ANTIMYCOBACTERIAL AGENTS: A STUDY OF LIPOSOMAL-ENCAPSULATION, COMPARITIVE PERMEABILITY OF BRONCHIAL TISSUE AND *IN VITRO* ACTIVITY AGAINST *MYCOBACTERIUM TUBERCULOSIS* ISOLATES

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DECLERATION

I, the undersigned, hereby declare that the work contained in this thesis is my original work and that I have not previously submitted it, in its entirety or in part, at any other university for a degree.

Lyné van Rensburg

SUMMARY

In this thesis, research results are reported on the role of dipalmitoyl phosphatidyl choline (DPPC) and DPPC-liposomes on the in vitro permeability characteristics of various antimycobacterial drugs across porcine bronchial tissue. The permeability flux values of the different compounds (isoniazid, ofloxacin and moxifloxacin) and their relevant DPPC formulations were determined using a continuous flow through perfusion system. Mean steady state flux values were compared statistically by means of a t-test at a significance level of 5% as well as an F-test using whole curve comparisons. The results indicated that the different formulations of drug and their DPPC combinations retard the permeation of drug through bronchial tissue. However, moxifloxacin permeation was significantly enhanced when in a DPPC-liposomal formulation. These results demonstrate the important role that molecular weight, electrostatic charge, partitioning of the molecules in DPPC and DPPC-liposomes play in transmembrane diffusion.

In addition, the effect of individual drugs and their DPPC combinations on the surface tension lowering property of DPPC was evaluated. The results obtained showed minimal decreases in the surface tension lowering capability of DPPC; however, the minimal increases in surface tension do not alter the integrity of DPPC to a large extent.

Drug susceptibility testing of Mycobacterium tuberculosis cultures against the individual antitubercular drugs and their DPPC combinations was done by using the Radiometric BACTEC 460TB™ system. Drug-entrapped DPPC liposomes were tested at concentrations comparable to their relative minimum inhibitory concentrations (MIC). The results for the BACTEC assay indicated that the

mycobacteria were susceptible to the developed drug entrapped liposomes; of which their encapsulation efficiencies for the relevant drugs were approximately ± 50%. It was concluded that drug-entrapped DPPC liposomes could fulfill the dual role of pulmonary drug delivery and alveolar stabilization due to antiatelectatic effect of DPPC which can improve the distribution of anti-tubercular drugs in the lung.

OPSOMMING

Hierdie tesis doen verslag oor navorsingsresultate met betrekking tot die rol van dipalmitoïel-fosfatidiel-cholien (DPPC) en DPPC-liposome in die in vitro-permeasiekenmerke van verskeie antimikobakteriese middels oor vark- brongiale weefsel. Die permeasievloedwaardes van die verskillende verbindings (isoniasied, ofloksasien en moksifloksasien) en hul betrokke DPPC-formules is met behulp van 'n deurlopende-deurvloei-perfusiestelsel bepaal. Gemiddelde vloedwaardes in 'n bestendige staat is statisties vergelyk met behulp van 'n t-toets op 'n beduidendheidsvlak van 5%, sowel as 'n F-toets met behulp van heelkurwevergelykings. Die resultate dui daarop dat die verskillende middelformules en hul DPPC-kombinasies middelpermeasie oor brongiale weefsel vertraag. Tog is die permeasie van moksifloksasien aansienlik versterk in 'n DPPC-liposomale formule. Hierdie resultate bevestig die belangrike rol van molekulêre gewig, elektrostatiese lading, die verdeling van molekules in DPPC sowel as DPPC-liposome in transmembraandiffusie.

Daarbenewens is die uitwerking van individuele middels en hul DPPC-kombinasies op die oppervlakspanningsverligtingsvermoë van DPPC beoordeel. Die resultate toon minimale afnames in die oppervlakspanningsverligtingsvermoë van DPPC. Die minimale toenames in oppervlakspanning het egter meestal geen noemenswaardige effek op die integriteit van DPPC gehad nie.

Voorts is die vatbaarheid van Mycobacterium tuberculosis-kwekings vir die individuele anti-tuberkulêre middels en hul DPPC-kombinasies met behulp van die radiometriese BACTEC 460TB™-stelsel getoets. Middel-ingeslote DPPC-liposome is getoets in konsentrasies wat met hul relatiewe minimum inhibisiekonsentrasies

(MIK) vergelyk kan word. Die resultate van die BACTEC-toets toon dat die mikobakterieë vatbaar was vir die ontwikkelde middel-ingeslote liposome, met 'n enkapsuleringsdoeltreffendheid van ongeveer 50% vir die betrokke middels. Die studie kom tot die gevolgtrekking dat middel-ingeslote DPPC-liposome die dubbele rol van pulmonêre middel-lewering en alveolêre stabilisering kan vervul weens die anti-atelektatiese werking van DPPC, wat die verspreiding van anti-tuberkulêre middels in die long kan verbeter.

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Gel-filtration chromatography of drug combined DPPC liposomal preparations on a 16 x 53 cm Superdex–75 column equilibrated with 50 mM phosphate buffer containing 100 mM NaCl.

DEDICATION

I dedicate this thesis to my family, for their love, support and guidance, without which this wouldn't have been possible. All of you have been a source of encouragement, perseverance and steadfastness throughout my life.

DISCLAIMER

Any opinion, findings and conclusions or recommendations expressed in this material are those of the author(s) and therefore the NRF does not accept any liability in regard thereto.

1. INTRODUCTION

An increasing amount of research is focused on optimizing drug delivery as a method of improving pharmacotherapy. As oral administration is probably a non-optimal delivery route for many therapeutic agents, growing attention is given to the potential of absorptive mucosae, including nasal, ocular, rectal, vaginal, buccal, sublingual and pulmonary tissue, as a non-invasive route of administration for systemic drug delivery. The pulmonary route seems most advantageous when taking into account that inhalation delivery may offer more rapid as well as a superior degree of drug absorption compared to that of other routes. However, with this route of administration, targeting a specific site of action requires the aid of a delivery vehicle that relies on the careful consideration of the drug to be delivered, as well as the unique properties of the tissue being targeted.[1]

The human respiratory tract has the potential to provide means for non-invasive drug delivery as it is a highly specialised organ with a surface area of more than 75 m^2 of which the alveoli constitute about 95%. The good vascularisation, immense capacity for solute exchange and ultra-thinness of the epithelium are unique features that can facilitate systemic delivery of drugs. Controlling the manner in which drugs disperse, particle size and the consistency of the delivery have been researched extensively. Advances in these parameters have clearly made pulmonary delivery a practical approach for therapeutic agents. With more clinical data being made available in support of the efficacy and safety of inhalable drugs, pharmaceutical developers are recognizing that pulmonary delivery offers unique advantages. This delivery route has substantial impact on four areas of pharmaceutical development: proteins and peptides, fast-acting drugs, localized

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lung infections and other pulmonary disease treatments (e.g. *Mycobacterium tuberculosis*).[2]

Pneumonia and other lung infections such as tuberculosis (TB) remain a leading cause of death worldwide.[2] A drug delivered by way of the pulmonary route can act directly at the infected site. It offers the potential for faster and more efficient therapy. This route also offers the reduced risk of drug resistance development as well as fewer side effects.[2] Lung diseases such as asthma, emphysema or cancer can also expect similar treatment benefits with local treatments with drugs.[3] However, the permeability barrier of the lungs presents a major problem for most drugs when formulated without an absorption enhancer/promoter.[4] To circumvent this problem, mixing of a pharmaceutically active agent with a pulmonary surfactant or a promoter have been tested for enhancing the systemic availability of drugs from the lungs. Many protease inhibitors, lipid or polymers have been tested for their efficacy in improving the systemic uptake of drugs from the lungs.[4]

The mechanism of action by which surface–active agents manipulate the alveolar–capillary transfer of solutes still needs to be elucidated. However, they are most likely to be involved in the increase in transcellular transport by way of an interaction and/or fluidisation of the cell membrane, a variation of tight junctions and an increase in paracellular permeability or a combination of both.[4]

Surfactant uses in treatments beyond the traditional role of Surfactant Replacement Therapy (SRT) for neonates are currently being assessed.[5] Some of the areas where surfactants are being utilised to improve drug delivery are being carried out in animal and clinical studies involving therapy of pneumonia.[6]

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Exogenous surfactant's particular spreading properties, ability to re-expand atelectatic areas in the lung and its ability to dissolve drugs that are insoluble in aqueous solutions suggests that it could be exploited to act as a pulmonary drug delivery agent. However, little is known about possible interactions between pulmonary surfactant and antimicrobial agents when combined.[7] Furthermore, pulmonary surfactant also has the ability to form lamellar structures, i.e. liposomes.[8] Surfactant-based liposomal delivery system have shown great potential of being effective drug–delivery vehicles. The development of liposomal formulations for aerosol delivery with jet nebulizers has expanded the possibilities for effective utilization of aerosol based therapies in the treatment of pulmonary diseases.[9]

Vesicle size is one of the critical components of liposome formulation for aerosol delivery as it determines site of impact. Liposomes impinging on the conducting airways will be cleared by mucocilliary action. However, when deposited in the alveolar region; liposomes will be subjected to different, slower clearance mechanisms.[10] In addition; phospholipid vesicles have perceived advantages over other proposed drug carriers in the lung as demonstrated by the ability of Type-II cells to utilize administered phospholipids in the production of lung surfactant. The physiological as well as pathophysiological environment of the lung will also affect deposition and the utilisation of liposomes. Therefore further developments directed at liposome delivery to the lung are warranted.[10]

2. LITERATURE REVIEW

2.1 Epidemiology of Tuberculosis

Tuberculosis (TB) is an infectious disease caused by the bacillus *Mycobacterium tuberculosis* (M.tb). It typically affects the lungs (pulmonary TB) but can affect other sites as well (extrapulmonary TB). There were an estimated 8.8 million incident cases of TB globally in 2010, 1.1 million deaths (range, 0.9 million – 1.2 million) among HIV-negative cases of TB and an additional 0.35 million deaths (range, 0.32 million – 0.39 million) among people who were HIV-positive. Even though the incidence of TB has been falling since 2006, by an incidence rate of 1.3% per year (per 100 000 population) since 2002, it still remains one of the leading causes of preventable deaths in more than 198 countries including South Africa.[11]

By making use of modern day DNA extraction and purification methods, molecular evidence for TB was found in an ancient Egyptian mummy from the New Kingdom which dated back to about 1550 — 1080 BC.[12] The molecular diagnosis of tuberculosis in historic specimens has thus far only been successful in a Peruvian mummy dating to about 1000 — 1300 AD.[13] Robert Koch, a German physician, was the first to identify and describe M.TB bacilli in 1882 where tuberculosis was generally referred to as "consumption" in the poor urban population in Britain and Europe in the early 19th century.[14] The only treatment available was to stimulate the body's own immune system by focusing on a healthy diet, fresh air, hygiene and the administration of expectorants and purgatives. This led to the opening of the first sanatorium in 1854 in Germany.[15] The nature of this treatment plan proved success compared to any previous treatment regimens and a further critical improvement of public health led to the reduction of tuberculosis cases before the discovery of streptomycin in the mid-20th century.

As indicated in the global tuberculosis control report of 2011 from the World Health Organisation [10], South Arica currently has one of the highest incidence rates of TB per 100 000 people (>300) which is illustrated in Fig. 1.[16]
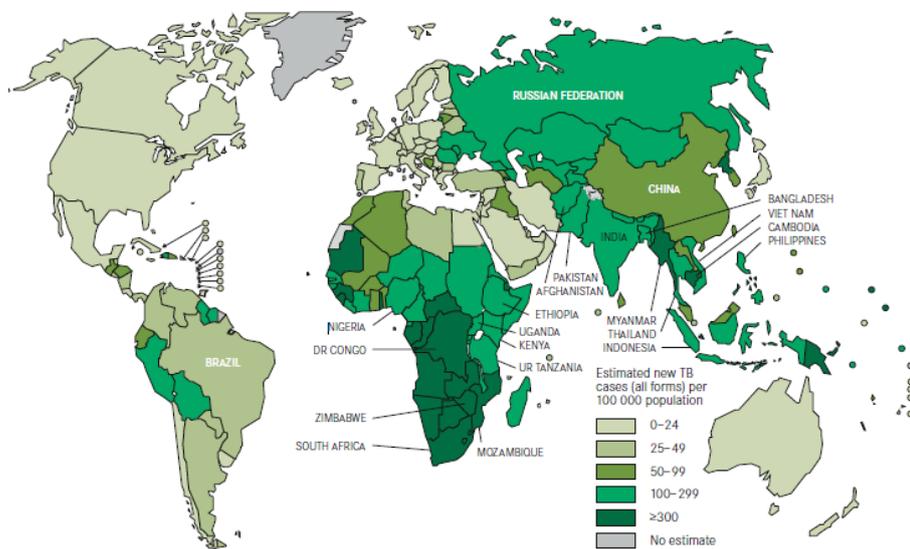
Figure 1: World map indicating the incidence of TB for 2010.[16]

2.2 Multidrug – and Extensively drug - resistant tuberculosis

Multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) occurs when the M.tb strain presents resistance to, at least isoniazid (INH) and rifampicin (RIF). In the case of XDR-TB it is compounded by the additional resistance to the second line drugs which can be any of the fluoroquinolones as well as at least one of the injectable drugs kanamycin, capreomycin or amikacin. This is a growing public health and clinical problem worldwide as well as a great concern in South Africa especially pertaining to individuals co-infected with HIV/AIDS.[17,18]

It is estimated that the 450,000 multidrug-resistant (MDR) TB cases that occur globally constitute 14% of the global burden of TB. Which is more, South Africa contributes approximately 1-2% to this 14% MDR-TB occurrence.[17,19] Concern over

MDR/XDR TB was extrapolated when, in 2006, an MDR/XDR TB outbreak involved 536 TB patients at the Church of Scotland Hospital in KwaZulu-Natal South Africa; 221 were found to have MDR-TB of which 53 were diagnosed with XDR-TB. Within 25 days, 52 patients died. This outbreak also involved a high proportion of HIV co-infection which was confirmed by 44 of the 53 patients who were found to be HIV positive. It was thought at the time that the co-infection with HIV led to the development of XDR-TB. However, at the 2nd TB conference in Durban (2010), it was indicated that it primarily originated through inadequate infection control. Patients who, with no prior TB or HIV infection, were hospitalised for other ailments contracted XDR- TB through inadequate infection control.[20]

2.3 Current TB Chemotherapy

Streptomycin was discovered in 1944 and only then did the treatment of TB become possible. This was then followed by the introduction of isoniazid (INH) in the 1950s which established the basis of anti-TB chemotherapy until the 1960s. Streptomycin (STM), INH and para-aminosalicylic acid (PAS) comprised the anti-TB drug regimen in the initial months during that period. This was followed by INH and PAS in succeeding months, resulting thus in a total duration of administration of 18 months. In 1965, rifampicin (RIF) was discovered and in the 1970s, a short-course treatment for TB was launched. The directly observed treatment, short-course (DOTS) strategy, when properly implemented with the necessary infrastructure, produces high TB cure rates and curtails the development of acquired drug-resistant TB.[21]

According to the South African tuberculosis control programme, the current standard first line treatment regimen of 2004 consists out of an initial (or intensive) phase of 2 months consisting of 4 drugs INH, RIF, pyrazinamide (PZA), and

ethambutol (ETH). To ensure complete elimination of mycobacteria, 4 months of INH and RIF continued treatment is followed after the intensive phase (Table 1).[22,23]

Pre-Treatment body weight	Intensive phase: 2 months	Continuation phase: 4 months	
	RHZA 150 / 75 / 400 / 275 mg	RH 150 / 75 mg	RH 300 / 150 mg
30 – 37 kg	2 tablets	2 tablets	-
38 – 54 kg	3 tablets	3 tablets	-
55 – 70 kg	4 tablets	-	2 tablets
≥ 71 kg	5 tablets	-	2 tablets

R = rifampicin; H = isoniazid; Z = pyrazinamide; E = ethambutol

Table 1: Treatment regimen for known drug sensitive TB.[22]

Although treatment of drug-susceptible TB under ideal conditions by DOTS may be successful in 95% of cases, cure rates in the field are often significantly lower.[24] However, the reality of the DOTS strategy has revealed that it is difficult to maintain in many national TB control programmes, especially over a long time. One specific example is the failure to ensure patient compliance. One of the most common reasons for patient non–compliance is due to the treatment associated adverse–effects. First line drugs have been known to cause nausea, vomiting and drug induced hepatitis. Non-compliance to the recommended chemotherapeutic regimen not only lowers cure rates, but leads to the development of drug-resistant TB, inclusive of the formidable scenarios of MDR-TB and XDR-TB.[25,26] Where moxifloxacin, a second line drug used in the treatment of MDR-TB, has been associated with gastrointestinal disturbances and neurological dysfunction.[27]

Most of the standard chemotherapy has proven to be ineffective for patients that have MDR-TB and although there is no cure for XDR-TB, a standardised regimen is under review.[22] Drug resistance is predominantly detected in patients who have previously been treated for TB. This could be due to the previously mentioned failure to comply with the extensive drug regimens resulting in the pathogens becoming resistant to the first line drugs, namely INH and RIF.[25-27,28,29] There are two treatment possibilities when faced with MDR-TB. Firstly, a combined treatment plan of aminoglycoside, a fluoroquinolone, PZA, and ethionamide or prothionamide can be used for MDR-TB with resistance to both INH and RIF. However, the second option of an aminoglycoside, fluoroquinolone, PAS, cycloserine, and ethionamide or prothionamide is required for disease with resistance to INH, RIF, ETH and PZA (Table 2).[21,22]

