

CHAPTER 5: THE POTENTIAL OF PHEROID™ TECHNOLOGY IN THE TREATMENT OF INFECTIOUS DISEASES

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5.1 Chapter summary

Advances in the elucidation of disease processes and pathologies often highlight the potential for improvement in the performance of a medication. Performance enhancement may include a number of factors: More options for administration, less frequent administration or simply providing medication that is more acceptable to the user. Possibilities also exist, depending on the kinetics and dynamics of drug action, and its dose–response relationships for improving efficacy or reducing side effects.

The properties of any drug are a composite of the innate activity and properties of the compound as modulated by the formulation in which it is to be presented. To optimise their clinical effectiveness, the design of these formulations is becoming an increasingly important part of the development of new agents. However, the application of formulation technologies has also enabled the resurrection of older molecules and produced some surprising shifts in usage. Crowley and Martini summarised how the understanding of physicochemical and pharmacological properties combined with formulation technologies has been applied to improve absorption (intestinal, buccal and transdermal) and prolong therapeutic effects in addition to allowing access to other routes of delivery (i.e. intra-bronchial). There are now multiple examples where the application of these principles has provided significant clinical benefit in terms of effectiveness, safety or convenience, and has clearly provided a novel path for many older antibacterial agents.

This study relates to pharmaceutical Pheroid™-based preparations for use in combating infective organisms. Some evidence to suggest this study was discussed in Chapter 4 under the section of Therapeutic Efficacy (16.4). The enhancement of known anti-bacterial, anti-fungal or anti-viral properties of known agents lies at the very heart of this study. As presented here, the study was performed with the aim of compiling a PCT patent application. Such a patent was indeed filed by the company Pitmy International (Pty) Ltd., but its continuance was not pursued in the PCT countries. The patent has been granted in South Africa. The wider meaning of the term infective organism is intended and pursued in the patent application. Some of the studies included in the patent is herein described and is meant as models or examples of the wider application of Pheroid™ technology to anti-infective agents. In addition, some legal definitions of compounds and processes are included.

5.2 Background to the study

It has been found that the Pheroid™ and media related thereto have the ability to enhance the action of known anti-infective agents. The expression “anti-infective agents” as used herein is meant to include the antimicrobial agents, the antihelmintic agents and the anti-ectoparasitic agents, including agents that serve to destroy and those that serve to inhibit the proliferation of the organisms. The expression “antimicrobial agents” is similarly intended to be understood in the wider sense of that word and hence to have the meaning ascribed thereto in The McGraw-Hill Dictionary of Scientific and Technical Terms 2nd Ed 1978, namely all chemical compounds that either destroy or inhibit the growth of microscopic and sub-microscopic organisms. This term is further specifically intended to include all the compounds falling within the Pharmacological Classification 20 set out as part of Regulation 5(1) of the General Regulations made in terms of the South African Medicines and Related Substances Control Act, Act 101 of 1965, as well as the active ingredients of all products falling within class 18 of the pharmacological classification employed in the Monthly Index of Medical Specialities (“MIMS”) published by Times Media in South Africa. It is thus intended to include:

- the anti-bacterial agents (including both antibiotics and substances other than antibiotics such as the sulfonamides, the erythromycins and other macrolides, the aminoglycosides, the tetracyclines, the chloramphenicols and the quinolones);
- the anti-fungal agents;
- the anti-viral agents (including anti-retroviral agents);
- the anti-protozoal agents;
- the tuberculostatics;
- the anti-leprotics;
- the germicides; and

➤ the spirochaetocides.

Of the above listed agents, enhancement of efficacy of all groups was investigated in one way or the other except for the germicides; and the spirochaetocides. No study investigated the anti-leptotics specifically, but since it is related to the tuberculostatics, and the same APIs are used in the treatment, it is included in the list of investigated compounds/ organisms. Tuberculosis, HIV and malaria were chosen as models within which to investigate the enhancement of drug efficacy by Pheroid™ technology in the treatment of infectious diseases for the following reasons:

- (i) It is representative of the three main categories of infective agents, namely a bacterium, a parasite and a virus.
- (ii) The incidence of these three diseases is very high in sub-Saharan Africa. While these diseases individually may not be the main causes of mortality, the combined impact of these diseases on mortality and morbidity is fearsome.
- (iii) The active pharmaceutical ingredients (API's) used in the treatment of these diseases are well-known – mechanisms of action, potential side effects and expected pharmacokinetic (PK) and pharmacodynamic (PD) parameters as well as therapeutic requirements have been described.
- (iv) The treatment regimes of each of these diseases are problematic in some way or the other.
- (v) Some drug resistance issues are of concern for each of these diseases.
- (vi) A number of groups and initiatives in South Africa could supply the background advice, expertise and facilities that made the study possible and its outcomes valuable.

Table 5.1: The various studies performed and institutions/companies involved

Infectious agent model	Formulation characteristics, dosage forms	Preclinical	Clinical
Antimicrobials with specific reference to tuberculosis	(i) Formulation studies; (ii) Oral dosage form studies, including disintegration of capsules (iii) Membrane diffusion and release studies (iv) Stability of oral dosage form	<i>In vitro</i> studies at the US/MRC Centre for Molecular and Cellular Biology	Phase I healthy volunteer trial, including (i) Bioavailability parameters; (ii) Safety parameters; (iii) PK/PD modeling (iv) <i>In vitro/in vivo</i> correlation studies

Malaria	Formulation studies	<i>In vitro</i> studies at the Dept of Biochemistry, University of Pretoria.	
HIV/AIDS	(i) Formulation studies (ii) Transfection studies	(i) <i>In vitro</i> studies at the Dept of Medical Virology, University of Stellenbosch. (ii) <i>In vitro</i> transfection studies at the Dept of Med Virology, UCT.	

5.2.1 Research objectives

The primary research aim of this study was an exploration of the potential of Pheroid™ technology in the treatment of infectious diseases with the purpose of the development and production of quality, accessible cost-effective medications for the treatment of non-resistant and drug resistant infectious diseases. As such the study had several objectives:

- To provide a method of enhancing the known action of anti-infective agents;
- To provide pharmaceutical preparations of such anti-infective agents, which preparations have enhanced action compared to the action of known formulations containing the same agents;
- To reduce treatment time;
- To produce a stable oral dosage form;
- To reduce the side effects of current treatment regimes;
- To develop TB treatment formulations that will prevent drug-drug interactions between rifampicin and anti-retrovirals by entrapment of rifampicin in Pheroid™.

These objectives stem from the observations made in respect of a selection of agents falling within the group of anti-active agents as herein defined, which can advantageously be formulated with nitrous oxide and long chain fatty acids, to elicit a more potent response or to evoke such response more rapidly than it does when used by conventional administration. Infectious diseases, especially those that are known to develop resistance to compounds are known to be difficult to treat due to insufficient penetration of the compound into the causative microorganisms. These compounds, or at least some of these appear to be particularly suited to the benefits of Pheroid™ technology.

5.2.2 Tuberculosis

There is a dread disease which so prepares its victim, for death: which so refines it of its grosser aspect, and throws around familiar looks, unearthly indications of the coming change – a dread disease, in which the struggle between soul and body is so gradual, quiet, and solemn, and the result so sure, that day by day, and gram by gram, the mortal part wastes and withers away, so that the spirit grows light and sanguine with its lightening load, and, feeling of immortality at hand, deems it but a new term of mortal life; a disease in which death takes the glow and hue of life, and life the gaunt and grisly form of death: a disease which medicine never cured, wealth warded off, or poverty could boast exemption from: which sometimes moves in giant strides, and sometimes at a tardy pace, but, slow or quick, is ever sure and certain.

Charles Dickens on tuberculosis, Nicholas Society; 1870

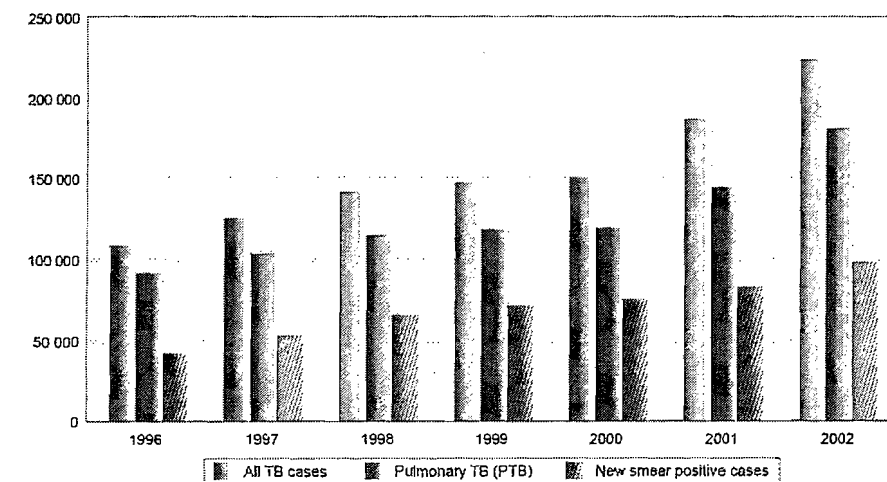
The present study is specifically, though not exclusively aimed at the enhancement of the action of anti-mycobacterial agents, and particularly those used in the treatment of patients infected with *Mycobacterium tuberculosis* (*M.Tb.*), one of most significant human pathogens. Microorganisms of the genus *Mycobacterium*, more specifically *M. tuberculosis*, has re-emerged as a serious public health problem. *M. tuberculosis* and *M. leprae* (the causative agent of leprosy) are the main causative microorganisms. The situation has been exacerbated by the lack of a wide array of chemotherapeutic agents, and the development of drug-resistant strains. Of particular concern is the emergence of tuberculosis (TB) as an increasing cause of morbidity and mortality among persons compromised by human immune-deficiency virus (HIV) infection.

Although the prevalence of tuberculosis in developed countries declined in the first few decades of the 1900's, this trend has reversed and an increased incidence of tuberculosis has been reported in many countries. Africa alone is estimated to have approximately 170 million TB patients. Various sources estimated that between 2000 and 2020 nearly 1 billion people will be newly infected with TB, 200 million will become sick and 35 million will die (WHO, Global Alliance for TB Drug Development, Stop TB Partnership). Even discounting the financial consequences of this disease for patients and their families, the cost to the healthcare systems and national economies is estimated to be US\$16 billion annually – \$4 billion for the costs of diagnosis and treatment and \$12 billion from lost income.

In South Africa the incidence of tuberculosis is also rising to a different degree in different population groups and in the various provinces (Chapter 15: Tuberculosis in the South African

Health Review of 2002 (available at <http://www.hst.org.za/sahr/>). In 2002, the WHO estimated that six countries had a higher TB incidence than South Africa (SA), with an estimated incidence of 556 cases per 100 000 population. Co-infection of individuals with *Mycobacterium Tuberculosis* and human immunodeficiency virus are common in Africa, with 55% of patients with smear or culture positive TB in SA also diagnosed as HIV-positive (Aziz *et al.*, 2004). Since South-Africa is ranked second in the world in terms of prevalence of HIV/AIDS, the catastrophic impact of co-infection of these two diseases requires disease management on a community or national level. This must also be undertaken at a time of severe global financial constraints.

Despite very effective anti-tuberculosis drugs and regimens that can lead to the permanent cure of more than 95% of patients, the incidence of tuberculosis continued to increase in South Africa as shown in Figure 1. Although figure 5.1 portrays the incidence during the years up to 2002, it appears from various reports (WHO, 2006) that the situation has deteriorated instead of improved. Of the twenty-two high burden countries with a combined load of approximately 80% of the world's TB cases, only two of these countries are estimated to have incidences of TB higher than the estimated figure for SA. The WHO has consistently reported case detection rates for new smear positive cases for SA of over 85%. According to the South African Department of Health (SADOH), this figure is probably an overestimate. Accurate prevalence figures for the country are currently unfortunately unavailable.



