release
characteristics in comparison to previously tested formulations.\textsuperscript{22,51}
5.1.6 Efficacy and Targeting

*In vitro* efficacy experiments illustrate that the activity of each drug is maintained when it is formulated in liposomes, as demonstrated by extracellular and intracellular efficacy assays. Efficacy seems to improve after 1 week, suggesting that release continues and maintains the drug concentrations above the MIC for *M. bovis* BCG.

Although our goals of passively targeting disease and enhancing the therapeutic index and response rate have to be validated in future animal studies, related studies involving pulmonary delivery of microparticle formulations of isoniazid and rifampicin have shown increased concentrations of drug uptake by alveolar macrophages. In addition, increased efficacy in the lung was achieved following pulmonary administration when compared to oral or i.v. administration.

The primary goal of developing liposome encapsulated anti-TB drugs is to significantly increase the half-lives *in vivo* to the order of days instead of hours. Favourably altering the pharmacokinetics of each drug in this manner will maintain drug concentrations greater than the MIC for periods that permit weekly or ultimately monthly administration instead of the current daily treatment regimen.

This goal corresponds to the first objective of the Global Alliance for TB Drug Development that states that improvement in therapy would occur upon decreasing the dose and duration of treatment, with the ultimate goal of eradicating TB in infected persons within two months. Significantly decreasing the dosing frequency would ease the burden of compliance enforcement for DOTS programs, particularly in developing countries where compliance is particularly low. Together, these factors would result in increased completion of treatment regimens, increased TB eradication and decreased
resistance development. In addition, the prolonged release of antibiotics at consistently higher concentrations would favour decreased resistance development due to the presence of greater than one active drug at any given time throughout the treatment period. Alternatively, these formulations also have the potential to significantly decrease toxicity related to these drugs, permitting increased dose administration and therefore less frequent administration.

Taken together, these results suggest that the formulations tested in these studies are promising candidates for the treatment of tuberculosis since pulmonary administration of liposome-encapsulated drugs has the potential to simplify treatment protocols. Further investigation of the formulations developed in this thesis will further this aim.

5.2 Future Directions

5.2.1 Formulation Development

Although the use of sphingomyelin/cholesterol liposomes decreases clearance and release rates, this composition may also inhibit macrophage digestion and fusion because of its decreased membrane fluidity. Further development of these formulations may include incorporation of negatively charged lipids such as cholesterol sulfate, dicetyl phosphate, or phosphatidic acid to enhance uptake by alveolar macrophages. When testing various formulations in vitro it was also noted that the incorporation of a negative charge increased efficacy against MAC. When developing any formulation, there must be a balance achieved between characteristics such as potency, release, and targeting to achieve the appropriate formulation. These must be ultimately validated in vivo.

Future work may also include the development of a combined isoniazid and rifampicin formulation since these are the two most potent anti-TB drugs and are the
most common components of primary tuberculosis therapy. Other liposomal formulations that may be of interest include pyrazinamide, which like rifampicin is active against non-replicating bacteria, and a new member of the rifamycin class such as rifapentine or rifabutin. These drugs have prolonged half-lives in comparison to rifampicin and their pharmacokinetic parameters may be improved further by encapsulation.

5.2.2 In vitro Characterisation

Experimentation combining various liposome formulations and free drug using the intracellular BCG assay may be beneficial to optimize potential therapeutic regimens. In addition, further characterisation of the interaction between the bacteria and liposomal delivery systems may also be carried out using the confocal microscopy procedures established in this thesis. Incorporation of dyes within the vesicular aqueous space (such as carboxyfluorescein), as a marker for drug, would allow visualization of release within the macrophage. Staining the cells with endosomal markers would also identify the exact location and trafficking pathways of bacteria and liposomes in relation to the other. Finally, it may be interesting to observe the differences in bacteria and liposome localization if THP-1 cells were first loaded with liposomes and then infected.

Further investigations into the kinetics of intracellular drug release may also be beneficial to explain the efficacy results. The use of encapsulated radiolabelled drugs would make this possible.

5.2.3 In vivo Investigations

Finally, and most importantly, animal studies are required to investigate in vivo pharmacokinetics and efficacy following pulmonary administration of each formulation in comparison to the free drugs. Deposition studies and toxicity studies should also be
performed. Although no animal models are completely appropriate as models of human TB infection, the commonly used models are guinea pigs and mice, where a low aerosol infection is established before carrying out the required manipulations. Mice are useful since their immune system has been well characterized, they have similar susceptibility to infection as humans, are relatively inexpensive, and a large number of compatible reagents are available for this model. However, they do not develop pathology like that found in the human lung. Guinea pig models are also used and show pathology that more closely mimics human pathology upon infection. However, guinea pigs are particularly susceptible to TB, they are more expensive and the model is not as well characterized as the mouse model. Testing encapsulated drugs against an in vivo latent TB model would also be interesting to determine if prolonged release would increase efficacy in such a model.

It is particularly important to carry out these experiments in order to test the pulmonary delivery aspect of our working hypothesis. In addition, in vivo studies will verify the potential of the formulations described in this report since there is always a degree of variability between in vitro and in vivo studies.
6.0 REFERENCES