Intensive phase: 6 months or at least 4 months				
Drug	**Body Weight**			
	< 33 kg	**33 – 50 kg**	**51 – 70 kg**	**> 70 kg**
Kanamycin[#]	15 – 20 mg/kg	500-750 mg	1000 mg	1000 mg
Pyrazinamide	30 – 40 mg/kg	1000 - 1750 mg	1750 - 2000mg	2000 - 2500 mg
Ethionamide	15 – 20 mg/kg	500 mg	750 mg	750 - 1000 mg
Ofloxacin[¥]	800 mg	800 mg	800 mg	800 - 1000 mg
Or				
Levofloxacin	7.5 – 10 mg/kg	750 mg	750 mg	750 - 1000mg
Or				

Moxifloxacin	7.5 – 10 mg/kg	400 mg	400 mg	400 mg
Terizidone$^{\phi}$	15 – 20 mg/kg	750 mg	750 mg	1000 mg
Or				
Cycloserine	155 – 20 mg/kg	500 mg	750 mg	750 - 1000 mg

Continuation phase: 18 months				
Drug	**Body Weight**			
	< 33 kg	**33 – 50 kg**	**51 – 70 kg**	**> 70 kg**
Ethionamide	15 – 20 mg/kg	500 mg	750 mg	750 - 1000 mg
Ofloxacin$^{¥}$	800 mg/kg	800 mg/kg	800 mg/kg	800 - 1000 mg
Or				
Levofloxacin	7.5 – 10 mg/kg	750 mg	750 mg	750 - 1000 mg
Or				
Moxifloxacin	7.5 – 10 mg/kg	400 mg	400 mg	400 mg
Terizidone$^{\phi}$	15 – 20 mg/kg	750 mg	750 mg	1000 mg
Or				
Cycloserine	15 – 20 mg/kg	500 mg	750 mg	750 - 1000 mg

Table 2: Treatment regimen for MDR - TB. [#] Kanamycin may be substituted with amikacin; [¥] Moxifloxacin (patients > 8 yrs) or levofloxacin (patients < 8 yrs); [ϕ] Pyridoxine (Vit B6) 150 mg to be given daily to patients on terizidone or cycloserine.[22]

Another characteristic of TB that makes treatment difficult besides prolonged treatment and acquired resistance (genetic resistance) is its persistence. M.tb can persist within the human host for years without causing disease, in a syndrome known as latent tuberculosis.[30,31] Mycobacteria are obligate aerobes however, they have been known to encounter hypoxic environments. This leads to the suggestion that the development of physiological resistance could possibly play a role in latent infection. Wayne and co-worker demonstrated that the bacillus, when oxygen is steadily depleted, terminates growth and develops into a defined non-replicating/dormant form; however, when oxygen is completely removed from the environment, the bacilli cannot survive.[32] These latent/dormant mycobacteria are able to survive within granulomatous lesions within the lungs that are surrounded by foamy macrophages.[33] As the current chemotherapy only works for growing, drug susceptible TB, the lengthy treatment time depends on the presence of dormant bacteria present in the old lesions that become active again.[34] Evidently, this model is a commonly accepted paradigm that still remains to be proven. However, if it turns out that this idea is a true reflection of aspects *in vivo*, the development of drugs that specifically target dormant bacilli could have a profound impact on tuberculosis therapy. The administration of conventional drugs along with the potential 'anti-dormancy' drugs might shorten the treatment duration as well as treatment efficacy regarding latent infection. [33, 34]

Patients who are co-infected with HIV present some major challenges when it comes to TB treatment. Firstly, drug-drug interactions between anti-retroviral drugs (ARVs) are included. RIF interactions with protease inhibitors and non- nucleoside reverse transcriptase inhibitors lead to decreased concentrations of ARVs, thus resulting in the loss of antiviral efficacy and the development of viral resistance. The

second complication for HIV-infected patients receiving TB treatment is drug toxicity. For example, a retrospective study done by Yee *et al* which involved patients treated for TB in Canada found that HIV co-infected patients were 3.8 times more likely to experience a significant drug-related adverse event.[35] Therefore, these overlapping toxicities may lead to treatment discontinuation, increasing the probabilities of nonadherence and drug-resistance. Thirdly, an immunopathological reaction, which is also termed "the immune reconstitution inflammatory syndrome" (IRIS), occurs most frequently when antiretroviral therapy is resumed in patients with tuberculosis.[23,30,36,37]

Improvements for new TB treatments will require the fulfilment of three major objectives. Firstly, to discover new chemotherapies that decreases the duration of treatment due to faster-acting drugs. Secondly, to develop novel antimycobacterials which can combat the emergence of MDR-TB and thirdly, to target dormant bacilli to treat latent TB infections as previously discussed.[37]

2.4 Prospects and Challenges for Future TB Chemotherapy

Besides the recent discovery of a diarylquinoline TMC207 (Bedaquiline) which has proven to be promising drug candidate for the treatment of TB; the past 30 years has left a crucial gap in drug development. This new drug incorporates a novel approach by binding to a new target, the mycobacterial ATP synthase.[38,39] The reason for this crucial gap in drug discovery is due to two kinds of obstacles. Firstly, the "scientific obstacles" or so-called "biological uncertainties" about M.tb is currently under debate. As previously mentioned, a universal understanding of the heterogenetic characteristic of TB lesions in patients is still sought. Likewise, understanding the critical mechanisms that underlie the survival of M.tb during the long periods of chemotherapy is not completely understood. Therefore, these gaps

in the knowledge of M.tb biology are making the identification and validation of potential targets relevant within the human host very difficult.[40,41] These uncertainties are also the reason why pharmaceutical companies are reluctant to invest in anti-TB drug development as it presents varied risks for their businesses. This results in the lack of funding for academic laboratories to run research projects that connect basic and applied science. I mention this for the specific reason that if faster progress is to be made with drug discovery for TB; then advanced knowledge of M.tb's metabolism, physiology and genetics need to be interpreted into the identification of new compounds and targets.[41] Currently, there is a lack of adequate animal models available that are able to accurately predict the required treatment duration with newly identified compounds. In this regard, the guinea pig model is currently explored as an alternative for the mouse model since it resembles TB pathology in humans more closely. [42]

It was stated by McKinney that " In the TB field, target identification and target validation projects are not carried out by biotech or pharmaceutical companies due to lack of interest and lack of expertise – there is little capacity and expertise on TB among pharmaceutical companies since there have not been on-going TB development projects. If this work cannot be done by academic laboratories, the process gets stuck".[43] There is the need to enable the academic sector to go beyond the proof of principle that traditionally is the final aim and it is necessary to re-think the traditional roles played by academia and pharmaceutical industry in the drug discovery and development.[43,44]

One prospect that has shown some promise in the advancement of TB chemotherapy is the repurposing of current chemotherapeutic agents.[45] Revising current drugs and reconstituting drug combinations have indeed improved

treatment outcomes. One such an example is a recent study done at Stellenbosch University, called NC001. It involved a new drug combination of an experimental medicine called PA-824 (nitroimidazole derivative) alongside bedaquiline, moxifloxacin and pyrazinamide. This cocktail which was dubbed PaMZ, proved to be safe and presented the potential to reduce the time needed to treat drug-resistant forms of the disease.[46,47] The study holds importance from a drug development perspective as it was using a novel approach. Another such case involved linezolid in the treatment regime and it achieved great success in patients with uncomplicated drug-resistant TB and for previously treated patients with XDR-TB.[48] However, a concern which one would need to take into consideration when including new derivatives in different drug combinations is the possibility of cross-resistance. [44]

Currently, a common belief to approach the investigation of drug treatment efficacy for tuberculosis lies in exploring novel systems for drug delivery.[43] One such an innovative approach was investigated during the course of this thesis where the feasibility of using phospholipid structured liposomes in drug delivery *in vitro* was investigated.

2.5 Liposomes as drug-delivery vehicles: Application and prospects

New approaches and new ways of thinking for TB drugs treatment are not only becoming necessary to fight this disease but essential; especially when it comes to patients with co-infections and drug resistance. As it stands, Sosnik *et al* stated that "nanotechnologies appear as one of the most promising approaches for the development of more effective and compliant medicines".[28]

Liposomes can be micro to nano–sized vesicles that consists of a phospholipid bilayer surrounding an aqueous core. The aqueous centre allows for the

encapsulation of hydroliphic drugs whereas the hydrophobic character of the membrane can entrap insoluble drugs.[49]

Academia has contributed more than industry regarding relevant research involving liposomes as drug carriers. One such example is the development of sterically–stabilized liposomes (SSL). This led to the combined efforts to develop SSL and their efficient remote loading with the drug doxorubicin (DOX) resulting in the development of DOXIL® (approved in the US since 1995).[50]

There have been other liposome - and liposome-like-based pharmaceutical products, intravenously injectables, approved by the US FDA such as DaunoXome®, Ableet®, Amphotech® and AmbiSome®.[50] This is evidence that there is potential for liposome technology to mature in sophisticated pharmaceutical products. However, the high cost of lipid raw materials used for liposome preparations still plays a crucial role in slowing down the development process of such products.

The main purpose of using liposomes as drug carriers is to achieve selective localisation of an active drug in the infected sites. This is where liposomes differ from other controlled release systems with various carriers as drug release can occur either in plasma or at the site of administration. For this form of therapy to be proven effective, the encapsulated drug in appropriate dosage should be made available to the target cells.[50] Passive or active targeting can be used to obtain selective localisation. When the physical properties of the liposome and the microanatomy of the target tissue determine drug selective localisation, passive targeting is obtained; whilst active targeting requires an antibody or ligand attached to the liposome's surface. This has the ability to act as a 'homing-devise' so that the

liposome can recognise the diseased cells in addition to reaching the disease site.[50,51,52]

Oh *et al* investigated the formulation of the antibiotics ciprofloxacin and azithromycin in liposomes and tested their antibacterial activities *in vitro* against *M. avium* using a conventional passive-entrapment as well as targeting of the reticuloendothelial system.[53] Both drugs showed enhanced antimycobacterial effect in liposomes. Similar results were obtained with different liposome-entrapped second-line antibiotics.[54] Another illustration of liposomes with enhanced affinity towards lung tissue was reported by Deol and co-worker for the development of more effective chemotherapy against TB.[55]

2.6 Local delivery of anti-TB drugs to the lung: Implications of Surfactant as a carrier

Local delivery to the lungs by inhalation has emerged as one of the most attractive administration routes to target pulmonary TB infection. Inhaled therapy directly delivers the drug to the diseased organ with a reduction in adverse effects associated with systemic toxicity.[56] The manipulation of particle characteristics including size, surface chemistry, surface charge, and surface area, allows for the possibility of synthesising particles specific for targeting to various cellular populations and organs including the respiratory tract.[57,58] In this context, different industrial technologies such as employing a spray drying technique to produce nanoparticles made of either natural (e.g. gelatin) or synthetic (e.g. polybutylcyanoacrylate) polymers are investigated.[59] These overviews display some of the nano-carriers explored for the local administration of anti-TB drugs.[28]

The use of exogenous surfactant as a drug delivery agent has shown immense potential in this regard. Pulmonary surfactant comprises of a complex mixture of

lipids (approximately 80-90%) and several specific surfactant proteins (10%, surfactant protein A, B, C and D) rendering it with unique spreading properties and dynamic surface tension behaviour. These characteristics portend to the ideal carrier of choice to instil therapeutic agents into the lung; holding to the theme, specifically antimycobacterial drugs. Besides mechanical stabilisation of lung alveoli, surfactant also transports mucus and inhaled particles, protects against lung oedema and can also act as a local defence against infection by aiding the defence properties of alveolar macrophages.[60]

In the advanced stages of pulmonary TB, patients develop pulmonary surfactant dysfunction resulting in alveolar collapse and respiratory distress. The M.tb bacteria reside in the alveoli and are in close proximity with the alveolar surfactant. Chimote *et al* proposed that the mycolic acid found within the bacteria's cell wall interact with the surfactant causing this dysfunction.[61,62] Furthermore, findings from Wang *et al* documented that TB contains multiple lipids that can directly impair the biophysical function of endogenous and exogenous pulmonary surfactants.[63]

Russia initially investigated pulmonary drug delivery in TB. They addressed issues ranging across drug selection, aerosol generation, clinical outcome and adverse effects. However, this literature is not readily available.[64-70] One such a particular study of interest was done by Lovacheva *et al*.[71] They demonstrated that a collective dose of 700 mg of a surfactant designated as "Surfactant-BL" over 4 weeks in 70 patients resulted in significantly decreased sputum counts in 70% of cases, complete sputum conversion in 83% in 2 months, and highly improved resolution of lesions.[71] The reasoning behind the efficacy of surfactant therapy in pulmonary TB infection was elucidated by Stoops *et al*.[72] These authors explained that bacteria are surrounded by a thick lipid coat primarily consisting of trehalose

dimycolate (TDM). This extensive coat shields the bacterium from the immune system's response and antibiotics. However, their studies revealed that exposing bacteria to certain surfactants enhanced the effectiveness of antibiotics and the immune system's response by disrupting the protective barrier (Fig. 2).[72]
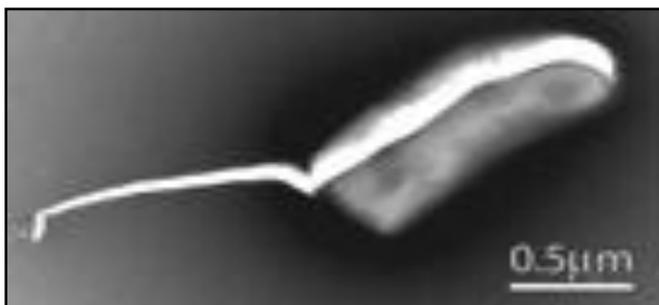
Figure 2: A bacterium which maintained a more extensive TDM coat (white) which has been partially dislodged in exposure to surfactant but remarkably maintains the bacterium's shape. This image illustrates the rigidity of the TDM outer layer.[72]

The innovative use of lipid surfactants offer two possible advantages. Lipids such as dipalmitoylphosphatidylcholine (DPPC), which comprises ± 90% of pulmonary surfactant, can be used to formulate drug-carrying liposomes due to the general spreading effects and the ability to form lamellar structures.[73] In addition to regional drug delivery, with the increased atelectasis and lung damage associated with advanced TB, exogenous surfactant can act as a replacement therapy to re-expand the atelectatic areas (restore alveolar stability) which are most likely to be infected areas (Refer back to dormant bacilli previously discussed).[74,75]

The progress of nanotechnology in developing inhalable nanoparticle encapsulated antitubercular drugs and drug-encapsulated liposomes, as previously discussed,

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are currently being investigated for improving drug delivery and decreasing the associated side-effects.[76,77] Zaru *et al* prepared drug-loaded freeze-dried liposomes, designed for the investigation of delivery to the lungs after rehydration.[78] They proposed that the interactions between the drug and membrane components (assembly, rigidity and integrity of the liposome membrane) should be taken into consideration when designing liposomal formulations intended for pulmonary delivery.[78]

Likewise, for the administration of drugs that are intended to act topically in the lungs, direct deposition to the lungs by some type of aerosol delivery technology needs to be taken into account when developing drug-loaded liposomes.[79] Previous studies have found that the lipid composition preparation and size could be a large determinant of its ability to be nebulized. More so, the shear forces experienced in the nebulizers may result in liposome damage and the effect of the droplet impact.[80-84]

2.7 Pulmonary Mucosa and Drug delivery

As pulmonary drug delivery receives acknowledgment, the need for validated test systems, methods and guidelines for regulatory purposes has become greater. Therefore the first step in drug profiling for delivery *via* the respiratory tract needs to address intrinsic physicochemical parameters and their impact on or correlation with absorption and solubility as well as taking into consideration the specific biology of the lung.[85] The respiratory tract is a highly specialised organ with a surface area of more than $75m^2$ and can be divided into two areas: the upper conducting airway and the lower respiratory regions. The conducting region consists of the nose, nasal passages, mouth, pharynx, oesophagus, larynx and trachea. The respiratory region consists of bronchi and alveoli.[86]

The solubility and lipophilicity of drugs may affect their behaviour in the bronchial and deep lung spaces (e.g. whether they reside for long periods in the respiratory system versus fast permeation across epithelial cell barriers). In addition, the properties of inhaled aerosolised drug particles such as size, shape, density, hygroscopicity, velocity and pressure of delivery, and charge should be taken into consideration. Moreover, the physiological state of the respiratory tract will also have an impact on the correct point of delivery inside the respiratory tract (upper airways versus deep lung).[87]