Source: NTCP, NDoH²

Figure 5.1: Number of TB, pulmonary TB (PTB) and smear positive (Sm+) cases in SA, 1996-2002. Reprinted with permission.

The stubborn persistence of TB points to some underlying defects in the current treatments. These defects can be listed as:

- (i) The present treatment requires extended periods of chemotherapy for at least 6 to 9 months of treatment;

- (ii) The treatment has a large number of side effects;
- (iii) Lower compliance is a feature of tuberculosis treatment = the current DOTS method of drug administration is aimed at ensuring patient compliance;
- (iv) Failure of tuberculosis programs to supply adequate support to patients during the prolonged period needed to achieve cure;
- (v) The development of multi-drug resistance due to non-compliance;
- (vi) The global disease trends reflect the inadequacy of the TB drugs and the current combination has failed to substantially reduce the overall levels of morbidity and mortality;
- (vii) The number of drug regimes available for the treatment of tuberculosis (TB) and HIV/AIDS is limited, especially in resource restricted settings. Drug-drug interactions have been described within the TB treatment regime with regards to rifampicin, as well as between drugs used in the AIDS/TB co-infection treatment regime, again between rifampicin and some of the antiretrovirals (AVRs). Such drug-drug interactions may result in sub-therapeutic levels of the drugs, which would in turn result in the development of drug resistance. In view of the limited number of treatment regimes, the increase in drug resistance against drugs within these treatment regimes is of grave concern in the public antiretroviral and TB roll-out programmes.

In an attempt to assist patients to complete their treatment the DOTS strategy (Directly Observed Treatment Short-course) has been devised. This is a 'package' which includes supervision of the actual taking of medication, but which should also include a variety of other interventions to support and encourage the patients to complete their course of treatment.

Implementation of the National TB Control Programme (NTCP) has helped to increase DOTS coverage. SADOH has developed a plan for the mobilisation of human and financial resources needed to expand TB control. In addition, clear TB policies and guidelines are in place, registers and monitoring tools have been developed and implemented and clear targets for control have been set. Yet, despite all these actions, the incidence of tuberculosis in South Africa continues to rise. Treatment failure may be a testimonial to the inequality in health care delivery that exists in the developing world; lack of management capacity, poor management systems, and inadequately trained and motivated staff at a grass root level have often been cited as the reasons contributing to the failure of the NTCP. What has become clear is that the cure rate is much lower than expected (figure 5.2).

A multi-drug resistant (MDR) TB survey undertaken between 2000 and 2002 revealed that 1.7% of new cases had MDR TB (Review, 2002). This level was not significantly higher than the global median of 1.1%, but because of the high burden of TB, SA had more MDR TB cases than any other country for which MDR prevalence data is available with the exception of Kazakhstan. Since that survey, XDR (extremely drug resistant) TB has made its appearance. Re-infection of patients is an ever-increasing problem and has been shown to be a function of reactivation of TB in patients not completing their therapy. It is also often associated with the appearance of drug resistant *M.Tb.* in the patient. The occurrence of MDR TB translates to at least 2 000 newly active cases of MDR TB in South Africa each year. MDR TB is extremely expensive to treat - R25 000 to R30 000 per patient for the drugs alone as opposed to less than R200/month for a new patient with ordinary TB. Such patients generally also require to be hospitalised for long periods of time (usually between six and eighteen months), adding significantly to the cost of their treatment.

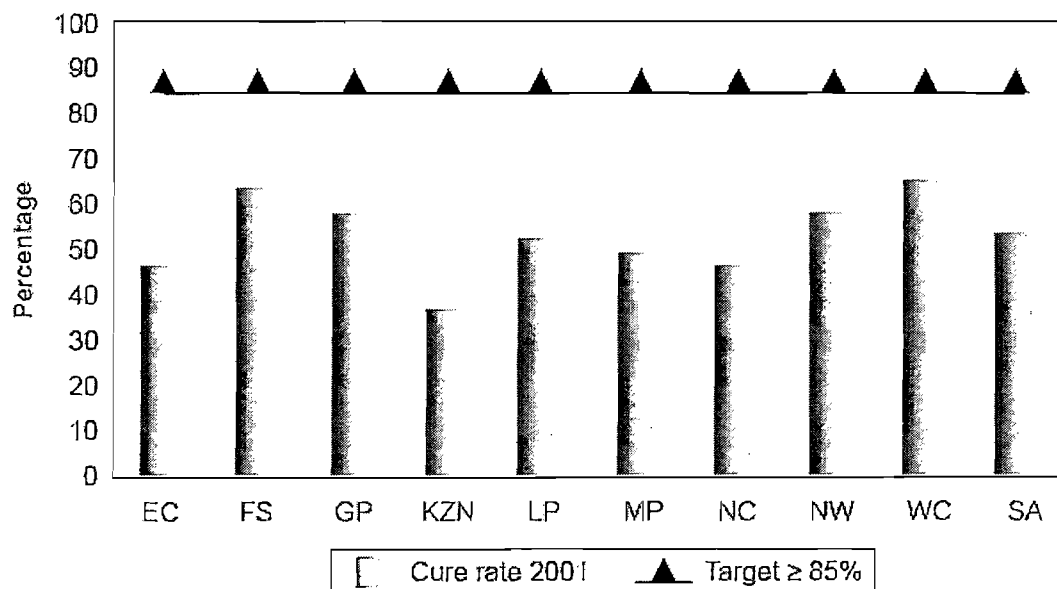


Figure 5.2: The cure rate of tuberculosis in the nine provinces in South Africa during 2001. The cure rate is defined as the percentage of new smear positive TB cases cured at the first attempt. The international target is 85%, a figure not nearly reached in any of the provinces. The abbreviations used: EC is Eastern Cape, FS is Free State, GP, KZN is KwaZulu-Natal, LP is Limpopo Province, MP is Mpumalanga, NC is the Northern Cape, NW is the North West Province. WC is the Western Cape and SA is the combined figure for South Africa. Although the cure rate in the Western Cape was higher than in any other province, the incidence of TB is still the highest in certain communities in that province with an estimated incidence is as high as 1400 per 100000 (WHO, 2002; reprinted with permission).

The high incidence of tuberculosis (TB) in developing countries, driven to a large extent by the HIV/AIDS pandemic, resulted in a global movement known as the “Stop TB Partnership”. This Partnership, established in 2000, has as its goal the elimination of TB as a public health problem and has formed several working groups to assist in the fight against TB. According to the Partnership, the modernization of TB therapy is a medical and moral imperative with direct public health benefits and significant socioeconomic returns.

The current TB treatment relies on a 6–9 month regimen of four drugs that date back to the 1960s or earlier to be taken in combination: isoniazid, rifampicin, pyrazinamide and ethambutol. In the standard regimens, these drugs are given daily for the first 2 months (intensive phase), followed by an additional 4-month therapy using only rifampicin and isoniazid (continuous phase). Combination therapy minimizes the threat of developing drug resistance. Each of the drugs has substantial disadvantages. The discontinuance of TB-treatments has been implicated in re-infections and the development of resistant strains, as the treatment interruption rate has been found to be unacceptably high.

The Global Alliance for TB Drug Development is the executive arm of the working group of the Global TB Partnership dedicated to the development of new drugs. Since the current arsenal of drugs cannot be regarded as effective, the aim of this Alliance is the development of new, affordable TB drugs that:

- (i) simplify or reduce the duration of treatment to 2 months or less;
- (ii) effectively treat MDR-TB;
- (iii) enable the simultaneous treatment of TB and HIV/AIDS; and
- (iv) provide treatment for patients with latent TB infection.

This group envisions that by 2015 an environment will exist that will allow for the sustained development of new TB drugs that can ultimately be combined into completely novel and revolutionary TB regimens. However, in the meantime, the occurrence of TB has reached pandemic proportions in SA with no new treatment directly available. Without new approaches, the TB pandemic will in the meantime grow, driven by its synergy with HIV/AIDS, complicated by multi-drug resistant strains, and amplified by the consequences of poverty.

5.2.1.1 *Mycobacterium tuberculosis* and its pathophysiology

Skeletal deformities in mummies and human remains from Egypt, Denmark, Hungary, Italy, and the Middle East suggest that tuberculosis (TB) has been infecting humans for the past 4,000 years. The generally-accepted hypothesis was that domestication of livestock caused *Mycobacterium bovis* that infects cattle but is relatively harmless to humans, to be passed to humans between 10,000 and 25,000 years ago, after which it evolved into *M. tuberculosis*

(Russo, 2004, Brosch *et al.*, 2002). However, the publication of the genome sequence of *Mycobacterium tuberculosis* in 1998 (Cole *et al.*, 1998) allowed investigation into the evolution of *Mycobacterium*, the genomic differences between laboratory and clinical strains of *M. tuberculosis* and some differences related to virulence and drug resistance. How the specific differences affect virulence is not yet clear.

According to genetic analysis, the *M. bovis* bacillus Calmette-Guérin (BCG) strain, an attenuated *M. bovis* strain used as vaccine for the last 50 years, lacks several genetic regions, including one called RD1. *M. africanum*, a less virulent strain that originated in Africa, similarly lacks some of the same regions. Since *M. tuberculosis* has no plasmids, it is unlikely to have gained large genomic regions as a result of lateral gene transfer (Russo, 2004, Brosch *et al.*, 2002). It seems more likely that the missing regions in *M. bovis* and *M. africanum* are the result of deletions from a common ancestor, which mean that *M. tuberculosis* may be much older than initially thought.

M. tuberculosis (*M.tb.*) forms part of the *M. tuberculosis* group of closely related organisms: *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium microti* and *Mycobacterium canettii*. *M. tuberculosis* is responsible for human infections. (Meltzer, 2006). Other non-tuberculous mycobacteria species include: *M. kansasii*, *M. scrofulaceum*, *M. marinum*, *M. fortuitum* complex, *M. leprae* and the *M. avium* complex (MAC) (Mandell & William, 1996:1167). The latter comprises of two species – *M. avium* and *M. intracellulare*, which is the most common cause for disseminated infection in immunocompromised individuals i.e. those suffering from AIDS. These patients usually develop disseminated MAC disease when their lymphocyte (CD₄) count falls below 50 cells/ μ l (Koirala & Harley, 2006). *M. leprae* is the causative micro-organism of leprosy (Hansen's disease) (Mandell and William, 1996:1159).

Mycobacteria are aerobic, non-sporeforming, paucibacillary, intracellular, curved rods measuring 0.2 - 0.5 by 2 - 4 μ m (figure 5.3). Their cell walls, comprised of phospholipoglycans and mycolic acid-rich long-chain glycolipids (see below) protect them against lysosomal attack and retain red basic fuchsin dye after acid rinsing (Ziehl-Neelson acid fast stain). They are not classified as Gram positive or Gram negative since they do not express the chemical characteristics of either (Meltzer, 2006).

The tuberculosis bacilli thrive in dark, oxygen rich environments. The lungs provide an ideal environment for the replication of specifically the slow-growth populations of *M.tb.* (Ait-Khaled and Enarson, 2003). More than 80% of new tuberculosis cases result when small airborne droplet nuclei, containing 1 to 400 bacilli each, are inhaled by an individual after a sputum smear-positive individual coughed, sneezed or talked nearby. These small bacilli-containing

particles can remain airborne for a few hours because of its small diameter (Ioachimescu, 2009).

Despite the fact that several unique features of both the structure and biosynthesis of the mycobacterial wall offer excellent targets for novel antibiotics, a growing number of mycobacterial pathogens are resistant to most common antibiotics and chemotherapeutic agents (Chatterjee, 1997). This phenomenon is thought to be related to the unusual structure and low permeability of the cell and the composition of the cell wall, which is thought to be responsible for *inter alia*, the small size of mycobacteria relative to other bacteria, their hydrophobicity, acid fast staining, and refractoriness to many existing antibiotics. Only those features that may have some relation to the current study will be highlighted.