Solubility, pKa, protein binding, polar surface area, and charge or rotatable bonds are also important properties of drugs that will influence their permeability across the lung epithelial barrier. It should also be taken into account that mucus and surfactant might influence solubility and permeability characteristics of a given drug.[85]
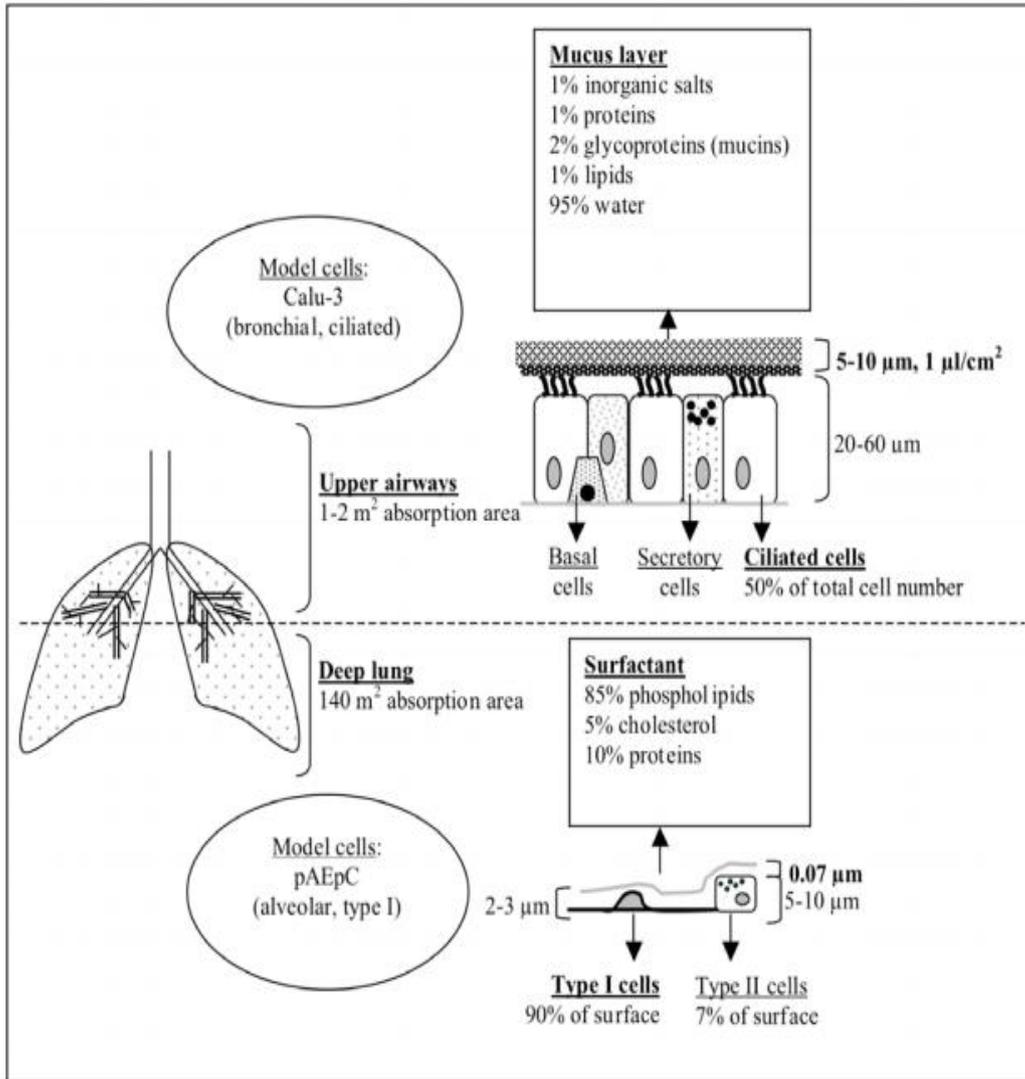
Figure 3: Comparison of the upper airways and the deep lung. Differences are found in the absorption area, cellular variety and height, lining composition (mucus vs. surfactant), thickness, and total volume. [85]
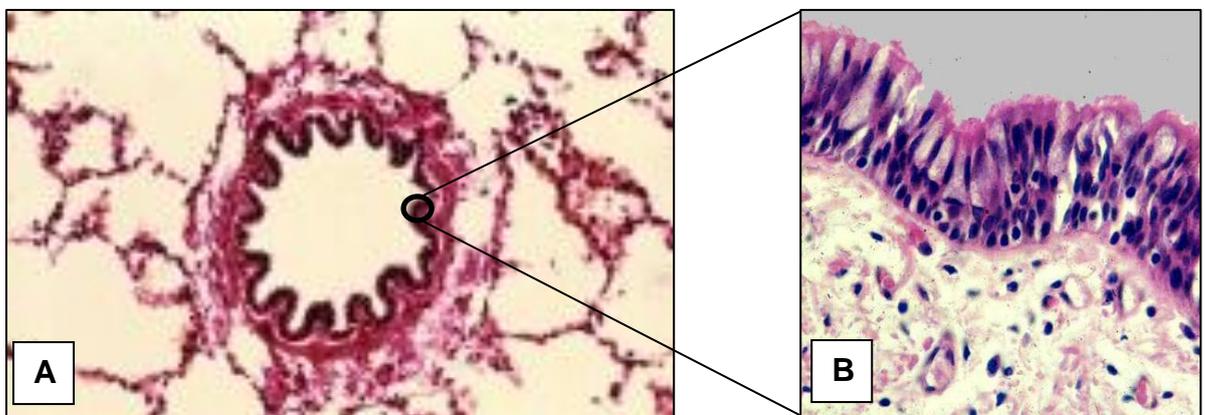

Figure 4: A; Cross section through a bronchiole in lung. B; The bronchial wall is lined by pseudostratified ciliated columnar epithelium with goblet cells. [88,89]

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2.8 Transmembrane diffusion processes

The manner in which substances diffuse through liquids, solids, and membranes is a process of great importance when considering drug delivery. The transmembrane diffusion process is passive and requires only a concentration differential as the driving force. In this manner, a molecule must first diffuse through the vehicle in which it is contained to the membrane interface and then partition from the vehicle into the upper lamina of the membrane. Molecules will then diffuse within the membrane, equilibrating laterally and reach steady state conditions, from the distal surface of the medium. It is also possible that there could be extensive adsorptive interactions resulting in the formation of a "reservoir". Finally the molecule partitions either to the adjacent membrane strata or into the receptor fluid (under the influence of the concentration gradient). Adsorption may occur once again. In this manner, diffusion through any one of the layers or any of the partitioning events may control the overall rate of permeation.[90,91]

Diffusion across mucosa can take place along two pathways i.e. transcellular (passage across cells) and paracellular or intercellular (passage between cells). Transcellular diffusion may occur through the use of a receptor mediated binding followed by vesicular transport (transcytosis) or independently by means of passive, absorptive transcytosis (fluid phase). Paracellular diffusion is commonly thought to occur by means of tight junctions between two or three cells. Another classification of diffusion processes is into polar (aqueous channels in mucosa) and non-polar (partitioning into the lipid bilayer of the mucosa) pathways. It would seem that the passage of most molecules across the mucosa appears to be a simple first-order diffusion process (Fig. 5).[91,92]
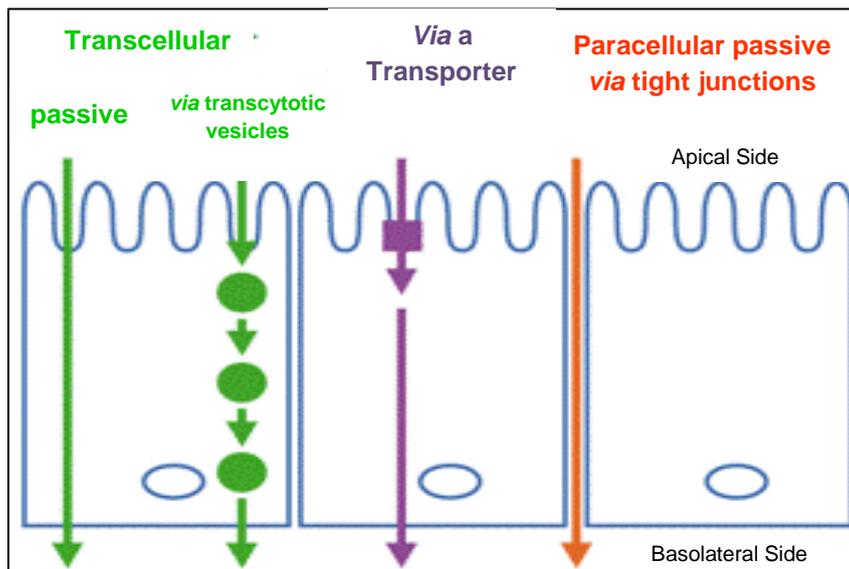
Figure 5: The main routes for transepithelial absorption are the transcellular passive diffusion and transcytosis, a transcellular route involving transport by vesicles, the transporter-mediated transport for small peptides, and the paracellular passive transport through tight junctions.[93]

As previously discussed, the physio-chemical properties of a drug such as partition coefficient, molecular weight, solubility, ionisation, and chemical nature will influence/determine its diffusion through any tissue membrane. On the other hand, the partitioning coefficient of the drug is the governing factor for which pathway it will follow during diffusion through the membrane. Hydrophillic molecules are expected to permeate predominantly *via* the intracellular route whereas lipophilic molecules are expected to permeate predominantly by means of the intercellular lipid domains.[94] A mixed permeation model has been proposed where drugs diffuse through tissue predominately by way of continuous intercellular lipid domains. According to this model, lipid and polar regions of the membrane could provide the micro-routes through which the permeant diffuses, depending on its partition coefficient.[94] On the other hand, surfactant molecules may enhance or retard partitioning by reducing the surface tension between the carrier vehicle and the membrane surface, but may also disrupt the barrier layers of the membrane.[95]

2.9 Aim

Therefore the aim of my study was:

i)   To determine the diffusion kinetics of two different classes of chemotherapeutic drugs used in TB treatment (Isoniazid, Ofloxacin and Moxifloxacin) through porcine bronchial tissue and the role of DPPC on their permeability (paying specific attention to liposomal preparation as drug carriers).

ii)   To evaluate the effect of individual drugs and their combinations on the surface tension lowering property of dipalmitoyl phosphatidyl choline (DPPC) as well as encapsulation efficiency of the drug-loaded liposomes.

iii)   To investigate drug susceptibility of *Mycobacterium tuberculosis* cultures against the individual antibiotics and their DPPC combinations by using the Radiometric BACTEC 460TB™ system

3. MATERIALS

1,2 Dipalmitoyl-L-α-phosphatidylcholine (DPPC) was purchased from Avanti polar Lipids Inc. Isoniazid (INH), Ofloxacin (Oflox) and Moxifloxacin (Moxi) used for the permeability studies as well as BACTEC 460™ experiments were purchased from the Sigma Chemical Company. (St. Louis MO, USA). Chloroform used was HPLC grade. All other chemicals used were of the highest analytical grade. Sterile water for injection was used in the BACTEC 460™ studies.

CHEMICAL STRUCTURES OF ANTITUBERCULAR DRUGS	
Isoniazid $C_6H_7N_3O$	**Ofloxacin** $C_{18}H_{20}FN_3O_4$
Mw = 137.139 g/mol	Mw = 361.38 g/mol
Moxifloxacin $C_{21}H_{24}FN_3O_4$ Mw = 401.431 g/mol	
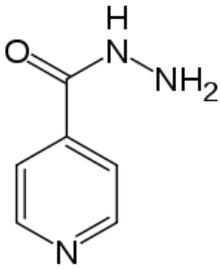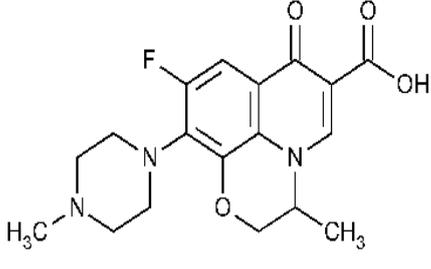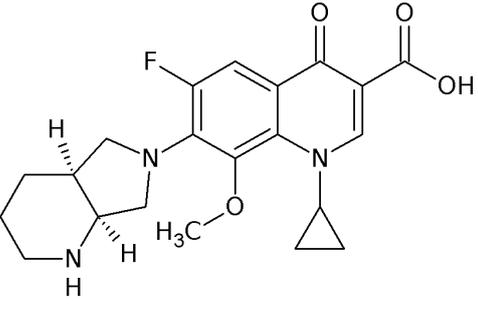
Figure 6: Chemical structures of Antimycobacterial drugs

4. METHODS

4.1 Liposomal Preparations

The antitubercular drug-loaded liposomes were prepared by a modified thin-film hydration method maintaining the drug to DPPC at a 1:1 w/w ratio. The final concentration of DPPC and drug was kept at 2.5 mg/ml.

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4.1.1 Drug Loaded liposomes

Thin Film Formation

The drug:lipid ratio was maintained at 1:1 ration w/w. Therefore, for every milligram of DPPC used for liposomes, a milligram of drug was added to the hydrating medium. DPPC was dissolved in a 2:1 chloroform: ethanol mixture, after which the organic solvent was removed at a temperature of 38ºC by vacuum rotary evaporation (Heidolph LABOROTA 4003). A homogenous thin lipid film was deposited during the evaporation of the solvent. The round bottom flasks were then kept in a vacuum pump dryer (Townson & Mercer Ltd) overnight. DPPC

Film Hydration

The individual drugs at a ratio of 1:1 by weight to DPPC were singularly dissolved in heated phosphate buffered saline (PBS pH 7.4) at 45ºC. The resulting dried lipid layers were then hydrated with this aqueous solution of PBS (pH 7.4) and drug combination. Small glass beads were added to the flask which was gently agitated for 30 min in a water bath at 45ºC until a homogenous suspension was formed. The suspension was kept at 4ºC overnight after being sealed under a N_2 atmosphere to prevent oxidation and ensure proper hydration of the lipids.

4.1.2 Size Reduction and Homogenisation of Liposomes

Ultra sonication

The liposomal suspension of Multi-Lamellar Vesicles (MLV) was heated to a temperature of 37ºC prior to further studies and placed in the middle of an ultra-sonication bath (Bransonic 220) and exposed to ultrasonic pulses for 3 min in total in 15 sec intervals whilst agitating the flask gently.

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Extrusion

The ultra-sonicated liposomal drug suspension was filtered-extruded through a polycarbonate membrane filter using the LiposoFast-Basic™ extruder consisting of Teflon Luer lock type Hammilton syringes (Avestin, Inc Canada). A maximum volume of 500 µl, at a time, was passed through eleven times, back and forth, through the 100 µm filter whilst the temperature was kept close to 45ºC.[96]

Optical Microscopy

To confirm the formation of liposomes, samples of the MLVs suspension as well as the extruded suspension were observed on an Olympus Cell^R system attached to an IX-81 inverted fluorescence microscope equipped with an F-view-II cooled CCD camera (Soft Imaging Systems). Images were acquired by using an Olympus Plan Apo N 60x/1.4 Oil objective and the Cell^R imaging software.

4.2 Tissue preparation

Fresh porcine bronchial tissue was obtained from Winelands Pork Abattoir. The tissue specimens were immediately transported on ice to the laboratory within 1 h. Excess connective and adipose tissue were trimmed away and tissue specimens were snapped frozen in liquid nitrogen and stored at -80ºC. No specimens were included in the study where there was any evidence of disease that might influence the permeability characteristics of the tissue. The Ethics committee of the Stellenbosch University granted approval for this study.

4.3 Permeability experiments

The diffusion kinetics of antituberculosis drugs (alone or as DPPC suspensions or as filtered-extruded liposomes) through porcine lung tissue was determined with a flow-through diffusion system (ISCO Retriever IV) (Fig 7).

Figure 7: Flow-through diffusion apparatus used in the present study

Bronchial tissue specimens were equilibrated for approximately 10 min at room temperature in phosphate-buffered saline (PBS, pH 7.4) before each permeability experiment. The specimens were then cut very carefully, as not to damage the epithelial surfaces, into 4 mm^2 sections. The individual sections were mounted in the flow-through diffusion cells (exposed area 0.039 cm^2). Permeation studies were performed on 7 tissue replicates for each experiment. Tissue disks were equilibrated for 10 min with PBS (pH 7.4) at 36.5ºC in both the donor and acceptor chambers of the diffusion cells. After equilibration, PBS was removed from the donor chamber and replaced with 0.5 ml of antimycobacterial drug and DPPC/DPPC-liposomes concentrations as described above. PBS at 36.5ºC was

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pumped through the receiving chambers at a rate of 1.5 ml/h (IPC High Precision Multichanger Dispenser ISMIATIC) and collected, by means of a fraction collector, at 2 h intervals for 24 h. The permeability studies were performed under sink conditions, i.e. at the completion of each run the concentration of permeant, in this case antituberculosis drugs, never reached 10% of that in the donor chamber.[97]

4.4 HPLC detection of drugs

Permeant containing effluent samples, collected from the acceptor compartments of the perfusion apparatus over the 2 – 24 h sampling intervals, were analysed using a Hewlett Packard 1100 series high–performance binary liquid chromatograph fitted with a diode-array UV detector (Agilent Technologies, Waldbron, Germany). The analytical parameters are summarised in Table 3.

4.5 Calculation of flux values

Flux values (J) of antimycobacterial drugs across bronchial tissue were calculated by means of the relationship: J = dQ/dt x 1/A ng x cm-2 x min-1), where Q = quantity of substance crossing membrane (ng), A = membrane area exposed (in cm^2), and t = time of exposure (in min).

4.6 Steady-state kinetics

Reaching of steady state (equilibrium kinetics) was assumed to have been reached for a particular drug compound, when no statistically significant differences ($p < 0.05$) (ANOVA and Duncan's multiple range test) between flux values were obtained over at least two consecutive time intervals.

4.7 Statistical analyses

Non-linear regression analyses (third order polynomial) using GraphPad Prism, Version 5 (GradPad Software Inc, San Diego, CA, 92130 USA) were performed.[98] A F-test was used to compare entire curves. An unpaired t test with Welch's correction was used to investigate possible differences between flux means of bronchial membranes at 2 h intervals. A significance level of $P < 0.05$ was used for all tests and comparisons.

Parameter	Isoniazid		Ofloxacin		Moxifloxacin	
Column used in all instances	Agilent Zorbax C18 150mm x 4.6mm x 3.5µ particle size					
Sample preparation	See below*		not required		not required	
Column flow rate		1.6 ml/min	0.75 ml/min		1 ml/min	
Column temperature		45°C	40°C		40°C	
Injection volume		60 µl	20 µl		20 µl	
UV detector setting		340 nm	-		-	
Florescence detector:	Ex	-	284 nm		287 nm	
	Em	-	460 nm		463 nm	
Solvent A	50 mM KH_2PO_4					
Solvent B	Acetonitrile : Isopropanol 4 : 1 v/v					
Gradient solvent B	0 - 1 min	40%	0 - 1.0 min	35%	0 - 0.75 min	55%
	1 - 6 min	linear to 60%	1.0 - 4.0 min	linear to 65%	0.75 - 2.0 min	linear to 70 %
			4.0 - 4.1 min	return to 35%	2.0 - 2.1 min	return to 55 %
			4.1 - 6 min	35%	2.1 - 6 min	55%
Retention time	2.08 min		2.43 min		1.62 min	
Run time		6 min	6 min		6 min	
Equilibrium time		3 min	not required		not required	

Table 3: HPLC detection of Isoniazid, Ofloxacin and Moxifloxacin

- ***Sample preparation of Isoniazid:** For the detection of INH, 100µl of 10% TCA (trichloroacetic acid) and 100µl of INH-containing effluent sample were mixed and vortexed for 30 seconds. Hereafter, 40 µl of 1% metanolic cinnamaldehyde was added to the 200µl for derivatisation.

4.8 BACTEC

All experiments with infectious material were carried out in a category-3 biosafety laboratory.

4.8.1 Culturing of *M. tuberculosis*

Clinical isolates of *M. tuberculosis* X51 XDR strain and *M. tuberculosis* H37Rv reference strain, banked in the Department of Medical Biochemistry, was cultured on L-J slant cultures and used for BACTEC analysis.

4.8.2 Solvents

Depending on the biological activity of the compounds as well as the specific strain, concentration ranges were adjusted in relation to the minimum inhibitory concentration (MIC) of the compound (Table 4):

	INH: H37Rv Reference Strain	Oflox: X51 XDR Strain	Moxi: X51 XDR Strain
MIC	0.02 µg/ml	2 µg/ml	1 µg/ml

Table 4: Minimum Inhibitory Concentrations of the relevant antimycobacterial drugs and their corresponding strains.

All of the above antimycobacterial drugs were prepared in PBS (pH 7.4). Liposomal-drug preparations were made following the same procedure as previously described in Section 4.1. A dilution series was made of the respective drugs and their liposomal combinations to create a concentration range above and below the MIC.

4.8.3 Mycobacterial cultures and BACTEC determinations.

Clinical and laboratory strains of *M. tuberculosis* were cultured in Middlebrook medium enriched with ADC enrichment medium. Continuous stirring was executed to ensure homogenous bacterial distribution and uniform aeration, allowing the cultures to grow reproducibly (< 1.0% difference). This procedure allows accurate assessment of manipulation of mycobacterial cultures with compounds. At a culture density of approximately A600 nm = 0.16 (1x McFarland), *M. tuberculosis* strain cultures was inoculated (0.1 ml) into 4 ml-BACTEC vials. Cultures in BACTEC were grown until a growth index (GI) of 500 - 700 was reached. This culture was used as the starter (primary) culture for BACTEC evaluation of antimycobacterial compounds in question on *M. tuberculosis* strains. Sterility of primary cultures was monitored by Z-N acid fast staining. The test was performed as follows: 0.1 ml of the primary culture was added to each of the fresh BACTEC vials along with 0.1 ml compound-containing-solution in their relevant concentrations according to their MICs. Controls were held at 1:100 dilution of the primary culture in the absence of the compound. The bacterial cultures were then incubated at 37ºC. The culture growth was monitored over a time period of approximately 14 days. When the control reached a maximum GI of 500, the experiment was completed, and the ΔGI values (the change in growth for every 24 h doubling period) were determined for each concentration value to detect a positive (growth was witnessed) or negative (no growth witnessed) growth response. Control assays (with no drugs) were brought to the same solvent concentrations.

4.9 Entrapment efficiency

An ÄKTA™ explorer FPLC (Amersham Pharmacia Biotech) system was used to remove free, untrapped drug from drug-loaded liposomes. An aliquot of the liposomal preparation was loaded onto a Superdex-75 column (Sigma), 16 x 53 cm (bead diameter of 24 – 44 µm; fractionation range 300 – 70 000 MW). Detection of drugs was carried out at 273 nm for INH and 287 nm for the fluoroquinolones (λ_{max}). Fractions containing drug-loaded liposomes were assayed using HPLC to determine the concentration (entrapment efficiency). The column was eluted with 50 mM phosphate buffer containing 10 mM NaCl (pH 7.4) at a constant flow rate of 0.25 ml/min.

4.10 Spectroscopic analyses

The spectral properties of the Superdex-75 fractions were performed on a Beckman DU 640 spectrophotometric system. Fluorescence scans were conducted with a Perkin–Elmer LS50B luminescence spectrometer fitted with a well-plate reader. Excitation and emission wavelengths of Oflox and Moxi were 287 nm and 474 nm respectively (excitation slit 2.5 nm; emission slit 5 nm; scan speed 500 nm/min)

4.11 Surface tension determination

Surface tension measurements of the drug/DPPC-liposomal preparations (All at same concentration values) were done using a DIGIDROP Contact Angle Meter by GBX. Hammilton syringes were used to calibrate the pixels by using a syringe needle of 0.73 mm. The syringe pump was set at rates varying between 0.005-0.0075 ml/min depending on the droplet-formation speed of the sample. The

density of each sample was also recorded as to ensure accurate readings. The right and left contact angles were measured and averaged by the GBX software which automatically calculates the tangent to the droplet shape and the contact angle and 7 repeat tests were performed for each adhesive. This allowed calculations of the spreading rates by logarithmic fitting of the variations in angles and determination of the standard deviations.[99]
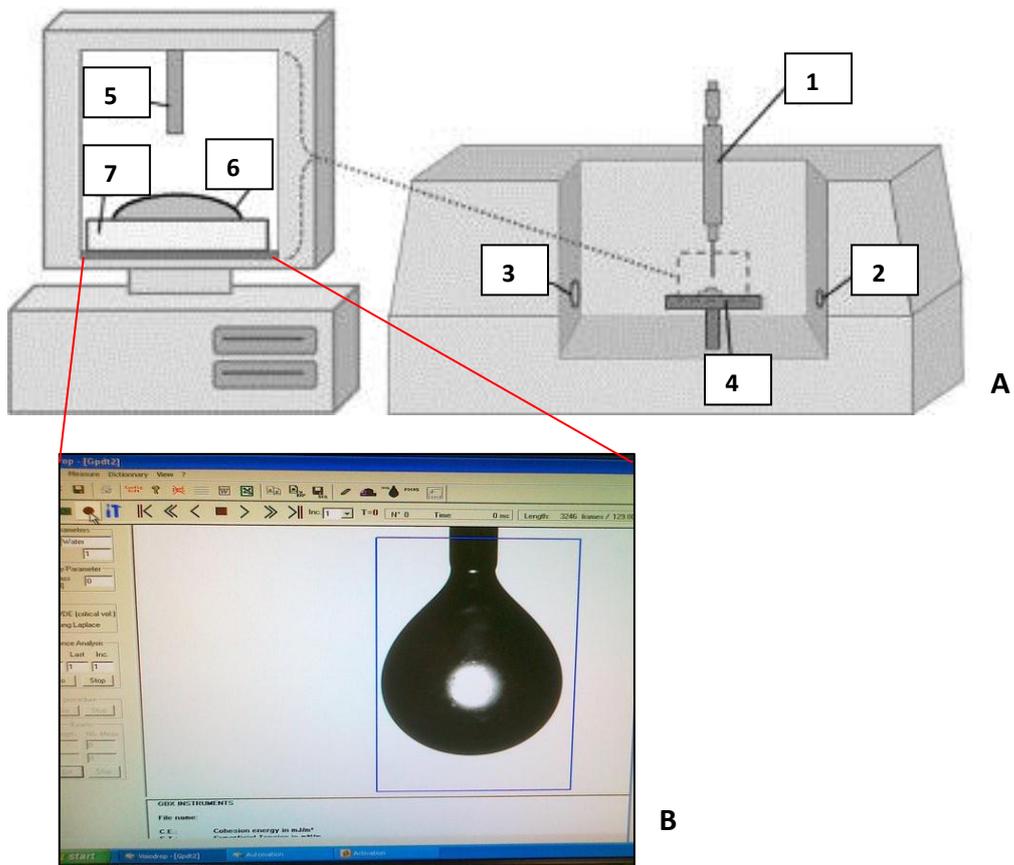
Figure 8: A; Apparatus used for contact angle measurements (DIGIDROP, GBX, Bourg de Peage, France). 1: vertical syringe, for the formation of calibrated volume drops, 2: light source, 3: video camera, 4: adjustable platform, 5: needle tip, 6: drop as registered on screen, 7: substrate surface B; Open window of the DIGIDROP program used to measure Surface Tension.[99]

5. RESULTS

The overall mean flux values of isoniazid (INH) across porcine bronchial tissue with and without DPPC/DPPC-liposomes are shown in Fig. 9. No statistically significant differences between the INH control (n = 4) and the INH with DPPC (n = 4) could be demonstrated (p = 0.9345). On average, the mean steady state INH flux rates of the DPPC-liposomes (n = 4) decreased by 43.3% when compared to the INH control (p = 0.0173) and 44.2% when compared to the INH-DPPC combination (p = 0.0124), respectively. Steady state for INH, INH-DPPC and INH DPPC-liposomes were obtained after approximately at 8 to 10 hrs (p > 0.05).
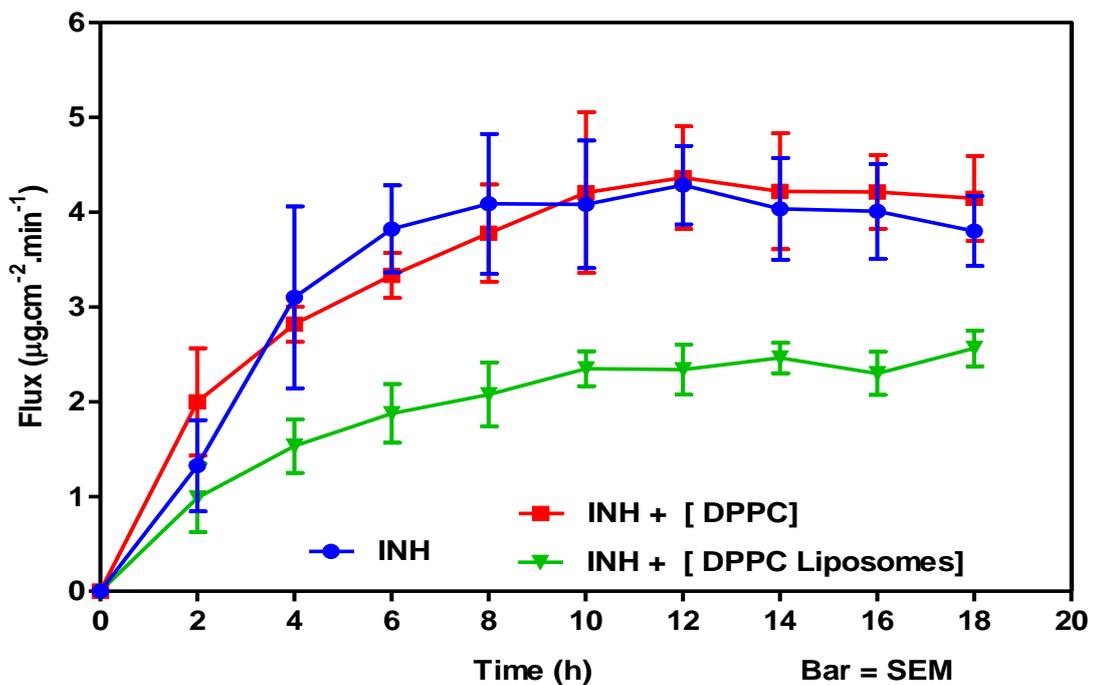
Figure 9: Overall mean flux values of isoniazid across porcine bronchial tissue with and without DPPC and DPPC-liposomes.

The overall mean steady-state flux values of Oflox across porcine bronchial tissue with and without DPPC/DPPC-liposomes are shown in Fig. 10. All three the preparations achieved steady state flux values within 2 hrs. On average, this is 4 – 5 times shorter than the time taken for INH to reach these values. No statistically significant differences between the mean steady state flux values of OFLOX control (n = 5) and Oflox with DPPC-liposomes (n = 3) could be demonstrated (p = 0.3122) with the latter having on average 16.4% lower flux values. Flux values for Oflox-DPPC (n = 6) were statistically significantly decreased by 35.1% when compared to the Oflox control (p = 0.0265) and non–significantly by 22.4% when compared to the Oflox DPPC-liposomes (p = 0.1724). Steady-state flux rates for Oflox and Oflox-DPPC were reached at approximately 8 hrs while Oflox DPPC-liposomes demonstrated a steady-state within 4 – 6 hrs (p < 0.05).
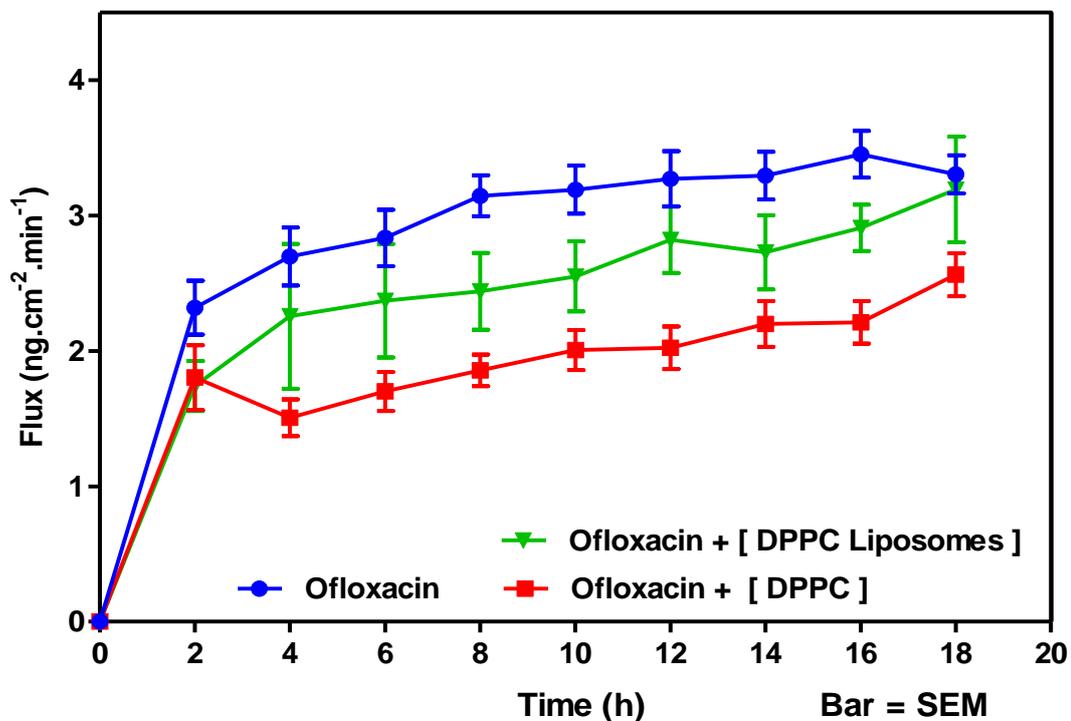

Figure 10: Overall mean flux values of ofloxacin across porcine bronchial tissue with and without DPPC and DPPC-liposomes.