The mycobacterial cell wall is comprised of three covalently linked macromolecules: peptidoglycans, arabinogalactans, and mycolic acids (McNeil, 1996). The three-dimensional and spatial arrangement of the primary components of the cell wall is less well known. The literature describes the framework of the bacterial cell wall as being formed by covalently linked peptidoglycan (P) and mycolylarabinogalactan (mAG). Covalently linked complexes of mycolic acids (C₇₀-C₉₀), which are α -alkyl branched, and β -hydroxylated branched fatty acids (Besra and Brennan, 1997) are present as tetramycolylpentaarabinofuranosyl clusters but only two thirds of these are mycolylated (McNeil *et al.*, 1991). The mycolic acids extend perpendicular to the mAGP framework, with other cell wall-associated glycolipids intercalated into the mycolic acid layer to form a 'pseudo' lipid bilayer as portrayed in Figure 5.4 below:

- (iv) The arabinogalactan polymer (Lee *et al.*, 1996) is comprised exclusively of two molecules rarely occurring naturally together: D-galactofuranoses and D-arabinofuranoses;
- (v) The fatty acids on the cell surface of slow-growing pathogenic mycobacteria such as *M. tuberculosis* is modified by cyclopropanation, whereas the fatty acids of rapidly growing saprophyte species such as *M. smegmatis* are not. They also occur within the fluid matrix in the form of free trehalose dimycolate (TDM) ('cord factor'); which has been implicated in the pathogenesis of tuberculosis (Yuan *et al.*, 1997);
- (vi) Mycolic acids are the major determinants of the impregnability of mycobacterial cell walls. The structural form and proportion of mycolic acid containing *trans*-substituents at proximal positions of the mycobacterial cell wall determine to a large extent the fluidity and is also related to the sensitivity of mycobacterial species to hydrophobic antibiotics (Yuan *et al.*, 1997). This may be important for the design of a fatty-acid and vitamin E-based nitrous oxide-containing delivery system (see below), since all three components is said to influence membrane fluidity. These three components may also play a similar role in the fluidity of the mycobacterial cell wall;
- (vii) Since the mycobacterial peptidoglycan is notoriously resistant to lysozyme, the *N*-glycolyl in the peptidoglycan may protect the organism from degradation. It may therefore be partly responsible for the prevention of fusion between phagosome and lysosome (Chatterjee, 1997);
- (viii) The dominant feature of the mycobacterial cell wall is the extremely heterogeneous lipoarabinomannan (LAMs) that are diffusely embedded into the cell wall framework. LAM and lipomannan (LM) are the multiglycosylated versions of the PIMs and both carries a phosphoinositol (PI) anchor at the reducing end (Hunter and Brennan, 1990; Chatterjee *et al.*, 1992). The PI mannan core of LAM and LM may carry additional fatty acylation, and the multiacylation nature may relate to the virulence of the organism.

The LAMS are directly implicated in the immuno-pathogenesis of leprosy and tuberculosis (Brennan *et al.*, 1990). The LAM arabinan is attached to the walls with part of the molecule located on the exterior of the wall. Two non-reducing motifs, a linear Ara₄ motif and a branched Ara₆ motif in LAMs from the human pathogens *M. tuberculosis* and *M. leprae*, as well as the vaccine strain *M. bovis* BCG, are capped with mannoses at the terminal residue (Chatterjee *et al.*, 1992; Chatterjee, 1997). In contrast, LAM from the rapidly growing *M. smegmatis* displays mostly uncapped arabinan termini with a small proportion terminating with a unique inositol phosphate (PI) cap (Chatterjee *et al.*, 1992). The mannose-capped LAMs are referred to as ManLAMs whereas the PI capped LAMs are called AraLAMs. AraLAM isolated from *M. smegmatis* is devoid of the C₁₆ and C₁₉ fatty acids normally present on the PI anchor and are sometimes referred to as PI-GAMs (phosphoinositols–glyceroarabinomannans). ManLAM could stimulate phagocytosis of *M.tb.* by macrophages through interaction with the macrophage

mannose receptor (Schlesinger, 1993; Schlesinger *et al.*, 1996). The mannose caps may in part be responsible for efficient binding and entry of the organism to the macrophage, and may thus regulate the initiation of phagocytosis, as well as the prevention of phagosome-lysosome fusion and thus survival within the host macrophages.

AraLAM, ManLAM and *M. leprae* LAM contribute to pathogenesis and many of the clinical manifestations of leprosy and tuberculosis by suppressing immune responses and mediating the production of macrophage-derived cytokines. The following immuno-modulating functions have been described for the LAMS:

- LAMS induce abrogation of T cell activation (Kaplan *et al.*, 1987);
- LAMS inhibit various IFN- γ -induced functions including macrophage microbicidal and tumoricidal activity (Sibley *et al.*, 1998);
- LAMS scavenge potentially cytotoxic oxygen free radicals (Chan *et al.*, 1991);
- LAMS inhibit protein kinase C activity (Chan *et al.*, 1991);
- LAMS induce a large array of cytokines associated with macrophages such as TNF- α (tumour necrosis factor- α), granulocyte-macrophage-CSF (colony stimulated growth factor), IL (interleukin)-1a, IL-1b, IL-6, and IL-10 (Barnes *et al.*, 1992; Chatterjee, 1997; Adams *et al.*, 1993; Roach *et al.*, 1995) and
- LAMS elicit immediate early response genes (including c-fos, JE and KC (chemotactic cytokines)) in murine bone marrow-derived macrophages (Roach *et al.*, 1995).

These responses may be tissue specific in terms of the source of macrophages. T cells have been shown to recognize mycolic acids in the presence of CD1-expressing antigen presenting cells and mycolic acids may mediate a pathway for T cell recognition of non-peptide antigens (Sieling *et al.*, 1985). Mycobacterial LAM seems to be implicated as antigen in at least one CD1c restricted proliferation of T cell lines. The biosynthesis of LAMS seems to be crucial to understanding the basis of the action of numerous antituberculous drugs (e.g. isoniazid, ethionamide). Four steps have been described in the formation of the mycolic acids (Besra and Brennan, 1997):

- Stage 1: The synthesis of C₂₄–C₂₆ straight chain saturated fatty acids to produce a mixture of C₁₄–C₂₆ fatty acyl-CoA derivatives;
- Stage 2: The synthesis of C₄₀–C₆₀ meromycolic acids to provide the main carbon backbone; the fatty acids are elongated to a range that varies from C₂₀–C₃₀ fatty acyl Co-A;
- Stage 3: The modification of this backbone to introduce other functional groups, and

Stage 4: The final condensation of a C₅₆ meromycolate with a C₂₂₋₂₄ fatty acid and reduction to the mature mycolic acid (Lee *et al.*, 1997). This fatty acid is attached to a carrier transferase that is responsible for the transport of mycolic acids through the plasma membrane (Belisle *et al.*, 1997).

5.2.1.2 Drugs against the mycobacteria and their targets

The three main functions of anti-tuberculosis drugs are its bactericidal activity, its sterilizing activity and the ability to prevent resistance. Most of the drugs have some specificity in terms of the tuberculosis bacilli populations.

Table 5.2: Treatment categories and treatments with their corresponding anti-TB treatment regimens (Compiled from Jakubowiak *et al.*, 2001).

Category	TB treatment regimen	Initial phase (2 months)	Continuation phase (4 months)
I	Regimen i (new smear-positive regimen)	E, INH, RMP, PZA or S, INH, RMP, PZA	INH & RMP
II	Regimen ii (smear-positive re-treatment)	S, INH, RMP, PZA & E or 1 month INH, RMP, PZA & E	5 months INH, RMP & E
III	Regimen iii (smear-negative regimen)	INH, RMP & PZ	INH & RMP
IV	Chronic cases (sputum-positive after supervised re-treatment in hospital).	Regimes used for drug resistance management.	

E = ethambutol, INH = isoniazid, RMP = rifampicin, PZA = pyrazinamide, S = streptomycin

This study is specifically concerned with the four anti-tuberculosis drugs prescribed by the WHO for primary tuberculosis treatment, namely rifampicin, isoniazid, ethambutol and pyrazinamide and discussion will centre on these four drugs (WHO, 2003). These drugs are combined into the fixed-dose combination preparations and are generally prescribed according to the disease characteristics and drug combinations listed in Table 5.2.

The administration of anti-TB drugs is split into two phases, their objectives being:

- ✦ initial (intensive) phase → to decrease the number of tubercle bacilli in actively multiplying sub-populations, therefore bringing about a rapid decrease in bacterial load.
- ✦ continuation (sterilising) phase → to eradicate remaining organisms or significantly decrease the number of bacilli in semi-dormant subpopulations (Jakubowiak *et al.*, 2001).

A simplified sketch of the primary sites of action of the listed four APIs in the bacterial cell wall and cytoplasm is shown in figure 5.5, with the exclusion of streptomycin.

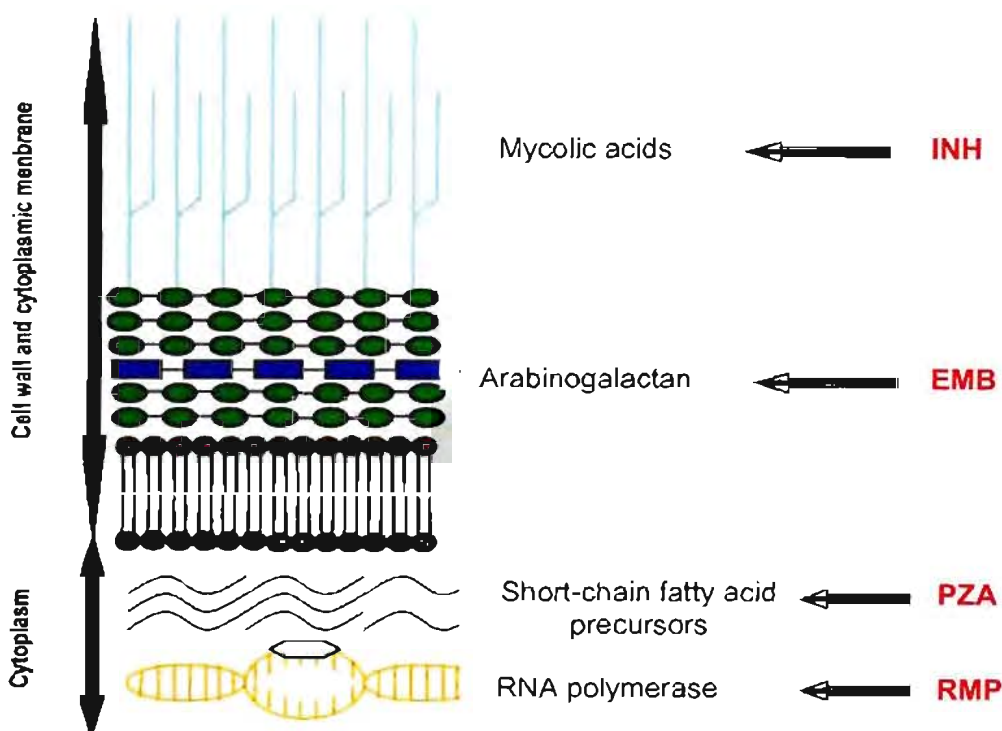


Figure 5.5: Target sites for relevant anti-tuberculosis drugs in mycobacteria. Arrows connect each of the four drugs (red) to the specific target. The targets are shown in relation to the physical site and structure of the bacterial cell wall.

Rifampicin (RMP) is the most potent sterilizing drug available and kills slow growing and possibly non-replicating organisms (Du Toit *et al.*, 2006). Pyrazinamide (PZA) has bactericidal activity in certain populations of bacilli since it is active only in acidic environments, while both isoniazid (INH) and RMP seem to act against most of the tuberculosis bacilli populations. INH and ethambutol (EMB) eradicate most of the rapidly replicating bacilli; PZA kills semi-dormant organisms in sites hostile to the penetration and action of the other drugs (Du Toit *et al.*, 2006). EMB is also used in combination with the other drugs to prevent emergence of resistant strains of *Mycobacterium tuberculosis* (Du Toit *et al.*, 2006; WHO, 2003). RMP also inhibits the growth of *M. kansasii*, the majority of strains of *M. scrofulaceum*, *M. avium* and *M. intercellulare* as well as *M. leprae* (Mandell and William, 1996:1159). Its application regarding the non-tuberculous mycobacterium species falls outside the scope of this study.

Some of these antimycobacterial drugs are known to inhibit the synthesis of the cell wall components (Winder, 1982). Ethambutol (EMB) inhibits the synthesis of arabinans of both the AG and LAM of the *Mycobacterium tuberculosis* cell wall. The mechanism of action of EMB is in fact the inhibition of the polymerization step of arabinan biosynthesis (Mikusov *et al.*, 1995), with truncated structural variants of lipoarabinomannan identified in EMB drug-resistant strains

(Khoo *et al.*, 1996). The prodrug INH is activated within the mycobacterial cell: INH is activated by *KatG* catalase and peroxidase activities and its biochemical effect is on mycolic acid synthesis (Zhang *et al.*, 1992). The gene *InhA* that encodes a reductase involved in mycolic acid synthesis confers resistance to INH clinical isolates. Step 1 and 2, i.e. the short chain fatty acid synthesis are not inhibited by INH in *M. smegmatis*, whereas mycolic acid synthase (MAS) is affected in a differential fashion. However, many resistant clinical isolates do not have *InhA* or *KatG* mutations indicating that there could be additional resistance mechanisms and indeed, Mdluli *et al.* (1996) showed that *InhA* is not the primary target for activated INH in *M. tuberculosis*.

Iron, heavy metals, and excessive alcohol consumption (an inherent feature of some identified high incidence TB communities) generate harmful reactive oxygen species which have been shown to be involved in the auto-oxidation of RMP, thereby generating more radical species. These free radicals are implicated in the liver toxicity experienced with the use of RMP. RMP is a known P450 enzyme inducer and is known to interact with protease inhibitors and non-nucleoside reverse transcriptase inhibitors used in the treatment of AIDS.

5.2.1.3 Possible interactions between Pheroid™, APIs and bacteria

The main components of the Pheroid™ are the unsaturated fatty acids, pegylated ricinoleic acid, nitrous oxide and α -tocopherol. The typical fatty acid distribution of this product is as follows:

< C₁₆: 0
 C_{16.0}: 8,3 %
 C_{18.0}: 3,5 %
 C_{18.1}: 21,7 %
 C_{18.2}: 34,8 %
 C_{18.4}: 28,0 %
 > C₁₈: 1,6 %
 unknown: 2,1 %

Figure 5.6 shows a hypothetical model of a membrane region of a Pheroid™ vesicle. The nitrous oxide and α -tocopherol are not accommodated in the model yet.

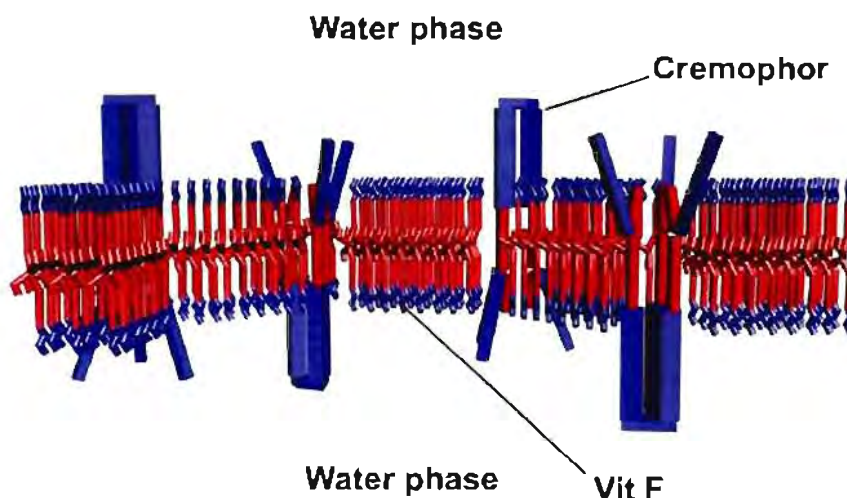


Figure 5.6: A schematic model of the fatty acid components of the membrane of the Pheroid™. The blue regions represent the hydrophilic domains whereas the red regions represent the hydrophobic domains. Each fatty acid contained in vitamin F ethyl ester (Vit F) is thus sketched as a red hydrocarbon chain with a blue ethyl ester attached. The hydrocarbon chains are bent where unsaturated C=C bonds occur. The pore structures or channels are formed by the Cremophor molecules.

The composition of Cremophor RH40 is shown in Figure 5.7. These molecules form the so-called channels or pores in the membranes.

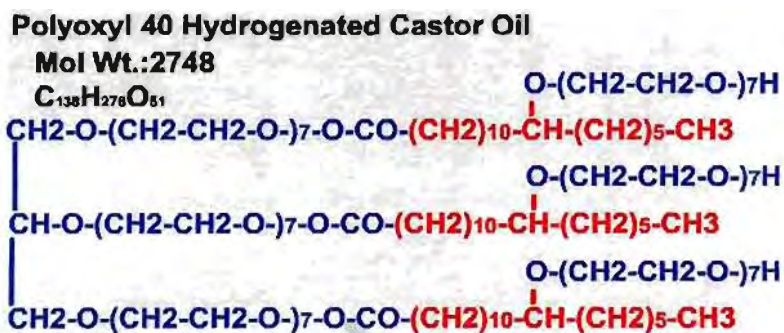


Figure 5.7: The molecular composition of Chremophor RH40. It is by far the largest molecule present in the Pheroid™ membrane, with its attached fatty acid/poly-ethylene chains. The blue regions are hydrophilic while the red regions are hydrophobic.

The discussion in sections 5.2.1.1 and 5.2.1.2 suggests a central role for fatty acids in *M.tb* growth and while no mechanistic studies were undertaken to investigate a specific effect of the fatty acid component on mycobacterial growth, there is a hypothetical chance that the fatty acids may be involved in growth inhibition/or therapeutic enhancement through mechanisms such as competition. From the literature, the involvement of nitrous oxide and tocopherol in combating disease-causing foreign microorganisms is clearer.

Nitrous oxide

Nitrous oxide is a natural gas that is also produced synthetically, and is known by the trivial name "laughing gas". It has been in use for many years as an inhalation anaesthetic and analgesic, particularly in dentistry and has been reported to have a synergistic or potentiating effect on halothane and other gaseous anaesthetics (Goodman & Gilman's:298-300). The use of nitrous oxide for all these purposes have been confined to the use of the gas itself as treatment and not to its incorporation in the production of a therapeutic formulation. Nitrous oxide is known to be soluble in water and it has been reported that at 20°C and 2 atm pressure one litre of the gas dissolves in 1.5 litres of water (The Merck Index:6499).

Nitrous oxide is also known for its use as a propellant gas, mainly as a substitute for propellant gases such as chlorofluorocarbons, and more particularly to produce a food product mousse such as whipped cream or chocolate mousse or quick-breaking foams for hair treatment preparations. Once again, the function of the nitrous oxide gas is thought to be a physical one, i.e. to expand on being depressurised and thereby to create a mousse or foam. In fact, nitrous oxide is typically regarded as an inert in these applications and useful due to the fact that it is colourless, odourless and tasteless but soluble in water and oils.

Other reactive nitrogen intermediates such as NO_• and exhibit cytostatic or cytotoxic activity against a remarkable variety of pathogenic microorganisms, including bacteria, viruses, helminthes and parasites (De Groote and Fang, 1995; Loskove and Frishman, 1995; Macmicking *et al.*, 1997a, 1997b). The literature does however not describe such a role for nitrous oxide (N₂O). Mammalian cells, including human cells, produce nitric oxide both constitutively and inducibly in response to inflammatory stimuli (MacMicking *et al.*, 1997a, 1997b; Liew and Cox, 1991). The antimicrobial response elicited in murine macrophages and neutrophils by infectious organisms involves the cytokine-dependent induction of nitric oxide synthase (iNOS). This enzyme catalyzes oxidation of the terminal guanidine nitrogen atoms of L-arginine to produce citrulline and NO_•.

NO_• seems to be an inorganic microbicidal molecule, as blocking the production of NO_• in iNOS knock-out animals infected with tuberculosis, pneumonia, or malaria, leads to enhanced severity of the infectious condition (De Groote and Fang, 1995, Ochoa *et al.*, 1991, Nicholson *et*

al., 1996; Stengler *et al.*, 1996; Anstay *et al.*, 1999; Macmicking *et al.*, 1997a, 1997b and Maclean *et al.*, 1998). Continuous exposure of gram positive, gram negative and multi-drug resistant strains of bacteria, yeast, and mycobacteria to exogenous gaseous NO (gNO) kills the infectious organisms in *in vitro* systems (Miller *et al.*, 2009). The minimum inhibitory concentration (MIC) was found to be 160 parts per million (ppm) during five hours of continuous exposure in these studies (Miller *et al.*, 2009). In *in vivo* studies, the same gNO dose reduced the bacterial load without it becoming toxic and this gNO treatment was used for the successful treatment of a critically colonized, non-healing, lower leg ulcer in a human subject (Miller *et al.*, 2009).

Although gNO may be effective as an inhaled antimicrobial treatment against pulmonary pathogens, continuous inhalation of the above dosage of 160 ppm gNO for 5h would lead to methemoglobinemia and unacceptable hypoxemia (Miller *et al.*, 2009). An alternative gNO inhalation regime - a high-dose of 160 ppm for a short duration (30 minutes) with 3.5 hours between treatments had the same antimicrobial effect as continuous gNO delivery but without severe methemoglobinemia. Multiple exposure of multi-drug resistant *Staphylococcus aureus* and *Escherichia coli* clinical isolates from the lungs of nosocomial pneumonia patients and of a lethal antibiotic-resistant strain of *Pseudomonas aeruginosa* to thirty minute treatments (4 cycles) every four hours to 160 ppm gNO led to a reduction in bacterial load (Miller *et al.*, 2009). The same intermittent regimen at 320 ppm gNO resulted in complete bacterial death without toxicity or inhibition of normal cell processes in the host human THP-1 monocytes, macrophages or pulmonary epithelial cells in *in vitro* studies. gNO treatment may become a viable treatment of tuberculosis in hospitalized settings but it is doubtful that it can be used in rural settings with the required degree of non-toxic dosage determination.

On the other hand, the nitrous oxide present in the Pheroid™ may contribute to bacterial death in conjunction with other antimicrobials. If the nitrous oxide is strongly associated with the fatty acids via its terminal nitrogen, the remaining –NO may be available for interaction and may indeed induce iNOS for instance.

α-Tocopherol

Some attention was given to the role of the fatty acids and nitrous oxide in the preceding chapters and section. In this section it is important to look at the antioxidant and other functions of Vitamin E, specifically because of the use of API's such as rifampicin that cause the formation of reactive oxidation species (ROS). Vitamin E is a collective name that includes the tocopherols and tocotrienols, the most common tocol being α-tocopherol, also known as vitamin E. Tocols are compatible with oils, surfactants and co-solvents, and has been used as excellent

solvents for water insoluble drugs; more specifically in parenteral emulsions, including the major chemotherapeutics such as paclitaxel (Constantinides, 2004).