The overall mean steady-state flux values of Moxi across porcine bronchial tissue with and without DPPC/DPPC-liposomes are shown in Fig. 11. As in the previous graph (Fig. 10), all three preparations achieved steady state flux rates within 2 hrs compared to the 8 – 10 hrs required for INH. No statistically significant differences between the Moxi control (n = 6) and the Moxi-DPPC (n = 6) mean steady-state flux values could be demonstrated (p = 0.1023) with the latter having on average 24.6% lower flux values. Flux values for Moxi DPPC-liposomes (n = 4) were statistically significantly increased by 43.8% when compared to the MOXI control (p = 0.0065) and by 57.7% when compared to the Moxi-DPPC combination (p = 0.0010). Steady state for Moxi and Moxi-DPPC were reached at approximately 4 hrs while Moxi DPPC-liposomes demonstrated a steady state within 4 – 6 hrs (p > 0.05).
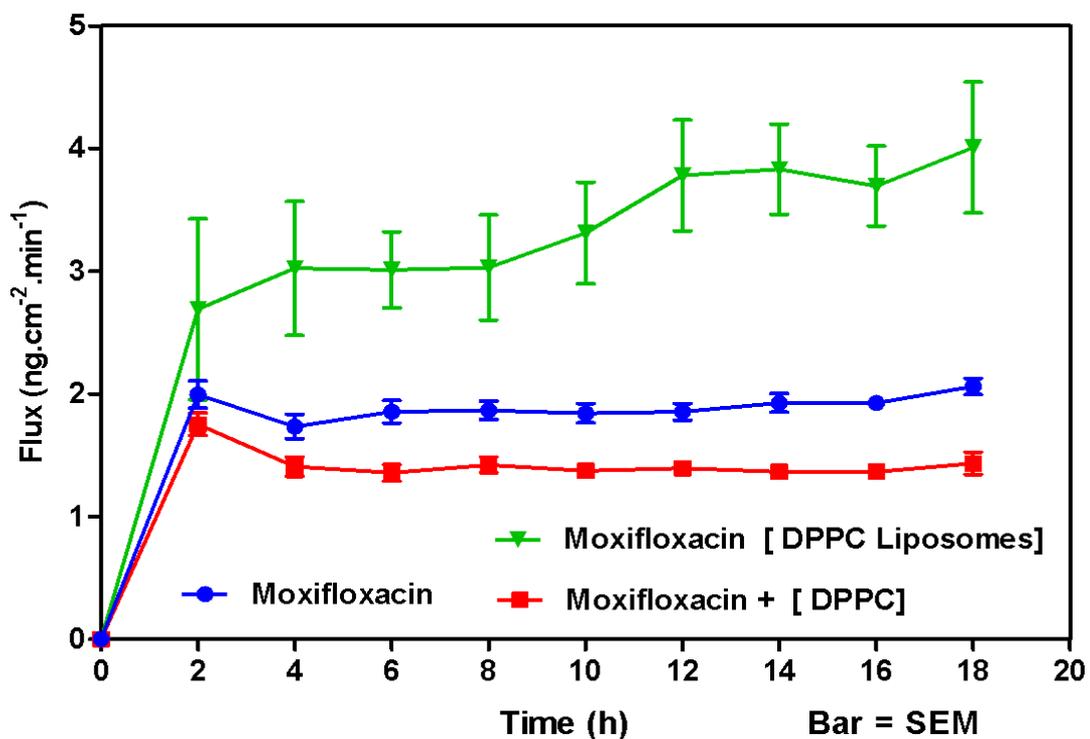
**Figure 11: Overall mean flux values of moxifloxacin across porcine bronchial tissue with and without DPPC and DPPC-liposomes.**

The overall growth index *M. tuberculosis* susceptibly testing of INH using the BACTEC 460™ system is shown in Fig. 12. The MIC of the H37Rv strain was confirmed at 0.02 µg/ml (See Table 5).
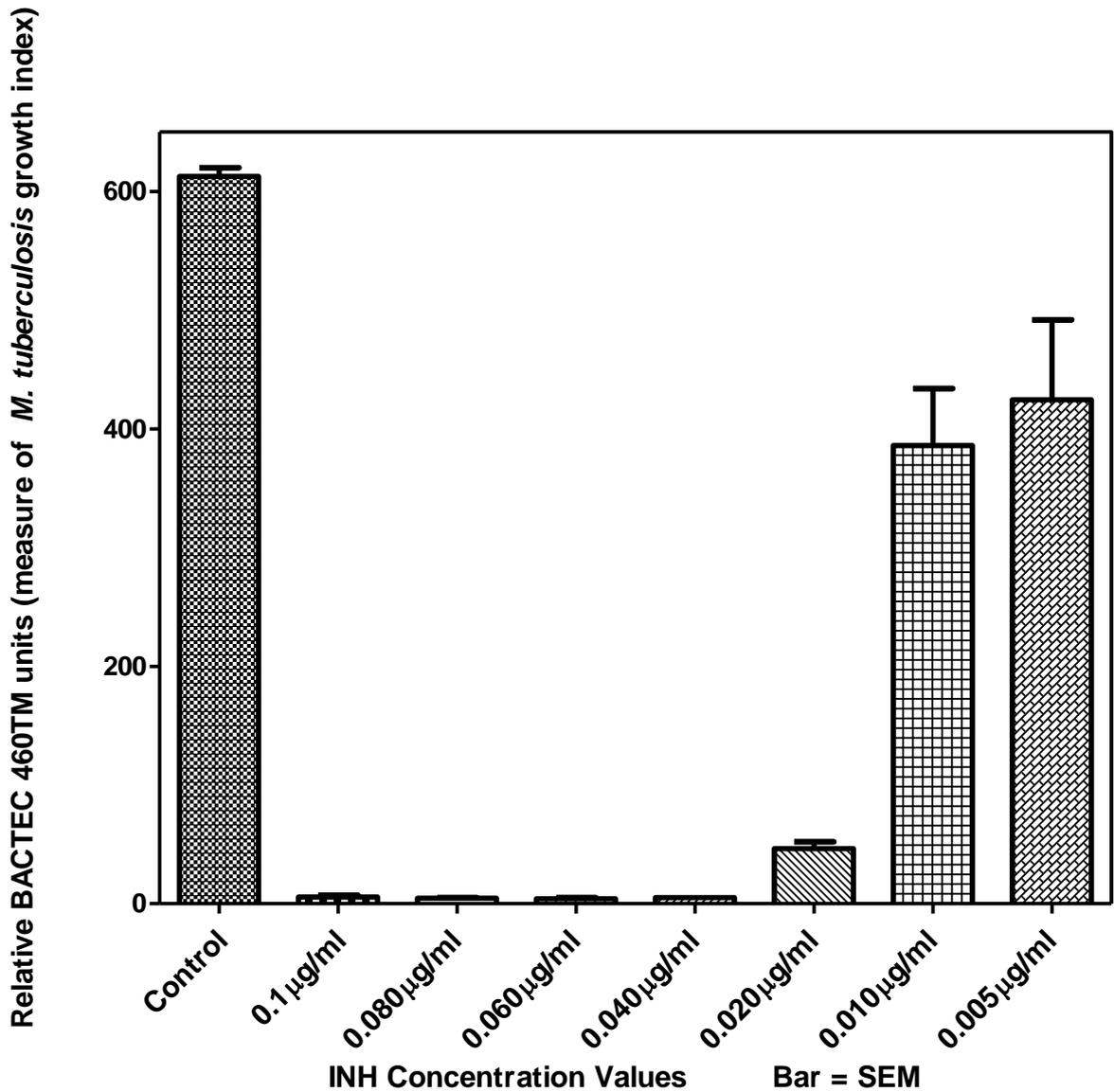
Figure 12: Extracellular drug testing at 1 x 10⁷ *M. tuberculosis* per BACTEC 460™ vial containing 4 ml Middlebrook 7H12 medium. For control, *M. tuberculosis* was inoculated into BACTEC 460™ vials with no inhibitor or drug added.

The overall growth index *M. tuberculosis* susceptibly testing of INH-DPPC liposomes using the BACTEC 460™ system is shown in Fig. 13. The MIC of the H37Rv strain was confirmed at 0.02 µg/ml (See Table 6).
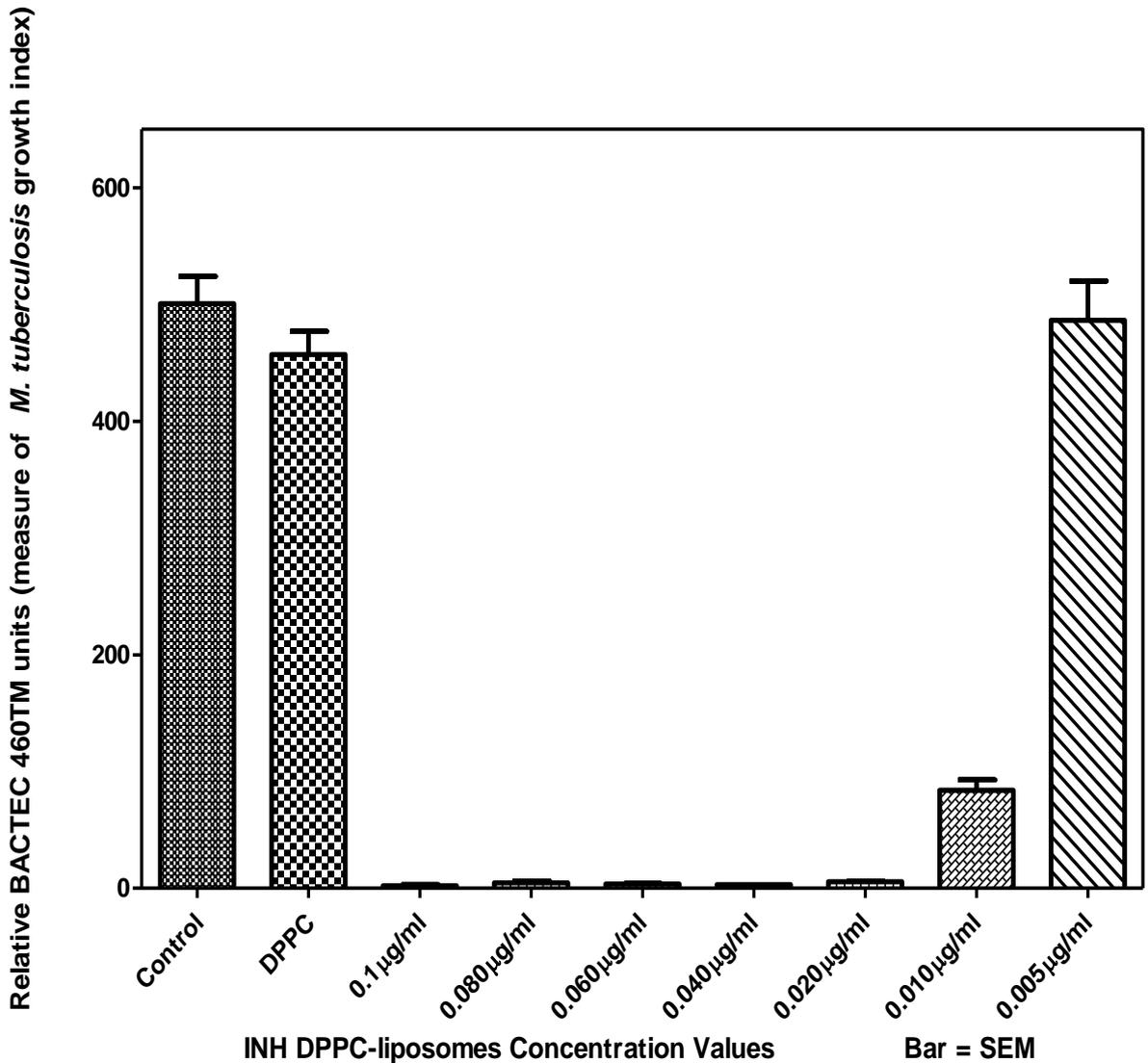
Figure 13: Extracellular drug testing at 1 x 107 *M. tuberculosis* per BACTEC 460™ vial containing 4 ml Middlebrook 7H12 medium. For control, *M. tuberculosis* was inoculated into BACTEC 460™ vials with no inhibitor or drug added. All drug concentrations displayed illustrate liposomal combinations.

In reference to Fig. 12 and Fig. 13, the overall susceptibly of the *M. tuberculosis*, H37Rv strain to various concentrations of isoniazid displayed in Table 5 and isoniazid, DPPC-liposomes is displayed in Table 6.

Samples	Isoniazid				
	GI.1	GI.2	ΔGI	% of Inhibition	R/S
Control	335	612.5	277.5	-	-
0.1 µg/ml	6.5	5.5	-1	100%	s
0.08 µg/ml	6	4.5	-1.5	101%	s
0.06 µg/ml	7.5	4	-3.5	101%	s
0.04 µg/ml	6.5	5	-1.5	101%	s
0.02 µg/ml	26.5	46.5	20	93%	s
0.01 µg/ml	279	386	107	61%	r
0.005 µg/ml	310	424.5	114.5	59%	r

Table 5: Susceptibility of *M. tuberculosis*, H37Rv strain, isolates as determined by the BACTEC 460™ system when exposed to various concentrations of INH without DPPC-liposomes. GI.1: Growth index day 1, GI.2: Growth index day 2, ΔGI: change in growth, R/S: resistant/susceptible.

Samples	Isoniazid DPPC-liposomes				
	GI.1	GI.2	ΔGI	% of Inhibition	R/S
Control	277.5	500.5	223	-	-
DPPC	241.5	457	215.5	3%	r
0.1 µg/ml	5.5	2	-3.5	102%	s
0.08 µg/ml	4	4.5	0.5	100%	s
0.06 µg/ml	3	3.5	0.5	100%	s
0.04 µg/ml	3.5	3	-0.5	100%	s
0.02 µg/ml	5	5.5	0.5	100%	s
0.01 µg/ml	45	84	39	83%	s
0.005 µg/ml	272.5	486.5	214	4%	r

Table 6: Susceptibility of *M. tuberculosis*, H37Rv strain, isolates as determined by the BACTEC 460™ system when exposed to various concentrations of INH with DPPC-liposomes. GI.1: Growth index day 1, GI.2: Growth index day 2, ΔGI: change in growth, R/S: resistant/susceptible.

The overall growth index *M. tuberculosis* susceptibly testing of ofloxacin using the BACTEC 460™ system is shown in Fig. 14. The MIC for the X51 XDR strain was confirmed to be at 0.2 µg/ml (See Table 7).
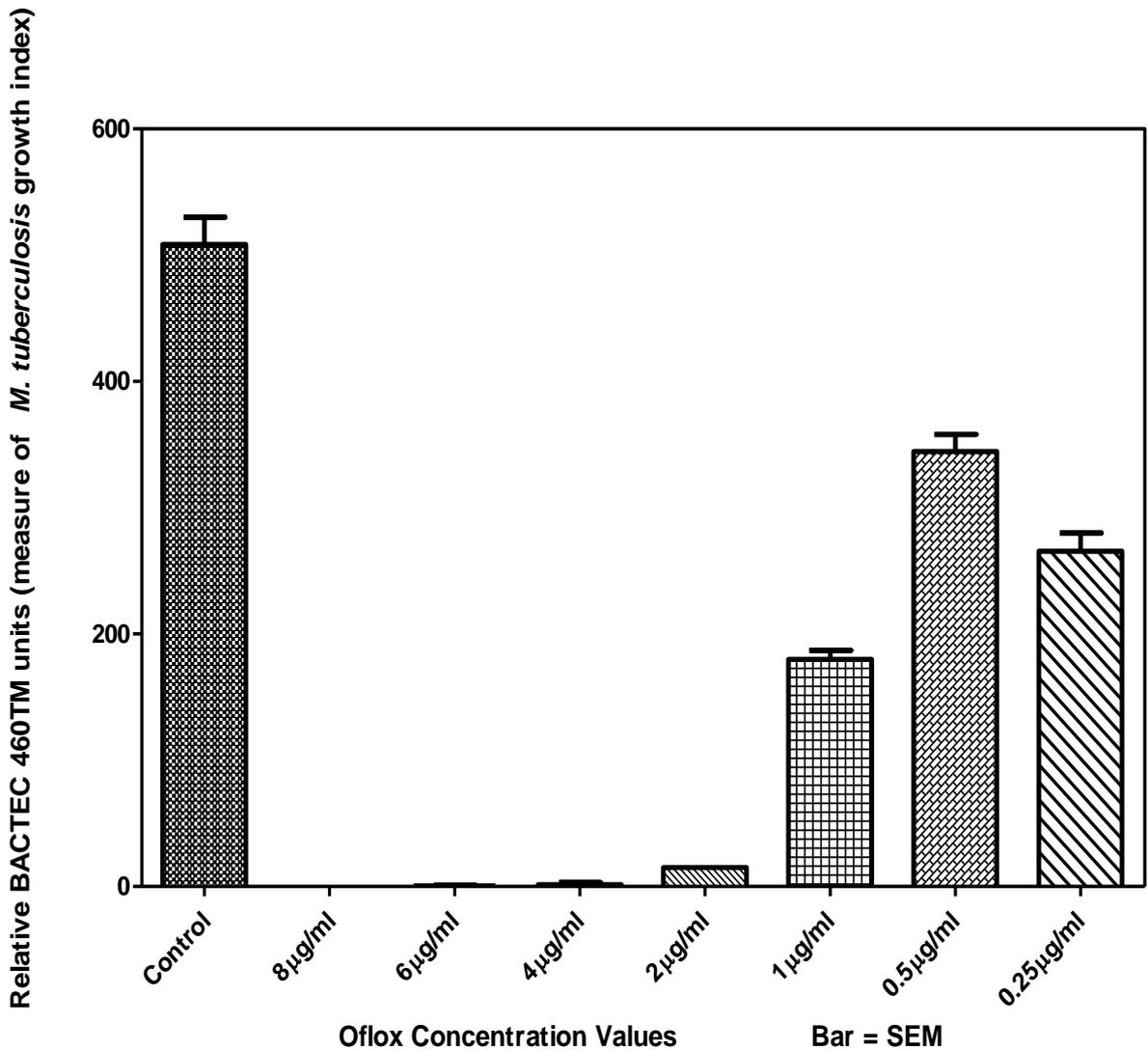

Figure 14: Extracellular drug testing at 1 x 107 *M. tuberculosis* per BACTEC 460™ vial containing 4 ml Middlebrook 7H12 medium. For control, *M. tuberculosis* was inoculated into BACTEC 460™ vials with no inhibitor or drug added.