Besides its role in solubilisation of drugs to be entrapped in Pheroid™ vesicles, vitamin E can act as antioxidant of both the APIs and the fatty acids of the Pheroid™ membranes. Vitamin E is a potent antioxidant according to the classical definition of being a chain-breaking free radical scavenger *in vitro*, and is widely used as a lipophilic antioxidant that protects membranes from being oxidatively damaged by acting as free radical scavenger. The rate constants of tocopherols scavenging reactions of hydroxyl and alkoxyl radicals are around $10^{10} \text{ M}^{-1} \text{ s}^{-1}$, while the rate constants of the reaction of the tocopherols with aryloxy and peroxy radicals lie in the range of 10^3 to $10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Brigelius-Flohe, 2009). The reactivity of tocopherols with radicals differs according to the methylation state of the chromanol ring and the saturation grade of the side chain (see figure 5.8), since the lipophilicity of the vitamers and, thus their incorporation into membranes is to some extent structurally determined.

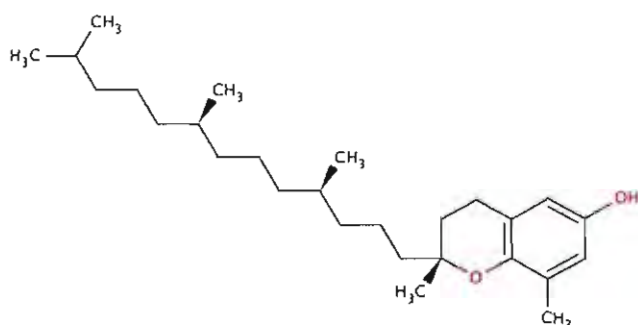


Figure 5.8: The molecular structure of α -tocopherol showing reactive oxygen-based groups and the methyl group on the chromanol ring.

Recent literature proposed the hypothesis that vitamin E may exert its functions in certain domains in membranes, and may influence signalling cascades with subsequent effects on the induction/suppression of genes (Brigelius-Flohe, 2009). Because of the very low concentration of α -tocopherol in soluble cellular fractions, it cannot compete with other cellular molecules such as proteins, DNA, or even lipids for highly reactive hydroxyl or alkoxyl radicals and Vitamin E is thought to act as an antioxidant only in the lipid fraction (Brigelius-Flohe, 2009). The formation of the initiating radical ($X\cdot$), which abstracts an H radical from unsaturated lipids can be triggered by light, heat, traces of transition metal ion and radicals or radical producing chemicals such as azo-dyes as shown in reaction 1 of figure 5.6. The reaction of a lipid peroxy radical ($LOO\cdot$) or a lipid radical ($L\cdot$) with lipids (LH) at about $10^2 \text{ M}^{-1} \text{ s}^{-1}$ is also shown in figure 5.9.

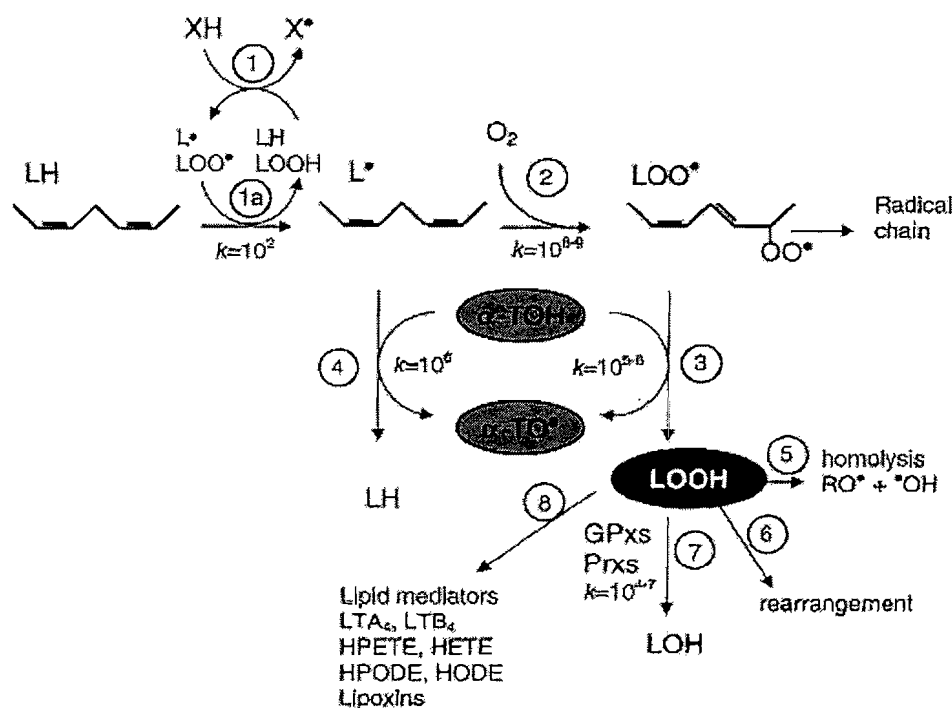


Figure 5.9: Lipid peroxidation and reactions of α -tocopherol using the following abbreviations: LH, polyunsaturated lipid; L^\bullet , lipid radical; LOO^\bullet , lipid peroxyl radical; $LOOH$ lipid hydroperoxide; α -TO $^\bullet$, α -tocopheroxyl radical; k , rate constants in $M^{-1} s^{-1}$; LTA_4 , leukotriene A_4 ; LTB_4 , leukotriene B_4 ; $HPETE$, hydroperoxyeicosatetraenoic acid; $HETE$, hydroxyeicosatetraenoic acid; $HPODE$, hydroperoxyoctadecadienoic acid; $HODE$, hydroxyoctadecadienoic acid (Brigelius-Flohé, 2009). Reprinted with permission.

L^\bullet reacts with oxygen leading to LOO^\bullet (reaction 2), resulting in the propagation of the radical chain if not scavenged. In figure 5.6, the scavenging is performed by α -tocopherol (reaction 3;), with much faster reaction rate than the reaction rate between LOO^\bullet and an unsaturated fatty acid (reaction 1a). According to this scheme, α -tocopherol would therefore generally prevent lipid peroxidation by interfering with the propagation of the free radical chain. This is however only true if the lipids and the α -tocopherol were present in similar concentrations, which is not the case: the concentration of α -tocopherol in membranes is generally about one molecule for every 100–1000 molecules of phospholipids, depending on the nature of fatty acids in phospholipids. In reality, LOO^\bullet probably reacts with lipids and α -tocopherol with comparable velocity (Brigelius-Flohé, 2009). While this scheme is based on cell membranes, it probably has application to the PheroidTM membranes as well.

The α -tocopherol is thought to accumulate in specific domains (reaction 4; Brigelius-Flohé, 2009).). A reaction between α -tocopherol and L^\bullet is possible but not physiologically probable for two reasons: the higher rate constant for the reaction between L^\bullet and the presence of molecular oxygen and the high concentration of oxygen. Therefore, the main antioxidant

function of α -tocopherol is the reduction of $\text{LOO}\cdot$ to LOOH , the last of which is in fact the most important chain-branching reactive oxygen species (ROS) and an α -tocopheroxyl radical or $\alpha\text{TO}\cdot$. ROS is cleaved to produce $\cdot\text{OH}$ and $\text{RO}\cdot$ (reaction 5), if it is not eliminated by reacting with a further scavenger such as phospholipid hydroperoxide glutathione peroxidase (GPx4) (reaction 7) or certain peroxiredoxins (Prx) (Brigelius-Flohé, 2009). On the other hand, LOOHs may, besides being ROS, also be important signalling molecules or sources of potent lipid mediators.

The hydrophilic $-\text{OOH}$ group can cause a rearrangement of lipids in the membrane (reaction 6) and might affect various membrane-associated enzyme reactions. The alcohol reduction reaction and concomitant oxidation of GPx4 and/or Prxs and their products can alter redox-sensitive signalling cascades (reaction 7). If hydroperoxy fatty acids (LOOH) are released, they are processed to hydroxy fatty acids, epoxides, diols, or possibly further peroxidized to result in lipoxins (reaction 8). These include the highly potent lipid mediators leukotriene A_4 and B_4 and their downstream derivatives from the arachidonic acid cascade, initiated by the α -tocopherol-regulated enzyme 5-lipoxygenase (Mousley *et al.*, 2007). In addition, oxidized fatty acids that form enzymatically or as by-products of spontaneous lipid peroxidation exert various signalling functions (Friedrichs *et al.*, 1999; Droge, 2002; Finkel, 2003; Chiarugi, 2005). Besides its antioxidant role, α -tocopherol probably has a function as sensor of oxidative membrane disturbances to trigger cellular responses by modulating lipid mediator production. It is important to note that the fatty acids used in the PheroidTM do not have free $-\text{OOH}$ groups as these groups have been replaced by ethyl groups and is therefore not expected to cause rearrangement of membrane lipids. However, the hydrocarbon hydrophobic group may align itself with those in the cellular membranes.

Can additional unsaturated fatty acids, as supplied in the PheroidTM, act to reverse the formation of the α -tocopheroxyl radical ($\alpha\text{TO}\cdot$), i.e. reverse reactions 3 and 4 between $\alpha\text{TO}\cdot$ and lipid hydroperoxides (LOOH) and fatty acids (LH) respectively? These reactions are generally too slow to substantially contribute to α -tocopherol recycling (Nagaoka *et al.*, 1990 and Bowry and Stocker, 1993), but at very high concentrations of fatty acids and α -tocopherol these reactions may enhance the pro-oxidative function of vitamin E (Upston *et al.*, 1999). It may also be noted that ascorbyl palmitate is included in one of the formulations used in this study. Ascorbate is thought to regenerate vitamin E by the reduction of $\alpha\text{TO}\cdot$ (Bisby and Parker, 1995). The ascorbyl radical is less reactive than the tocopheroxyl radical and oxidation would not be restarted by this radical. Rate constants of reduction of $\alpha\text{TO}\cdot$ by ascorbate vary with the chemical environment and the nature of fatty acids when investigated in model membranes (reviewed in Chan, 1993).

Protein-tocopherol interactions

α -Tocopherol is transported to its site of action by “lipid-binding proteins” reminiscent to the fatty acid binding proteins (see Chapters 3 and 4) that bind and transport small lipophilic molecules with some specificity. These transport proteins belong to the Sec14 proteins, a superfamily with about 500 distinct proteins identified (Saito *et al.*, 2007). Individual Sec14 proteins bind lipids involved in signal transduction, lipid transport, and membrane trafficking, as well as phosphatidylinositols and phosphatidylcholine (Mousley *et al.*, 2007). These proteins may thus bind to the fatty acids in Pheroid™ or to phosphatidyl-based groups present in liposomal systems. The proteins have the so-called Sec14 domain that forms a lipid-binding pocket. Not all members have similar lipid-binding domains (Mousley *et al.*, 2007). At least four members of the Sec14 family bind α -tocopherol: the α -tocopherol transfer protein (α -TTP), and three tocopherol-associated proteins (TAPs).

α -TTP shows differential affinities for non- α -tocopherol forms of vitamin E: 38% for β -, 9% for γ -, and 2% for δ -tocopherol, 11% for the synthetic *SRR* form, and 12% for α -tocotrienol compared to α -tocopherol, which explains the preference of mammalian organisms for the α -form of tocopherols, and our use of the α -form in Pheroid™. In the liver, α -TTP facilitates release of α -tocopherol from the alimentary tocol mixture, followed by its redistribution to peripheral tissues (Traber and Arai, 1999). In *in vitro* systems, α -TTP transfers α -tocopherol between membrane vesicles (Verdon and J.B. Blumberg, 1988), with the α -tocopherol extracted from lipid bilayers by α -TTP. The inter-membrane transfer is completed by the subsequent dissociation of the α -tocopherol-loaded α -TTP from the membrane (Morley *et al.*, 2008). α -TTP is responsible for the distribution of α -tocopherol to tissues such as brain, placenta and lung (Brigelius-Flohé, 2009). α -TTP gene mutations result in ataxia in man and neurological defects and infertility in mice (Brigelius-Flohé, 2009).

Vitamin E and signalling cascades

The role of vitamin E in regulating the various signalling cascades will not be discussed in depth. Suffice it to mention that protein kinase C (PKC) has been shown to be inhibited specifically by α -tocopherol (Mahoney and Azzi, 1988). Other enzymes that are regulated by vitamin E include NADPH oxidase, phospholipase A₂, PKB/Akt, 5-lipoxygenase (5-LO), and cyclooxygenase-2 (COX-2; Brigelius-Flohé, 2009). Most of these regulatory events involve complex recruitment processes and are differentially regulated by the different forms of tocopherol. Lipo-polysaccharide (LPS)-stimulated TNF release is decreased by α -tocopherol and other 5-LO inhibitors (Brigelius-Flohé, 2009). This is probably important in the treatment of infectious diseases and vaccines, where LPS is generally found to increase TNF levels. LPS- or IL-1 β -stimulated COX-2 activity is decreased by the tocopherols (Jiang *et al.*, 2006).

Novel functions of vitamin E

α -Tocopherol activates the enzymes PP2A, diacylglycerol (DAG) kinase and HMG-CoA reductase (Brigelius-Flohé, 2009). Activation of DAG kinase (DGK) by α -tocopherol decreases the level of diacylglycerol in thrombin-stimulated endothelial cells and increases phosphatidic acid (PA) (Tran *et al.*, 1994). PA strongly facilitates the vesicle scission by dynamin in the removal of clathrin-coated vesicles from the plasma membrane (Burger *et al.*, 2000 and Simonsen *et al.*, 2001) and the presence of α -tocopherol may facilitate uptake of Pheroid™ vesicles through clathrin-coated pits. Gene expression studies points to a pivotal role for α -tocopherol in membrane fusion. The specific functions include the release of preformed compounds from vesicles in general, transmitter release in the nervous system, cell adhesion, endocytosis, recycling of vesicles, phagocytosis, or fusion of cells and organelles. (Brigelius-Flohé, 2009).

Absorption, intracellular trafficking and distribution of α -tocopherol

All forms of vitamin E are taken up in micellar form in the intestine. Some of the tocopheryls have to be hydrolyzed in the intestinal lumen before uptake, which is mediated by SR-B1 and the Niemann-Pick C1-like protein-1 (NPC1L1); (Reboul *et al.*, 2006; Narushima *et al.*, 2008; see also Chapter 2). Tocopherol is released into the lymph in chylomicrons or into the portal venous circulation via ABCA1, where it is taken up by HDL (Anwar *et al.*, 2006; 2007). In plasma, the chylomicrons are metabolized by lipoprotein lipases to chylomicron remnants containing vitamin E. These remnants are absorbed by LDL receptors (LDLR) in liver cells or by the scavenger receptor SR-BI if it is incorporated in HDL as illustrated in figure 5.10.

The interaction between α -tocopherol and α -TTP is largely responsible for the high bio-potency of α -tocopherol compared to other forms of vitamin E as it prevents vitamin E degradation. α -Tocopherol-deficiency (AVED) in patients presents with extremely low plasma α -tocopherol levels, resulting in severe α -tocopherol deficiencies in peripheral tissues. This condition has been shown to result in electrophysiological abnormalities of visual and neural functions and altered cortical gene expression patterns. It seems that the distribution of α -tocopherol within the organism is α -TTP's most important function (Brigelius-Flohé, 2009).

The lipophilic vitamin E can be present in membranes in a variety of conformational states: the chromanol ring may be directed towards the membrane surface but its depth within the bilayer may vary, which in turn will affect ongoing processes in membranes. As with the fatty acid binding proteins, α -tocopherol and presumably also α -TTP, are found in specific membrane domains that serve as platforms for signalling complexes, such as lipid rafts (Brown

and E. London, 1998) where its impact can be localized while proteins outside the domains remain unaffected (see also Chapter 4).

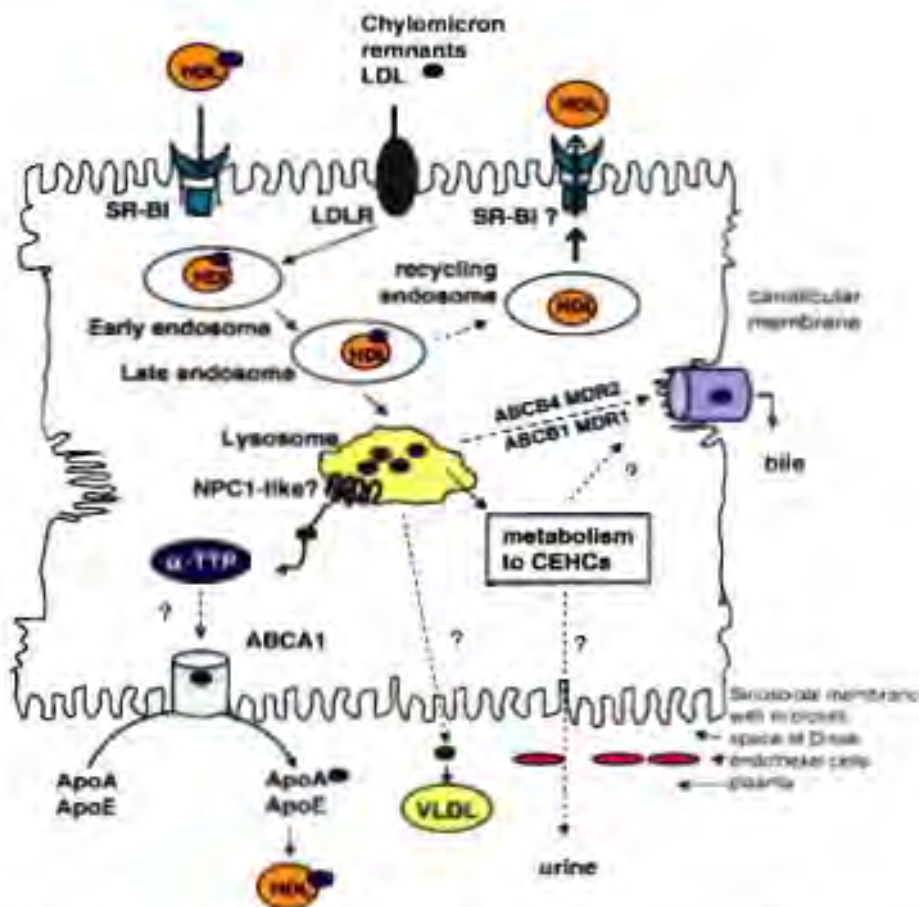


Figure 5.10: Cellular uptake and transport of vitamin E in liver cells. Chylomicron remnant-bound vitamin E is taken up by the LDL receptor (LDLR), and HDL-bound α -tocopherol by the scavenger receptor BI (SR-BI). The receptors are endocytosed and transported via the endocytic compartment to late endosomes/lysosomes where α -tocopherol is split off. Receptors and lipoproteins are either recycled or degraded in lysosomes. Arrows with dotted lines represent still unknown pathways. α -Tocopherol is recycled through α -TTP, probably requiring a Niemann-Pick type C-like protein. α -Tocopherol associates with the ABCA1 transporter and is transported through Golgi mediation to the extracellular leaflet at the cell membrane and from there to the space of Disse, where it is incorporated in the very low density lipoprotein (VLDL) fraction or into extracellular high density lipoprotein (HDL) particles with the support of apolipoproteins A or E. The MDR1 and MDR2 transporters transport excessive amounts of α -tocopherol to the bile. The remaining intracellular α -tocopherol is hydroxylated and oxidized in the endoplasmic reticulum. The final metabolites, carboxyethyl hydroxychromans (CEHC), are excreted into the urine (Brigellus-Flohe, 2009), reprinted with permission.

Lipid rafts are micro-domains that create cholesterol- and sphingolipid-rich micro-environments that are stabilized by structural components such as caveolin, activated

receptors, lipid-anchored proteins or ceramides generated by sphingomyelinase (Kusumi, *et al.*, 2004; Dumitru *et al.*, 2007). Multiple signalling molecules are recruited to these micro-environments, including the fatty acid binding proteins, PKC, PKB/Akt and NADPH oxidase (Brigelius-Flohé, 2009).

α -Tocopherol plays a role in the redistribution of signalling proteins in the membrane micro-environment (Marko *et al.*, 2007). This function of α -tocopherol is presumed to be of importance for inter-cellular transport of PheroidTM vehicles as well as for cellular uptake of the vehicles. The side chain of vitamin E arrange itself in an orientation parallel to the lipid hydrocarbon chains of preferentially unsaturated fatty acids present in cell membranes and may well have a similar orientation in the membranes of PheroidTM. α -Tocopherol has been shown to rotate around its long axis perpendicular to the surface, to diffuse laterally within fluid bilayers and to flip-flop between the two leaflets of the bilayer (Atkinson *et al.*, 2008).

5.3 Research methodology

5.3.1 Formulation of PheroidTM–entrapped anti-tuberculosis drugs

5.3.1.1 Raw materials

The components and manufacture of PheroidTM and pro-PheroidTM are discussed in some depth in both Chapters 3 and 4. Polyethylene glycol 40 (PEG) was used as a pharmaceutically acceptable carrier solvent for the gas and to decrease the viscosity of the formulation for encapsulation in soft gel capsules. Potassium hydroxide was used to adjust the pH. The PheroidTM formulations thus in general contain ethyl ester complexes of esterified essential fatty acids, a polyoxy-ethylated plant fatty acid, such as that found in the different Cremophors, nitrous oxide, α -tocopherol, ascorbyl palmitate and butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) as additional anti-oxidants. These components are recognized as pharmaceutically safe, as is evidenced by the fact that products based on this delivery system have been approved by the regulatory authorities of the US, most of the European Countries and Canada.

The safety data available indicates that Cremophor EL or RH administered intravenously may elicit certain dose-dependent toxicity characteristics. There is no evidence to suggest that the same is true for oral administration forms. In fact, in a study using oral administration of the drug paclitaxel in combination with Cremaphor EL, results showed undetectable plasma Cremaphor levels. It was concluded that this finding would have a beneficial influence on the safety of treatment with the PheroidTM, especially when it is used in its pro-PheroidTM form for

oral administration. In this form, any Excipient (see Textbook of Excipients) may be added according to the prescribed dosages.

The Pheroid™-based formulation thus become a pharmaceutical preparation comprising an anti-infective agent formulated with an administration medium which in turn comprises a solution of nitrous oxide in a pharmaceutically acceptable carrier solvent for the gas and which includes unsaturated fatty acid esters from the group consisting of oleic acid, linoleic acid, alpha-linolenic acid, gamma-linolenic acid, arachidonic acid, eicosapentaenoic acid [C20: 5 ω 3], decosahexaenoic acid [C22: 6 ω 3], ricinoleic acid and the derivatives thereof selected from the group consisting of the C1 to C6 alkyl esters thereof, the glycerol-polyethylene glycol esters thereof and the reaction product of hydrogenated natural oils composed largely of ricinoleic acid based oils with ethylene oxide. The carrier solvent for the nitrous oxide gas may be water or buffer or growth medium or pharmaceutically acceptable polymers such as a polyethylene glycol. The oil may be an organic essential oil based on long chain fatty acids having between 14 and 22 carbon atoms in the fatty acid. The oil may also be of either natural or synthetic origin and, if of natural origin, it may be either plant oil or animal oil. As plant oils those rich in gamma linolenic acid [GLA] are preferred.

The fatty acid component of the composition is constituted by the complex known as Vitamin F and in this regard it is preferred to make use of the ester form of Vitamin F known as Vitamin F Ethyl Ester. This product is commercially available under the trade description of Vitamin F Ethyl Ester CLR 110 000 Sh.L. U/g from CLR Chemicals Laboratorium Dr.Kurt Richter GmbH of Berlin, Germany.