The overall growth index *M. tuberculosis* susceptibly testing of ofloxacin DPPC-liposomes using the BACTEC 460™ system are shown in Fig. 15. The MIC for the X51 XDR strain was confirmed to be at 0.2 µg/ml (See Table 8).
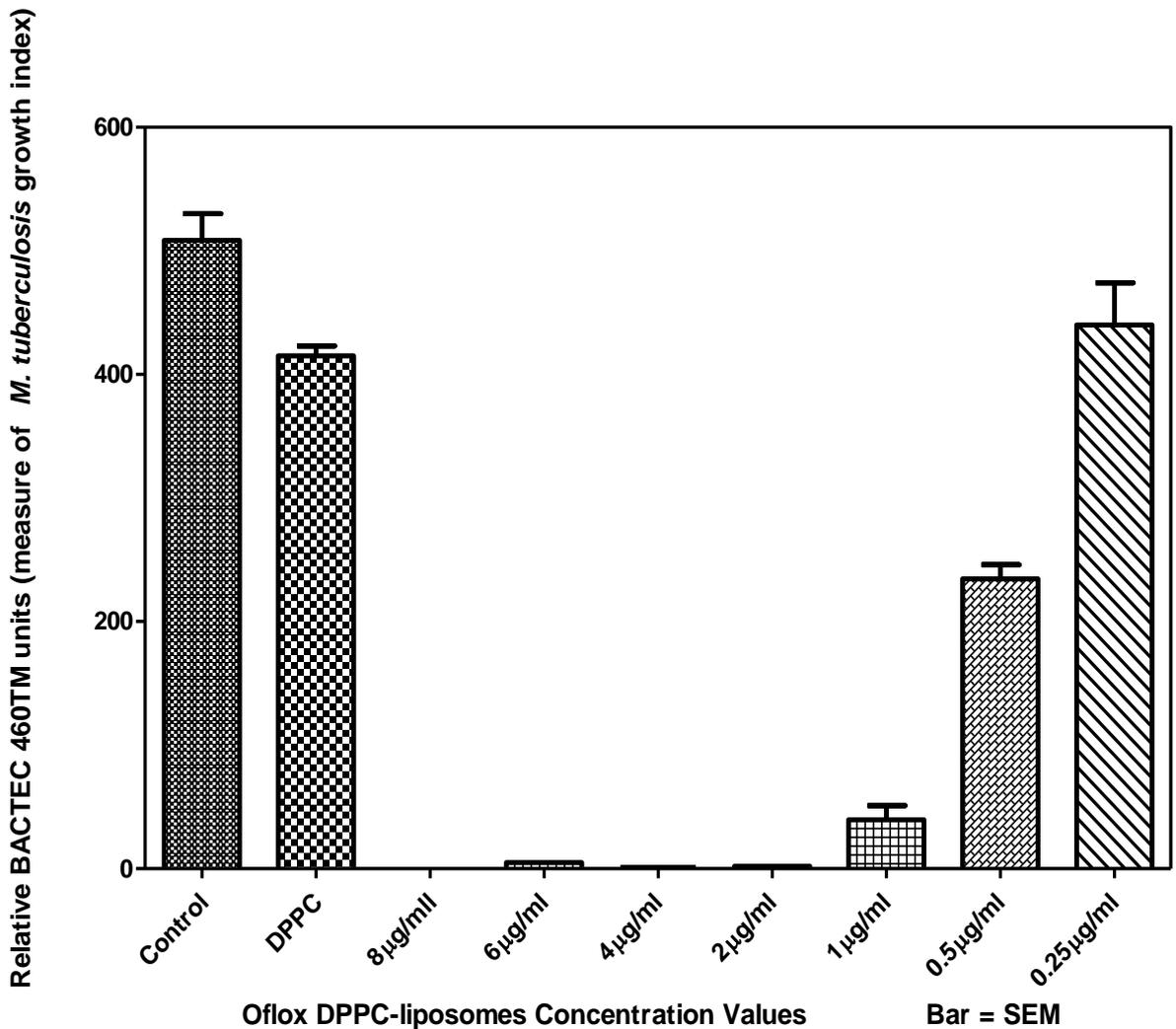

Figure 15: Extracellular drug testing at 1 x 107 *M. tuberculosis* per BACTEC 460™ vial containing 4 ml Middlebrook 7H12 medium. For control, *M. tuberculosis* was inoculated into BACTEC 460™ vials with no inhibitor or drug added. All drug concentrations displayed illustrate liposomal combinations.

In reference to Fig. 14 and Fig. 15, the overall susceptibly of the *M. tuberculosis*, X51 XDR strain to various concentrations of ofloxacin is displayed in Table 7 and ofloxacin with DPPC-liposomes is displayed in Table 8.

Samples	Ofloxacin				
	GI.1	GI.2	ΔGI	% of Inhibition	R/S
Control	191	508.5	317.5	-	-
8 µg/ml	0	0	0	100%	s
6 µg/ml	0	0.5	0.5	100%	s
4 µg/ml	2.5	1.5	-1	100%	s
2 µg/ml	13.5	22.5	9	97%	s
1 µg/ml	80.5	180	99.5	69%	r
0.5 µg/ml	151.5	344.5	193	39%	r
0.25 µg/ml	143	265.5	122.5	61%	r

Table 7: Susceptibility of *M. tuberculosis*, X51 XDR strain, isolates as determined by the BACTEC 460™ system when exposed to various concentrations of ofloxacin. GI.1: Growth index day 1, GI.2: Growth index day 2, ΔGI: change in growth, R/S: resistant/susceptible.

Samples	Ofloxacin DPPC-liposomes				
	GI.1	GI.2	ΔGI	% of Inhibition	R/S
Control	191	508.5	317.5	-	-
DPPC	161	415	254	20%	r
8 µg/ml	1	0	-1	100%	s
6 µg/ml	3	5	2	99%	s
4 µg/ml	27.5	1	-26.5	108%	s
2 µg/ml	13.5	2	-11.5	104%	s
1 µg/ml	22	39.5	17.5	94%	s
0.5 µg/ml	115	234.5	119.5	62%	r
0.25 µg/ml	177	440	263	17%	r

Table 8: Susceptibility of *M. tuberculosis*, X51 XDR strain, isolates as determined by the BACTEC 460™ system when exposed to various concentrations of ofloxacin with DPPC-liposomes. GI.1: Growth index day 1, GI.2: Growth index day 2, ΔGI: change in growth, R/S: resistant/susceptible.

The overall growth index *M. tuberculosis* susceptibly testing of moxifloxacin using the BACTEC 460™ system is shown in Fig. 16. The MIC for the X51 XDR strain was confirmed to be at 0.1 µg/ml (See Table 9)
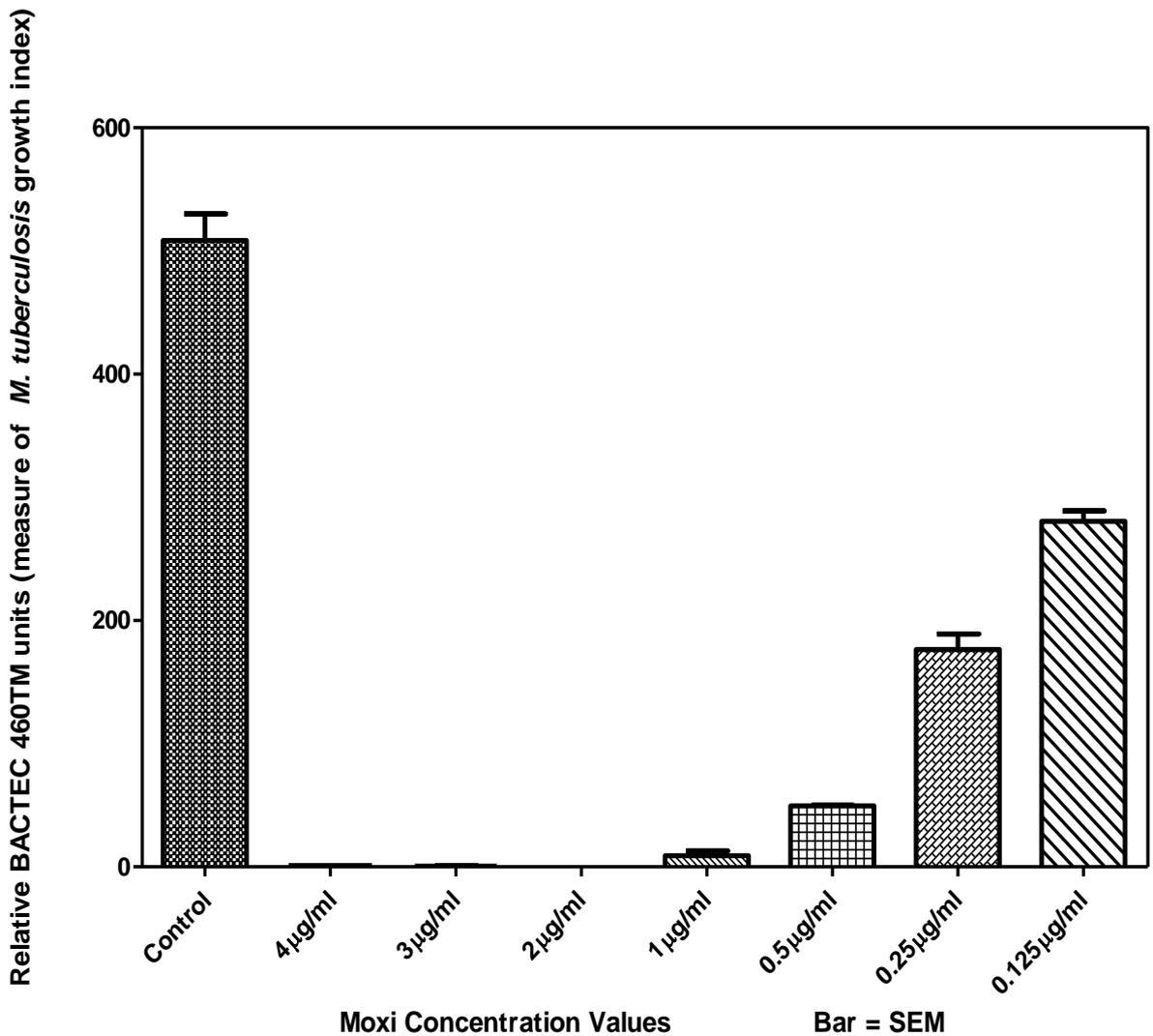

Figure 16: Extracellular drug testing at 1 x 107 *M. tuberculosis* per BACTEC 460™ vial containing 4 ml Middlebrook 7H12 medium. For control, *M. tuberculosis* was inoculated into BACTEC 460™ vials with no inhibitor or drug added

The overall growth index *M. tuberculosis* susceptibly testing of moxifloxacin DPPC-liposomes using the BACTEC 460™ system are shown in Fig. 17. The MIC for the X51 XDR strain was confirmed to be at 0.1 µg/ml (See Table 10)
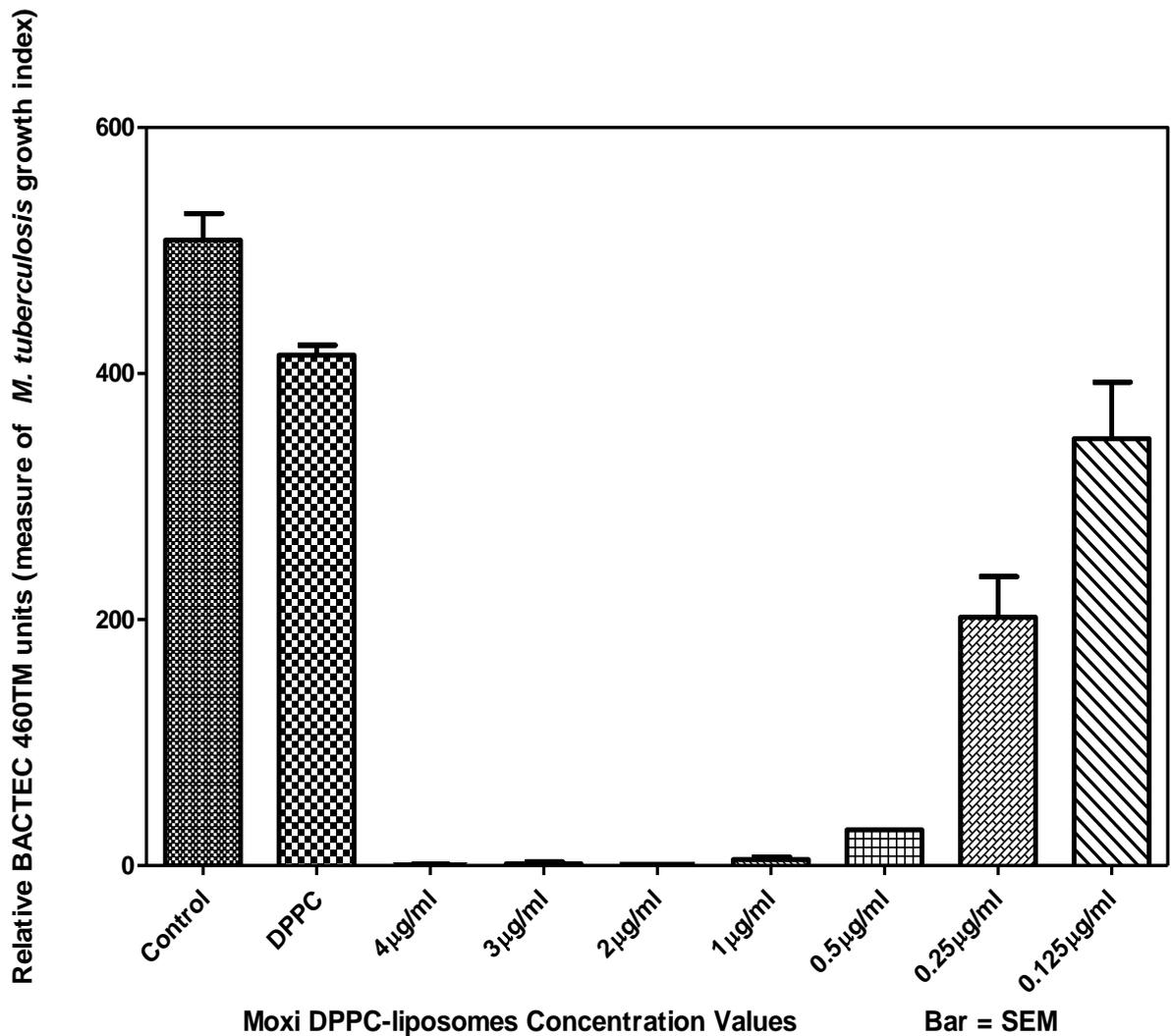

Figure 17. Extracellular drug testing at 1 x 107 *M. tuberculosis* per BACTEC 460™ vial containing 4 ml Middlebrook 7H12 medium. For control, *M. tuberculosis* was inoculated into BACTEC 460™ vials with no inhibitor or drug added. All drug concentrations displayed illustrate liposomal combinations.

In reference to Fig. 16 and Fig. 17, the overall susceptibly of the *M. tuberculosis*, X51 XDR strain to various concentrations of ofloxacin is displayed in Table 9 and ofloxacin DPPC-liposomes is displayed in Table 10.

Samples	Moxifloxacin				
	GI.1	GI.2	ΔGI	% of Inhibition	R/S
Control	191	508.5	317.5	-	-
4 µg/ml	0	1	1	100%	s
3 µg/ml	0.5	0.5	0	100%	s
2 µg/ml	0.5	0	-0.5	100%	s
1 µg/ml	6.5	9	2.5	99%	s
0.5 µg/ml	23.5	49.5	26	92%	r
0.25 µg/ml	79.5	176.5	97	69%	r
0.125 µg/ml	118	280.5	162.5	49%	r

Table 9: Susceptibility of *M. tuberculosis*, X51 XDR strain, isolates as determined by the BACTEC 460™ system when exposed to various concentrations of moxifloxacin without DPPC liposomes. GI.1: Growth index day 1, GI.2: Growth index day 2, ΔGI: change in growth, R/S: resistant/susceptible.

Samples	Moxifloxacin DPPC-liposomes				
	GI.1	GI.2	ΔGI	% of Inhibition	R/S
Control	191	508	317	-	-
DPPC	161	415	254	20%	r
4 µg/ml	0.5	0.5	0	100%	s
3 µg/ml	0.5	1.5	1	100%	s
2 µg/ml	0.5	1	0.5	100%	s
1 µg/ml	3	5	2	99%	s
0.5 µg/ml	17.5	29	11.5	96%	s
0.25 µg/ml	86.5	202	115.5	64%	r
0.125 µg/ml	144	347	203	36%	r

Table 10: Susceptibility of *M. tuberculosis*, X51 XDR strain, isolates as determined by the BACTEC 460™ system when exposed to various concentrations of moxifloxacin DPPC-liposomes. GI.1: Growth index day 1, GI.2: Growth index day 2, ΔGI: change in growth, R/S: resistant/susceptible.