Since the constituents in vitamin F comprise the n-essential fatty acids, which are fundamental to normal physiological processes, it is unlikely to be inherently toxic. It could theoretically be toxic if consumed at levels far higher than those normally available to the body. For example, the estimated endogenous rate of formation of the intermediate, gamma linolenic acid within the body of a normal adult was put at 100 – 1000 mg, or around 2-20mg/day. It was also estimated that daily intake of GLA in a human fully breast fed infant is of the order of 20-80mg/kg/day. Based on these estimates, it seems unlikely that doses of less than 100mg/kg/day will have any toxicity (Horrobin, 1992). Long term animal toxicity studies have shown that doses of evening primrose oil of up to 5 ml or 10ml/kg/day given for up to two years has no toxicity (Everett *et al.*, 2009).

The anti-infective agent has to be formulated in a liquid dosage form into which the nitrous oxide is dissolved and in which the fatty acid or ester thereof is either dissolved or suspended or emulsified. It may be formulated in a liquid form for oral administration such as in a suspension, an emulsion or a gelatine encapsulated liquid. The liquid may also be formulated in

a nasal or bronchial or pulmonary spray or in the form of an injectable formulation. Likewise, the anti-infective agent can be administered to the patient as a topical, buccal or vaginal cream or ointment, or as a suppository, using such additional excipients and carriers as are conventionally used in the pharmaceutical trade in making up such dosage forms. Such a liquid formulation suited to transdermal application may also be in the form of a skin patch that provides a reservoir for the formulation.

Unlike the PheroidTM, which is based on formulations containing long chain fatty acids and derivatives thereof, the liposomes are based on a clearly distinguishable group of compounds namely the phospholipids, and generally also contain cholesterol as a stabilising agent and may further contain a form of lecithin. The names and origins of other raw materials generally used in the formulations are shown in Table 5.3.

Table 5.3: Raw materials, lot or batch numbers and manufactures/suppliers		
Raw material	B/N OR LOT	Supplier/manufacturer
Propylene Glycol bn 2/6235	1583359	Savannah
Polyethylene Glycol 400 Sentry	0/440873	Savannah
Rifampicin IP/BP	RD98165	Marsing & Co, Themis Bios Ltd.
Rifampicin USP/BP	RD99256	Anant & Co/ Gujarat Themis Bios Ltd.
Loratadine micronized	OLT020	Cadila Healthcare Ltd; Savannah
Potassium hydroxide pellets	B561932	Merck KGaA
Ethambutol HCL B.P.	151200	Shamrock; Savannah
Pyrazinamide BP	/P-004/016142	Marsing & Co
Isonazide BP 98	T00910	Marsing & Co

5.3.1.2 Manufacturing of the formulations for *in vitro* studies

For the *in vitro* studies, the raw materials, culture media and reagents were obtained from the following manufacturers: Ethambutol (EMB) and Isoniazid (INH) from Sigma, Rifampicin (RMP) from Gujarat Themus Biosynth Ltd or Sigma, Pyrazinamide (PZA) from Anant & Co, mycobacterial 7H9/ Middlebrook medium from Difco, ADC Middlebrook enrichment from BMMTM; RPMI, Fetal Bovine Serum from BRL. Fluorophores for laser scanning confocal microscopy (CLSM) were obtained from Molecular Probes or now Invitrogen (Holland). Mainly Nile Red and BacLight were used for visualization of drug encapsulation and bacterial labelling

respectively. Comparative commercial drug products used for bioavailability studies were Rifadin (600mg) and Rifafour e-200 from Aventis.

The Pheroid™ used in these studies were prepared according to the general protocol described in Chapter 3 in the following way:

The applicable pressure vessel was charged to its operating volume with water at 20°C [ambient temperature]. The vessel was connected to a supply of nitrous oxide via a flow control valve and pressure regulator. The closed vessel is supplied with nitrous oxide at a pressure of 2 kPa for a period of 96 hours, it having been determined that at the aforementioned temperature the water is saturated with nitrous oxide over such period of time under the above-mentioned pressure.

18.9g Vitamin F ethyl ester as identified and described above was mixed with 4.5g Cremophor RH40 (which is the trade name used by BASF for a product which it describes as the reaction product of hydrogenated castor oil with ethylene oxide which product is also known by the INCI name as PEG-n-Hydrogenated Castor Oil), 0,08g butyl hydroxyanisole, 0,23g butyl hydroxytoluene with stirring at 80° C. This mixture was emulsified into 942,5g of the nitrous oxide-saturated aqueous solution with high-shear stirring. This mixture, sometimes referred to as "Lindil", constituted the stock nitrous oxide/Vitamin F ethyl ester emulsion used for the mycobacterial *in vitro* studies herein described. The Pheroid™ formulation was sterilized by forced passage through a 0.22µm syringe filter for use in bacterial and cell culture experiments. The resultant solution was bottled as stock solution for use in the formulations and applications set out below.

5.3.2 Bacterial growth *in vitro* studies

BACTEC is an internationally accepted technique for the identification of the *M.tuberculosis* complex. Since the *in vitro* studies included the growth and evaluation of live *M.tb.* strains, all BACTEC and bacterial culture studies had to be performed in a Biosafety class 3 laboratory. For that reason, all BACTEC and bacterial culture studies (see below) were performed at the MRC Centre for Molecular and Cellular Biology, Department of Medical Biochemistry, University of Stellenbosch Medical School, Tygerberg, South Africa under the auspices of Prof. Paul van Helden and Dr. Ian Wiid.

BACTEC items were purchased from BACTLAB Systems and used according to the BACTEC manual (12). Pheroid™ was diluted with sterile distilled water, nitrous oxide gassed water [obtained from MeyerZall Laboratories (Pty) Ltd] or mycobacterial 7H9 medium. Drugs were entrapped in Pheroid™ by sonication, using a Cuphorn probe of a Virsonic Digital 475

Ultrasonic Cell Disrupter or an UMC 5 or Elma ultrasonic bath). Pheroid™ was labelled with fluorochromes by incubation in the sonicating bath for 30 minutes.

5.3.2.1 *M.tuberculosis* strains

For these studies it was necessary to establish reliable, reproducible cultures, growth curves and antibiotic sensitivity levels (MIC) of *M.tuberculosis* strains. The H37Rv *M.tb.* strain was isolated from a human patient in 1905. The strain has since been used extensively in research and has been designated as the neotype of *Mycobacterium Tuberculosis* in 1972 (Kubica *et al.*, 1972). It may be obtained from ATCC as reference strain (see below). In contrast to some of the clinical strains, the use of H37Rv as reference strain has the following advantages: H37Rv is well characterized, it displays typical growth characteristics and responses; it retains full virulence *in vitro* and *in vivo*; and it shows typical drug susceptibility and biochemical activity (Kubica *et al.*, 1972). Other strains used proven distinct, clinical strains, some of whom were drug sensitive and some drug resistant. These strains were cultured from patients isolates. The culturing and characterization of the clinical strains was undertaken by Dr Ian Wiid at the Biohazard Class 3 Laboratory dedicated to tuberculosis research at the MRC Centre of Cellular and Molecular Biology, Medical School, Tygerberg.

M.tuberculosis strains were selected according to their genetic and epidemiological type from a bank of 1800 clinical isolates genotyped according to their IS6110 insertion sequence profiles, as determined by the MRC Centre of Cellular and Molecular Biology. The insertion sequence ranges from 1 to 23 copies per strain. These strains have been clustered into families according to their genetic patterns and represent recent transmission clusters because of their most frequent appearance in the community. Some of these strains may also represent more virulent strains although virulence factors in *M.Tb.* have not yet been clearly identified. Multi drug resistant (MDR) strains were selected over a range from mildly resistant to highly resistant for INH, RMP and EMB. The clinical isolates of *M.tuberculosis* (sensitive and resistant to INH, rifampicin and ethambutol) and *M.tuberculosis* H37Rv reference strain (ATCC 25618), were cultured on L-J slant cultures and used for BACTEC analysis. Pyrazinamide resistance was not evaluated in these BACTEC studies, since the methodology for the use of PZA did not exist in the BACTEC system.

Mycobacteria tuberculosis MDR strains V79 & V25 were selected for further studies and grown as described for reference strain H37Rv. Strains TV25 and TV79 were determined to be resistant to RMP, INH and also streptomycin. The catalase activity of TV79 was found to be negative and that of TV25 to be 5mM. The Tokyo *M.Bovis* BCG strain was cultured in Middlebrook medium, enriched with ADC as described below.

5.3.2.2 *M. tuberculosis* culturing and drug challenges

Clinical and laboratory strains of *M.tuberculosis* were cultured at 37°C in 7H9 medium enriched with ADC (Middlebrook (1977)). Cultures were stirred continuously to ensure homogenous bacterial distribution and uniform aeration. Under these conditions, cultures grow reproducibly (<1.0% difference). When cultures reached a density of approximately 0.16 at A600nm (one McFarland; Siddiqui, 1995) as spectrophotometrically determined, aliquots of 0.1ml were inoculated into BACTEC 12B vials. These primary cultures were incubated at 37°C until a growth index of between 500 and 600 was reached. From these stock cultures, 0.1ml was inoculated into new BACTEC vials for drug sensitivity testing and the average of three measurements of radioactivity were generally used to calculate bacterial growth. INH and EMB were dissolved in sterile water and added to the BACTEC vials at concentrations ranging between 0.05-0.2µg/ml and 0.0325-1.0µg/ml respectively. Rifampicin was dissolved in 50% ethanol and was used in a concentration range of 0.03125-0.125µg/ml in BACTEC. The solvent did not have any effect on bacterial growth at the amounts used. The minimal inhibitory concentration for INH, RMP, and EMB was established such that the growth rate of the treated culture was the same as a 1.0% dilution of the control culture. From the primary cultures, 0.1ml was used per BACTEC vial for drug susceptibility tests. Growth indices were monitored every 24 hours over the indicated period (see results). Results obtained on day 4 of incubation were used for calculation of the GI ($GI = [\text{Growth index (GI) of day (n)}] \text{ minus } [\text{growth index (GI) of day (n-1)}]$). Sterility of mycobacterial cultures were monitored by Ziehl-Nielsen staining.

For the *in vitro* drug challenges, RMP was dissolved at the concentrations indicated in the results of the different studies. The drug preparations were sterilized by filtration through 0.45 micron filters so as not to give background contamination by other bacteria. Rifampicin was made up in 50% ethanol at a concentration of 10µg/ml, after which it was diluted to the required concentration with either water or Pheroid™ and sonicated to ensure entrapment. Of this diluted mixture, 0.1 ml was added to a BACTEC vial to give the end concentrations of both Pheroid™ and drug.

To establish optimal doses of Pheroid™ formulations for human studies, the various formulations were first compared in an *in vitro* human macrophage infection model. Conventional live/dead assays as well as confocal microscopy were used to determine death of bacteria infecting the macrophages.

5.3.2.3. Microscopic Analysis

Confocal Laser Scanning Microscopy (CLSM) was used to investigate the entrapment efficiency of RMP in Pheroid™. All CLSM studies described here were performed by the author in a Biohazard Class 2 laboratory. A Nikon PCM2000 microscope, with Kr/Ar and He/Ne lasers

and a Zeiss LSM410, with the same laser set, were used in the investigation. Generally, small pinhole sizes (<5µm) and Plan Apochromat oil-dispersion objectives of 60x and 100x magnification were used, enhanced by digital zoom. Measure bars were digitally captured as an inherent feature of the micrographs. The fatty acid component of Pheroid™ was visualised by labelling with the lipophilic dye, Nile red. Nile red fluoresces or emits light photons at a specific wavelength only when in association with lipid-based molecules or structures. RMP is auto-fluorescent, and emits photons in the green or red wavelength spectra after excitation by laser, depending on the pH of the solution. At the neutral pH used in the formulations, the entrapped RMP was visualized as green crystalline material inside the Nile Red-labelled Pheroid™. To investigate the uptake of Pheroid™-entrapped RMP by the bacilli, free and encapsulated RMP were separated by centrifugation in a microfuge at 13000 rpm for 10 minutes. To remove the free label, the samples were washed twice and the Pheroid™-entrapped drug was resuspended in Middlebrooks at the indicated concentrations.