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Entrapment efficiency of drug–liposomal preparations were determined on eluted fractions after chromatography on Superdex–75 as shown in Fig. 18. Analyses, using HPLC, of eluted INH fractions showed entrapment efficiency for INH to be 53%. For Oflox and Moxi, entrapment efficiency was calculated to be 46.6% and 43.3% respectively.
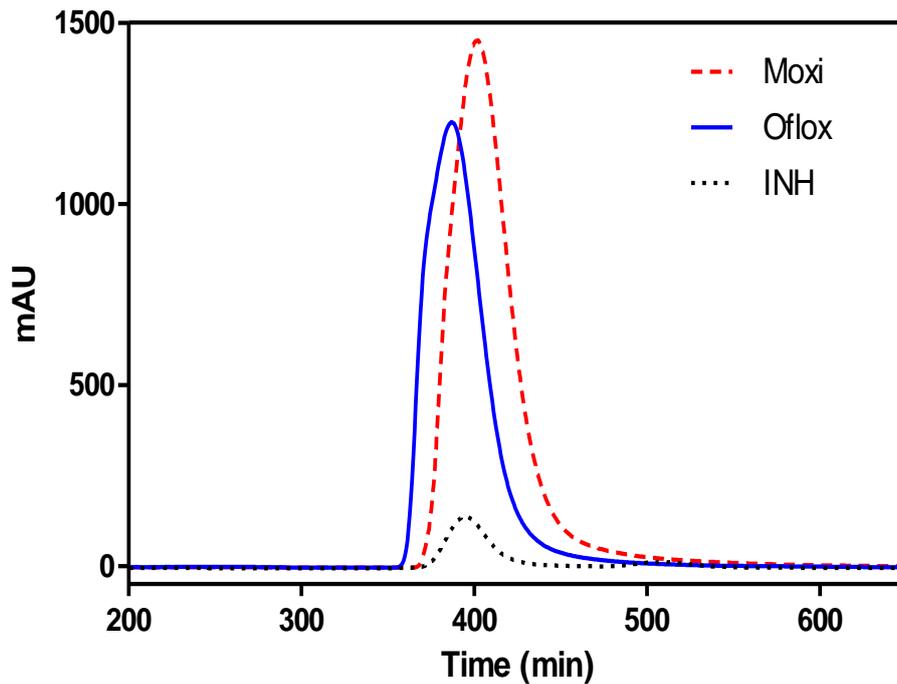
Figure 18: Gel-filtration chromatography of drug combined DPPC liposomal preparations on a 16 x 53 cm Superdex–75 column equilibrated with 50 mM phosphate buffer containing 100 mM NaCl.

Ultraviolet spectrophotometry of INH and INH–DPPC entrapped liposomes after gel filtration is shown in Fig. 19. Spectrum (b) showed the characteristic UV absorbance maximum of INH at 273 nm as depicted in (a).
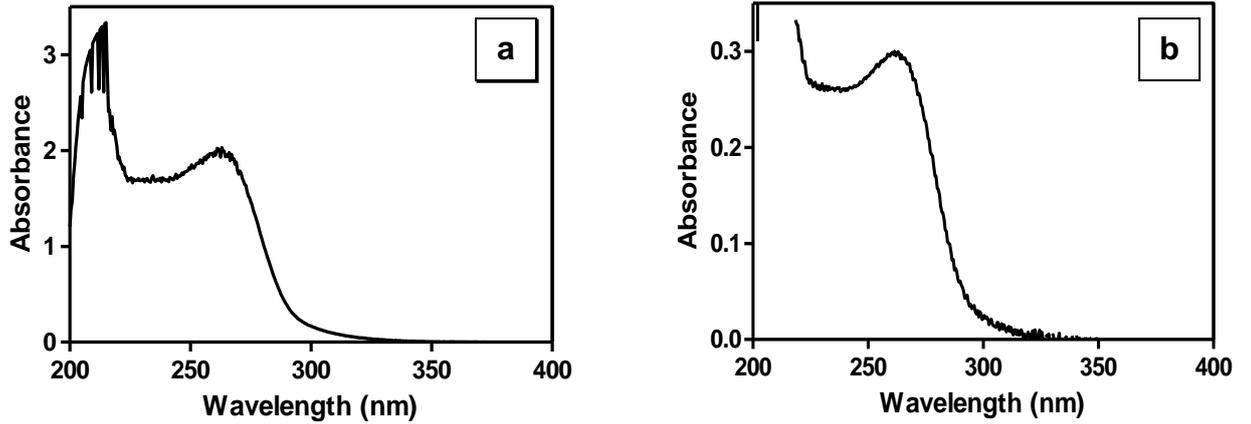
Figure 19(a): Absorption spectra of INH. (b): Absorption spectra of INH Liposomes (273 nm λ$_{max}$) after gel filtration on a Superdex-75 column.

To verify the presence of liposomes in the fractions collected after gel filtration, microscopy was done. Fig. 20 clearly depicts liposomes and the sizes ranging from 4.5 µm in diameter to smaller ones of approximately 780 nm in diameter. Liposomal clusters were also seen in the image.
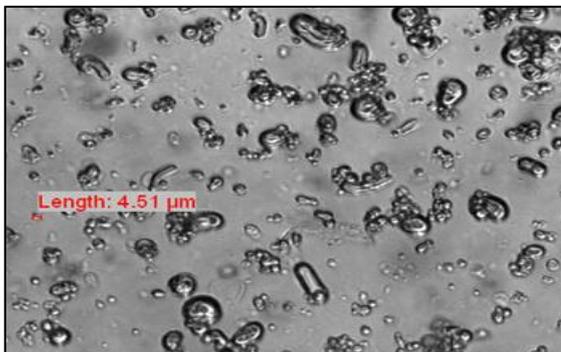
Figure 20: DPPC liposomes visualised by optical microscopy.

Ultraviolet spectrophotometry and fluorescence spectrometry of eluted fraction after Superdex chromatography verified Oflox and Moxi entrapped liposomes (Fig. 21 – 24).
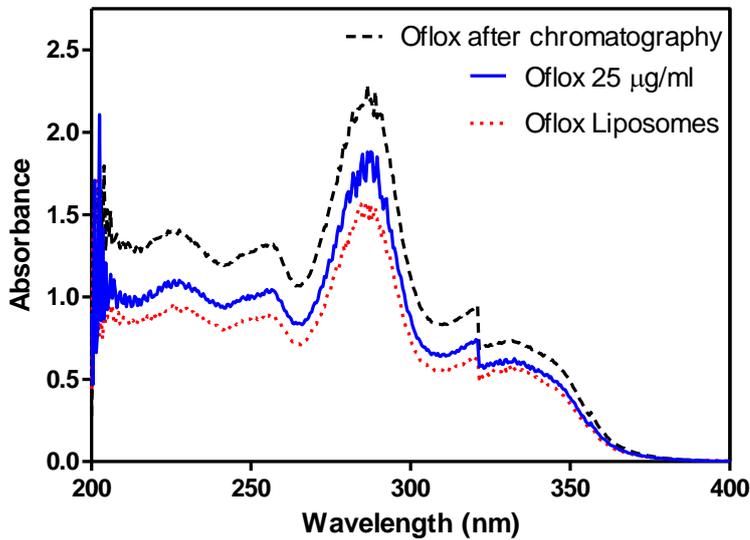
Figure 21: The absorption spectra of Oflox, Oflox liposomes before and a representative fraction of Oflox–liposomes after fractionation on a Superdex–75 column.
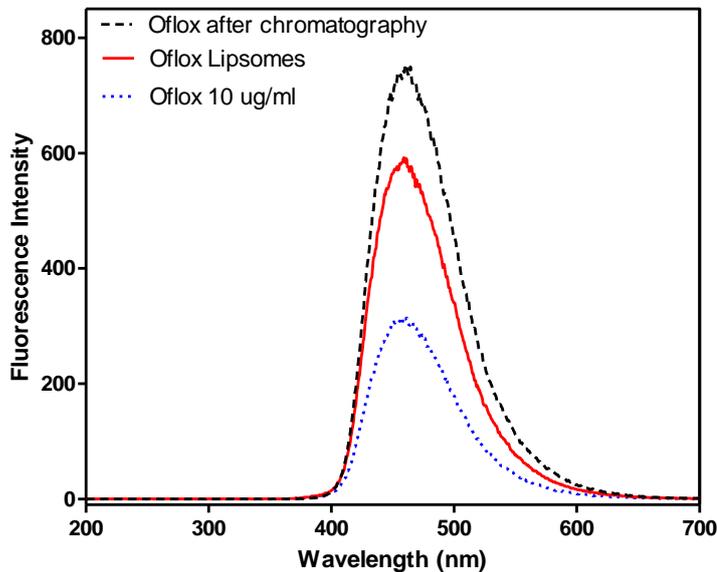
Figure 22: The fluorescence spectra of Oflox, Oflox liposomes before and a representative fraction of Oflox–liposomes (excitation at 287 λ$_{max}$) after fractionation on a Superdex–75 column.
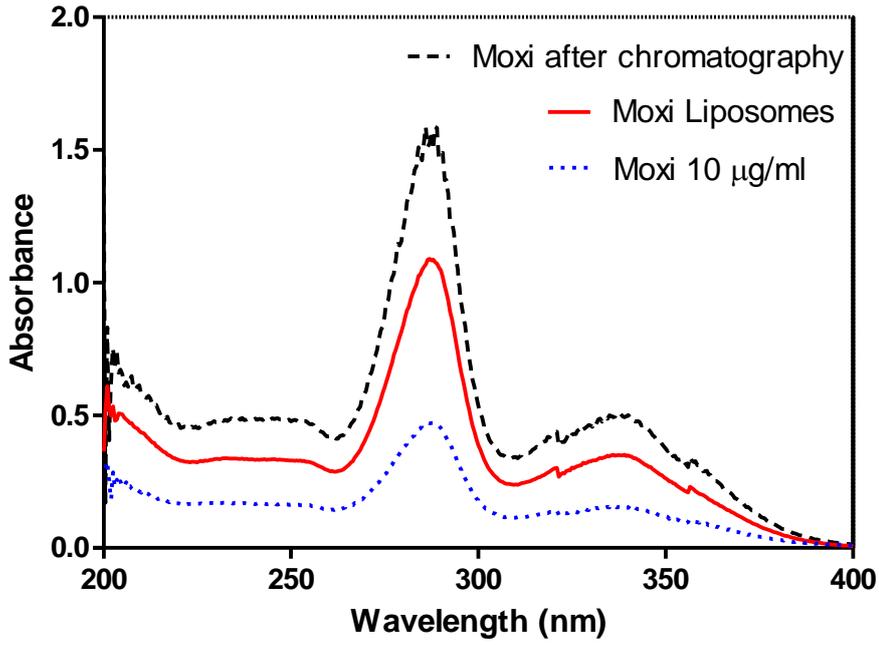
Figure 23: The absorption spectra of Moxi, Moxi liposomes before and the representation fraction of Moxi–liposomes after fractionation on a Superdex–75 column.
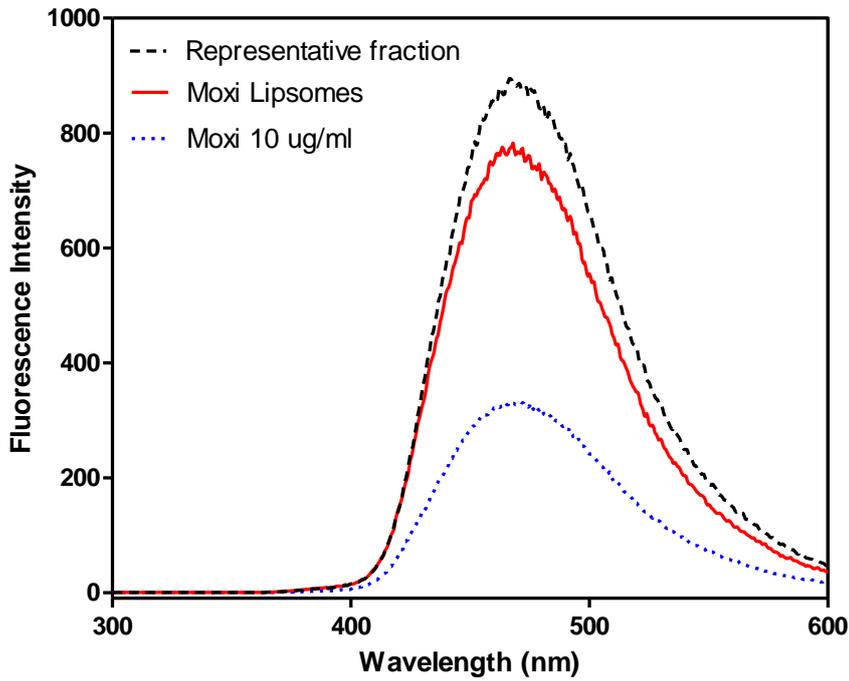
Figure 24: The fluorescence spectra of Moxi, Moxi liposomes before and the representation fraction of Moxi–liposomes (excitation at 287 λmax) after fractionation on a Superdex–75 column.

Figure 25 displays the surface tension of INH, Oflox and Moxi within their DPPC-liposomal combinations compared to that of DPPC-liposomes only, DPPC and PBS. The increases of surface tension seen for the drug-liposomal combinations compared to that of the DPPC-liposomes were found to be statistically significant. However, when taking into account the surface tension values of PBS and water (±72 mN/m), the increases were not found to be significant.
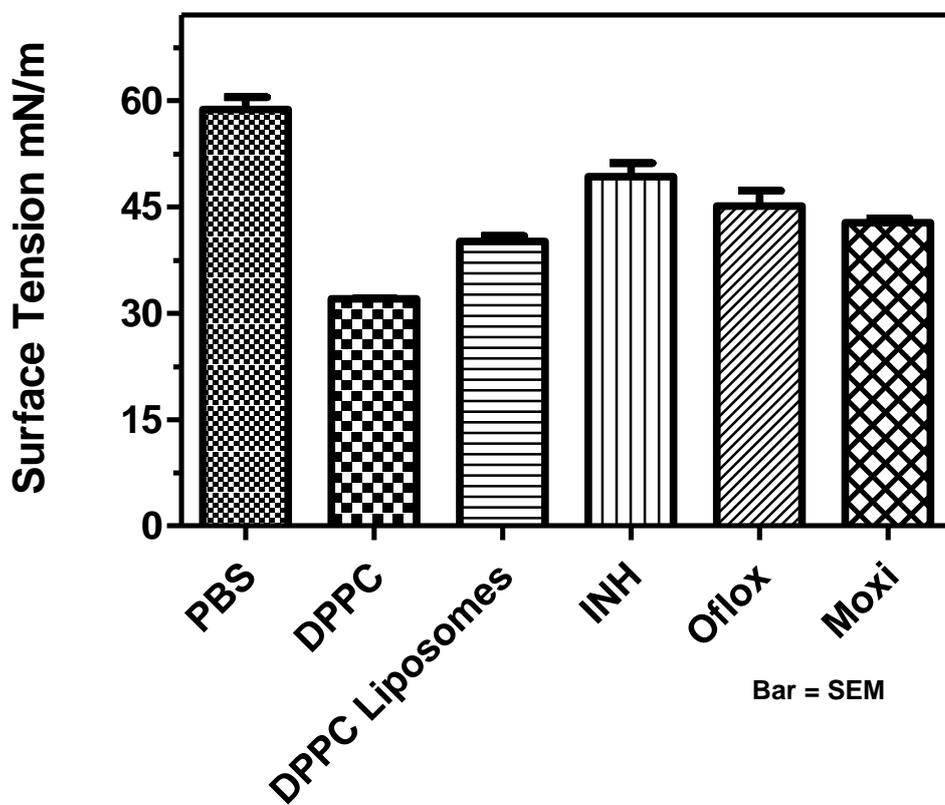
Figure 25: The surface tension values of the different drug-DPPC liposome combinations in reference to PBS and DPPC. INH: Isoniazid entrapped DPPC-liposomes, Oflox: Ofloxacin entrapped DPPC-liposomes, Moxi: Moxifloxacin entrapped DPPC-liposomes.

Fig. 26 & 27 displays images of drug-liposomal preparations, before and after extrusion. Multi–lamellar vesicles (MLV) are seen in high magnification before extrusion through a 100 nm pore size polycarbonate filter. In Fig. 27, the sample is more homogenous after extrusion and the liposomes are smaller in diameter and single–lamellar vesicles (SLV) are noted.
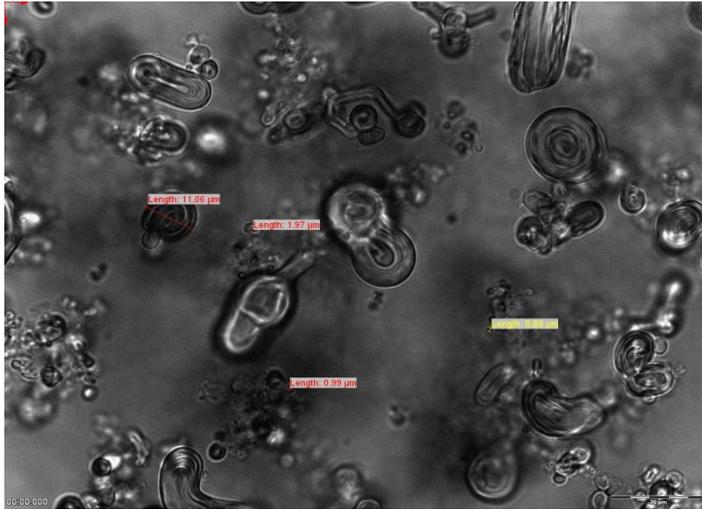
Figure 26: Un-extruded drug containing liposomes under high magnification illustrating MLV.
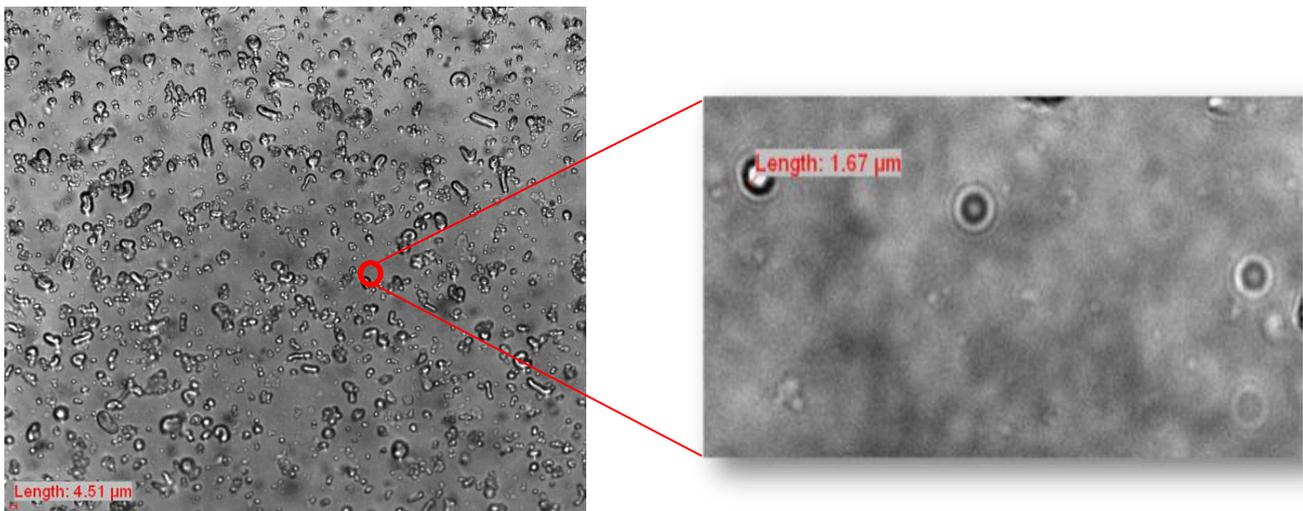
Figure 27: Extruded drug containing SLV.

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6. DISCUSSION

The future of pharmacotherapy for many disorders may lie in drug delivery routes other than oral administration. In particular, growing interest has been given to the lung as well as other absorptive mucosae as non-invasive administration routes for systemic delivery for therapeutic agents. Which is more, further interest has been shown in targeted drug delivery. Site specific drug delivery pursues to concentrate medication in the tissues of interest while reducing the relative concentration of the medication in the remaining tissues, improving the efficacy of the drug, minimizing systemic exposure and therefore circumventing adverse effects.[100] The possibility of inhalable formulations through the pulmonary route allows for faster onset and the drug is delivered directly to the target organ. There has been an increasing amount of interest in the transmembrane diffusion kinetics of a variety of chemical compounds across different tissues. This can be credited to a better understanding of factors that influence or govern transmembrane diffusion processes of chemical compounds in the context of: rates of transmembrane absorption, permeability enhancer characteristics and actions as well as therapeutic-agent delivery formulations. We decided to study the *in vitro* diffusion kinetics of antitubercular drugs across bronchial tissue as there is only a limited amount of information available on this subject. Since pulmonary tuberculosis (TB) continues to be a major infectious burden worldwide, the relevance of this study holds great value when looking at the possibility of pulmonary administration of drug loaded surfactant.

The ability of substances to diffuse through epithelial barriers depends not only on the properties of the barriers involved, but also on the chemical nature, size and

conformation, lipid/water partition coefficient, and degree of ionisation of the permeant molecules tested.[94,101,102] One of the major difficulties limiting the effectiveness of absorptive mucosa is the cellular membrane. It acts as a barrier to macromolecules and to most polar molecules, but it is relatively permeable to water and small lipophilic molecules. Rojanasakul *et al.* compared the permeability of the membrane barrier and charge selectivity in various rabbit epithelia and they concluded that pulmonary epithelium is one of the most effective mucosal surfaces for drug delivery purposes.[103] This is probably due to its high membrane permeability and large absorptive surface area.

The molecular diffusion of drugs across bronchial tissue can be viewed as consisting of a continuous epithelium, collagen fibre network and interstitium. Depending on the physiochemical properties of a permeant, the diffusional resistance offered by the individual layers can vary greatly. The main barrier offering high resistance to the diffusion of hydrophilic and charged molecules is the lipoidal nature of bronchial epithelium which consists of pseudostratisfied, ciliated columnar epithelium.[88,89,104]

The primary drug used in the current chemotherapy of TB is INH, the hydrazide of isonicotinic acid. It is a small molecule (Mw = 137) and it highly water soluble. The two second line drugs, Oflox (Mw = 361) and Moxi (Mw = 401) are included in the treatment regimen when patients are diagnosed with MDR-TB. Both of these drugs are large molecules from the fluoroquinolone antimicrobial class that are amphoteric/amphiphillic in nature. The main difficulties associated with treatment include dose related adverse effects due to toxicity, prolonged treatment duration and the fact that drugs may not reach atelectatic areas of the lung. Thus, the administration of INH, Oflox, and Moxi *via* the respiratory route is an exciting

possibility. Recently, the idea of incorporating the spreading properties of DPPC as a drug delivery agent of INH was studied by Chimote and Banerjee.[62,74,105] This approach can serve the dual purpose of alveolar stabilisation by surfactant action and drug targeting. For this reason, we decided to investigate the permeation and enhancement of the three antimycobacterial drugs used in the current TB regimen with DPPC/DPPC liposomes through porcine bronchial tissue.

The flux values of INH and INH-DPPC display no significant differences in permeability through bronchial tissue (Fig. 9). Due to the hydrophilic characteristic of INH, it will most probably take the intracellular route of diffusion but when in combination with DPPC, the disruption of intercellular lipids could possibly reduce restriction to molecular movement allowing for intercellular movement instead. However, when DPPC is present alone at physiological temperatures, it adsorbs slowly to the pulmonary air-liquid interface to form a surfactant monolayer that might explain the significant decrease of INH flux value of 43.3% with the liposomal combination to that of INH, in our studies. This is indicative of the possible interference of diffusion processes by the formulation nature of DPPC with drug. Due to the hydrophilic nature of INH along with the slow adsorption of DPPC in ripple phase (at 36.5 °C), it will reside within the hydrophilic nucleus of the liposome offering the explanation of the retarded diffusion.[106]

Considering the importance of fluoroquinolone drug delivery and localisation in bronchial tissue during infection; the diffusion kinetics of Oflox and Moxi were studied. Both drugs, and their relative DPPC preparations, achieved steady state flux values within 2 hrs compared to that of INH (Fig.10 and 11). A possible explanation for this difference may be that the permeation of these drugs through the epithelium could be ascribed to transcellular diffusion compared to the

paracellular route followed by INH. Also, less drug was able to diffuse compared to that of INH through the bronchial tissue. As the magnitude of the flux rates of the various permeants across bronchial tissue are related to molecular size, with the larger compounds having lower flux rates. These results are in agreement with previous permeability studies reported for human vaginal, small intestinal and colonic mucosa.[107]

When Oflox and Moxi were in combination with DPPC, the retardation found could be due to the fact that the DPPC may have aided solubilisation of the lipophilic fluoroquinolones, thereby negatively affecting their partitioning from solution into the tissue. Moreover, DPPC may merely have increased competition with Oflox and Moxi for the transmembrane permeation routes. Another explanation for the retardation of Oflox across porcine bronchial tissue in combination with DPPC and DPPC-liposomes could also be that Oflox flux rates were already at a maximum.

The liposomal preparation of Moxi significantly enhanced the flux values by 43.6%. This is contrary to in the DPPC-drug combination where retardation in flux was found. A study done by Neves *et al*. investigated moxifloxacin in liposomes of dimyristoyl-L-α-phosphatidylcholine or dimyristoyl-L-α-Phosphatidylglycerol.[108] They observed that the partition coefficient of moxifloxacin is larger than those reported for most of the other fluoroquinolones This could be a consequence of the structural changes observed in the molecule itself, which in turn changes its acid/base properties. The authors further stated that the molecule must be located near the phospholipid headgroups, similar to other fluoroquinolones, but they suspect contributions from a hydrophobic component as well. Furthermore, at physiological pH, Moxi exists 93% in the zwitterionic and 7% in the cationic form whereas Oflox exist 90% in the zwitterionic and 10% in the anionic form. These

small differences have important consequences when considering their partition coefficient within liposomal suspension. The different flux values observed for Moxi within the liposomal form to that of Oflox liposomes can also be explained by the associated presence of the substituent groups in their structures (Fig. 6). These groups change the pKa values of these drugs, meaning that at physiological pH the drugs exist not only in a zwitterionic form but also in a cationic form that interacts much more strongly with the negatively charged lipid heads. On the other hand, the presence of these substituent groups may require that these drugs insert themselves into the bilayers in a different way, thus enhancing electrostatic interactions. In such a situation, hydrophobic and electrostatic interactions may reinforce each other. [108]

The liposomal formulations containing the antimycobacterial drugs were tested against MTB strains for the accurate determination of susceptibly. Unlike the traditional methods of observing visible mycobacterial growth in culture media, this technique monitors mycobacterial growth by measuring $^{14}CO_2$ produced by the culture medium due to mycobacterial growth (^{14}C labelled palmitic acid present in the growth medium). The standard H37Rv strain was used for the susceptibility testing of MTB to INH (as it is a first-line drug) and the XDR strain X51 was used for the susceptibility testing of MTB to Oflox and Moxi due to their position as second line drugs in the treatment of MDR-TB. Furthermore, all three drugs displayed increased antimycobacterial activity to their relevant strains when in combination with DPPC-liposomes that was as effective as their unformulated counterparts in terms of their *in vitro* efficacy. INH, having a MIC of 0.02 µg/ml, displayed a 22% increase in bacterial susceptibility when in combination with the DPPC-liposomes at 0.01 µg/ml (half of MIC). The same increased bacterial

susceptibility was found for the fluoroquinolones. Oflox–DPPC liposomes displayed a 25% increase in susceptibility at 1 µg/ml (half of MIC) and, although the population was still seen to be resistant, 0.5 µg/ml (quarter of MIC) displayed an increased susceptibility of 23%. Moxi–DPPC liposomes, with increased susceptibility of 4%, did not alter the MIC to such an extent as Oflox and INH. However, one interesting factor to take into consideration is the bactericidal activity of DPPC alone. Un-loaded DPPC liposomes displayed a 3% decrease in mycobacterium growth for the H37Rv strain and a 20% decrease in growth for the XDR strain X51.

There are many possible suggestions for the increased susceptibility of MTB to drug-loaded DPPC liposomes. One explanation is presented by a study done by Stoops *et al* where the authors stated that "surfactants are proficient in removing MTB's shield (TDM coat) and, because they are well known as cell permeabilising agents, they may also enhance the effectiveness of antibiotics".[72] This view refers to the increased concentration/permeability of drug into the intracellular space of the bacteria rather than the increase in its activity. A supporting explanation is brought to the table by Neves *et al.*[108] With all the considerations to take into account for the permeability of these drugs through bronchial tissue (i.e. partition coefficient), these susceptibility results suggest that the enhanced activity of the antimycobacterial drugs may be related to a more facilitated entrance into the bacterial cell. Perhaps by including a mediator step involving electrostatic interaction with a hydrophobic component (DPPC liposome); this step then controls the extent or orientation of insertion and improves the electrostatic interaction.[108,109]

Entrapment efficiency depends on the interaction of the drug molecules and their partition coefficient. Hydrophillic drugs like INH are readily entrapped within the aqueous core of the liposomal vesicle, whereas hydrophobic/amphoteric molecules like Oflox and Moxi are localised within the bilayers of the vesicles. The higher entrapment of INH (53%) to that of the fluoroquinolones (46,6% for Oflox and 43,3% for Moxi, respectively) could be attributed to the entrapment of INH in the multi-lamellar compartments of MLVs.[62] Even with these relatively low entrapment percentages, drug susceptibility testing of MTB was not hindered. Even more so, the liposomal preparations increased MTB sensitivity to the relative drugs at concentrations below their respective MICs.

Different temperatures can alter the thermotropic behavioural nature of DPPC in the presence of a foreign molecule in the biomembrane of the vesicle as a function of its own physicochemical properties. Under physiological pH, ofloxacin is in its higher hydrophobic form and hence, able to permeate bilayers, slightly perturbing the hydrophobic zone of the membrane.[110] Furthermore, DPPC in the presence of higher temperatures (±45°C) offers a drug-rich domain between the transition of the ripple and liquid crystal phase. The ofloxacin carboxyl group can be twisted (might be applicable for Moxi as well) out of plane as in the zwitterionic form due to orientation and change in pK_a within the liposomal membrane. The situation can possibly be exacerbated further due to the heterocyclic ring strain and its orientation when a biological target is nearby such as living bacteria (MTB) that maintain a proton driving force across the cytoplasmic membrane. For these reasons, Fresta *et al* stated that in the case of amphoteric fluoroquinolones, an improved activity as a function of the charged/un-charged species of drugs on the cytoplasmic drug accumulation and on antimycobacterial efficacy is more likely the

effect rather than the accumulation of uncharged forms.[110] This allows for the understanding of the connection to the supporting explanation by Neves *et al.*[105] Conversely, this is in difference to Stoops *et al.*[72] However, all three authors do agree that the efficacy and permeation are influenced by the physicochemical properties of the drugs as well as the membrane properties of MTB. DPPC thus alters the membrane to become more fluid enabling increased permeation.

By itself, DPPC exhibits slow and poor interfacial adsorption at temperatures below its gel to liquid transition temperature (41 °C). DPPC adsorption yielded a surface tension value of 44.7 ± 0.6 mN/m. The surface tensions attained by the drug-loaded DPPC liposomes are as follows: INH–DPPC (1:1 ratio by weight) ± 46 mN/m, Oflox–DPPC (1:1 ratio by weight) ± 45 mN/m and Moxi–DPPC (1:1 ratio by weight) ± 43 mN/m. Thus, a statistically significant increase ($p < 0.05$) in the DPPC adsorption was observed on addition of antitubercular drugs to unloaded DPPC liposomes (± 35 mN/m). The isoniazid molecules could probably interact with the positively charged polar head groups of the DPPC molecules. Since very little amount of INH is adsorbed at the interface its interaction with the hydrophobic tails of the DPPC molecules seems less of a possibility. At physiological pH, INH is neutral and hence does not cause a direct difference in the ionised status of the DPPC molecule. Furthermore, the presence of the large fluoroquinolone molecules can possibly decrease the proximity between the DPPC molecules deterring their reorientation and packing of the hydrocarbon tails.[105] When taking into account the surface tension of water ±72mN/m, these minimal increases in surface tension do not alter the integrity of DPPC to a large extent that its ability to reopen atelectatic areas within the lung will be compromised.

In conclusion, the possibility of pulmonary delivery of INH, Oflox and Moxi along with an exogenous surfactant as a drug carrier was the primary theme of this study. Site-specific drug administration to improve the reach of antimycobacterials allows for lower dosages and can prevent undesired adverse-effects due to systemic toxicity. The drug loaded DPPC liposomes indicated a wide variation in their permeability characteristics compared to their counterparts through porcine bronchial tissue. This is indicative that there are definitive interactions between the drug and DPPC liposomes, drug and tissue, as well as DPPC liposomes and tissue. This study thus demonstrated the usefulness of the *in vitro* flow-through perfusion model to study the absorption characteristics of bronchial tissue in this area of research.

Furthermore, the drug loaded liposomes retained antimycobacterial activity in the BACTEC system thus proving that drug loaded DPPC liposomes exhibited a potential of being effective *in vivo* as surfactant-based drug delivery system. This study has been one of the first steps towards developing surfactant based drug delivery systems for pulmonary TB.

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8. ADDENDUM

This study has been presented in part and in its entirety at national and international conferences.

National conference: 45th Annual Congress 2011 of the SASBCP held in Durban, hosted by the University of Kwa-Zulu Natal, September 2011

Abstract Title:

The Influence of a Synthetic Lung Surfactant on the Permeability of Antimycobacterial drugs Through Porcine Lung Tissue.

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International conference: 5th AFRICAN INTERNATIONAL CONGRESS OF BASIC AND CLINICAL PHARMACOLOGY, held at La Palm Royal beach hotel and hosted by University of Ghana, Accra, Ghana, July 2012

Abstract Title:

Studies of Liposomal Preparations of Anti-Tubercular drugs

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