Penetration of Pheroid™ through the bacterial cell wall and membrane was illustrated by following the auto-fluorescent Pheroid™-entrapped RMP when the formulation was added to growing *M. Bovis BCG* cultures that was pre-labelled with the *Baclight* bacterial viability fluorescent stain. The unlabelled Pheroid™ with entrapped RMP was added to the bacterial cultures to a final concentration of 25 µg/ml. Free fluorophores were removed by washing. In the parallel control, un-entrapped or free RMP was added to cultures to the same final concentration. The same procedure was followed for INH, ETH and PZA. The bacilli were labelled with *Baclight* according to manufacturer's instructions. *Baclight* stain results in the emission of light in the green spectrum by live bacteria, while light are emitted in the red spectrum by dead cultures. Labelling was for 30 minutes at 37°C under normal culturing conditions, after which free *Baclight* was removed by washing of the cultures. Either Pheroid™-entrapped RMP or free RMP was added to the labelled live bacterial cultures by means of a flow cell, in which the bacterial growth conditions were optimized. The samples were subjected to CLSM. All determinations were in duplicate.

5.3.3 Results of mycobacterial *in vitro* investigations

5.3.3.1 The efficiency of Pheroid™ entrapment of anti-tuberculosis drugs

To evaluate the entrapment efficacy of anti- tuberculosis drugs by Pheroid™, RMP, INH, PZA and EMB were entrapped in Pheroid™ vesicles by sonication and entrapment efficiency was investigated by Confocal Laser Scanning Microscopy (CLSM).

The confocal micrograph in figure 5.11 shows a single Pheroid™, entrapping RMP. The entrapment efficiency of all four drugs by Pheroid™ is very high; at therapeutic concentrations entrapment is nearly complete, as determined by the ratio of free to entrapped drug.

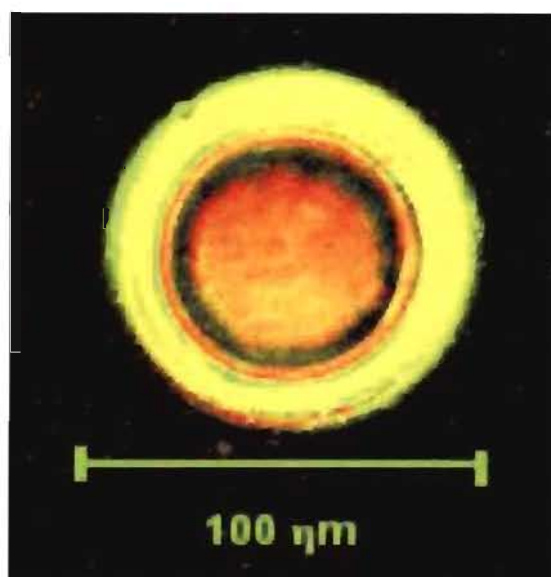


Figure 5.11: A confocal micrograph of RMP entrapped in a Pheroid™ vesicle. The multiple layers of the multilamellar vesicle is visible in yellow as a result of fluorescent labelling with Nile red at low pH, while the red interior fluorescence is that produced by RMP at a low pH.

The *M. Bovis* BCG vaccine is used extensively for vaccination against tuberculosis infection. It is classified as a non-pathogenic mycobacterial strain and is a widely used investigative cellular infection model. The infection studies described here were performed by the author in a Biohazard Class 2 laboratory (see also 5.3.3.5). To determine the uptake of entrapped drugs by the BacLight-labelled viable bacteria, the bacteria were investigated before and after addition of entrapped RMP. Uptake of entrapped RMP by the live bacteria was fast and efficient; within 15 minutes more than 80% of the bacteria had taken up the entrapped RMP. In the parallel control with free RMP, uptake of the RMP was relatively slow with few bacteria showing the presence of RMP in the same time period. Uptake of entrapped RMP by the bacteria was associated with decreased viability of the cultures, as reflected by the change in bacterial fluorescence from green to red as a result of BacLight staining (figure 5.12), as well as by a change in the fluorescence of the RMP inside the bacteria. Since the fluorescence of RMP is pH-dependant, the changes in the fluorescence are presumably due to a change in the pH environment of the RMP after uptake into the bacteria. The bacillus shown is not even 3 μm in length and the micrograph is at the edge of resolution of this imaging system.

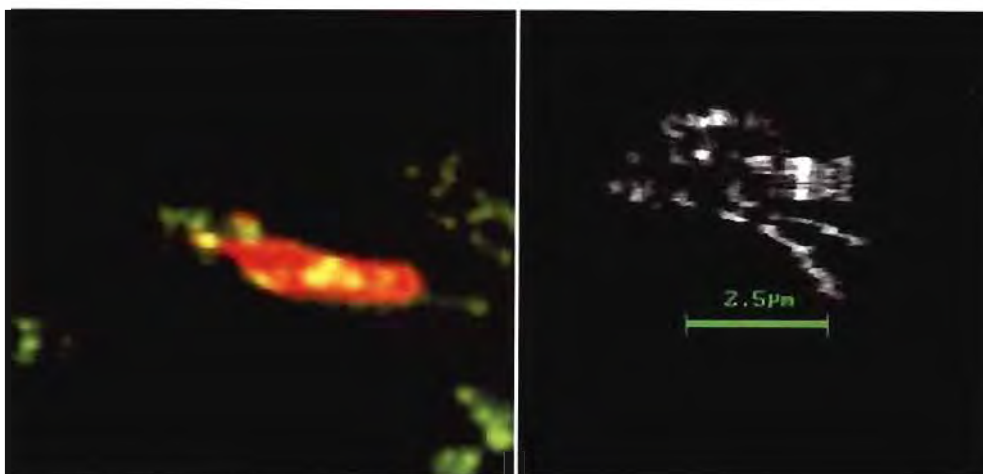


Figure 5.12: Confocal micrographs of optical longitudinal sections through Baclight-labelled BCG bacteria, with RMP (yellow and white in the micrograph on the left and right respectively) inside the bacteria. The red interior of the bacterium on the left indicates that the bacterium is dying or dead. The optical section shows the presence of other live bacteria just outside the optical plane. The fluorescence in the micrograph on the right shows that the Pheroid™-entrapped RMP is concentrated in specific sites in the bacilli. The excitation used for visualisation of entrapped RMP determined by fluorometry, was at 400nm and emission was detected in the orange to red wavelength in a window of between 480 and 560nm at the neutral pH used in the Pheroid™ formulations.

5.3.3.2 Inherent antimycobacterial activity of Pheroid™

To evaluate the effect of Pheroid™ itself on mycobacterial viability, 0.1ml of the Pheroid™ preparation was added to BACTEC vials (equivalent to a 1:40 dilution). The vials were inoculated with *M. tuberculosis* H37Rv strain. Pheroid™ was added to obtain final dilutions ranging from 1:40 to 1:2560. The growth index (GI) of the *M. Tb.* cultures was monitored over the indicated periods. The GI values were calculated and are shown relative to the Pheroid™ dilution (Figure 5.13). Pheroid™ at 1:40 dilution strongly inhibited growth of the *M. Tb* culture (GI<10) for 3 days following treatment when compared to the control incubation, which was positive (GI=27) after only 24 hours incubation. This inhibitory effect on growth could be seen at dilutions as great as 1:2560, but most strongly at dilutions of 1:640 or less. The period of time before conversion to positive growth was proportional to the dilution factor, thus a typical dose response. By day 6 of incubation, all cultures with the exception of the 1:40 dilution, showed growth increases to GI values of 400 whereas the 1:40 dilution showed only a marginal increase in growth on day 6 of incubation (results not shown).

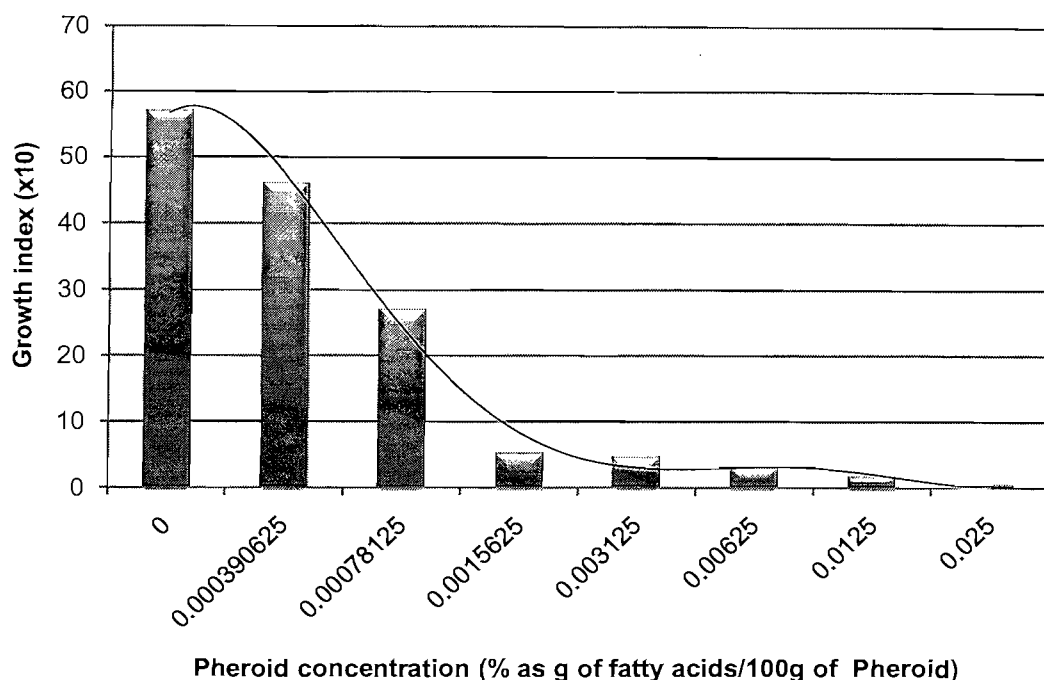


Figure 5.13: The average in vitro Pheroid™ dosage response curve of *M.tuberculosis* H37Rv. The bacteria were cultured using BACTEC. Pheroid™ was filter sterilized (0.22µm filter) and added to the cultures at various concentrations. Each of these bacterial preparations was incubated for 3 days. Growth indices were monitored every 24 hours over the indicated period as described in 3.2.1 and the average delta growth indexes calculated from three determinations ($n=3$) for each concentration.

Dilutions of the Pheroid™ with water, mycobacterial growth medium, or water saturated with nitrous oxide showed no difference in the dose-dependent inhibition of *M.tuberculosis* growth (results not shown).

In order to evaluate the ability of Pheroid™ to sustain growth inhibition of *M. tuberculosis* H37Rv, duplicate cultures (Figure 5.14) were treated with a single administration of Pheroid™ (1:40 final dilution) and incubated at 37°C for 7 days, at which stage the cultures became BACTEC positive. An untreated *M.tb.* culture was included to follow normal growth, and acted as control. A repeat Pheroid™ administration was added to one of the initial Pheroid™-treated duplicate cultures on day 7 and the incubation was continued. Figure 5.14 shows that the initial treatment of the culture (curve B) resulted in a 93% inhibition in mycobacterial growth during the first 7 days of incubation compared to the control untreated culture (curve A), after which the culture grew exponentially up to day 12. However the duplicate culture treated with a second Pheroid™ administration showed additional inhibition of growth (fig. 5.14 curve C) with inhibition of growth up to day 12 of incubation (GI=163).

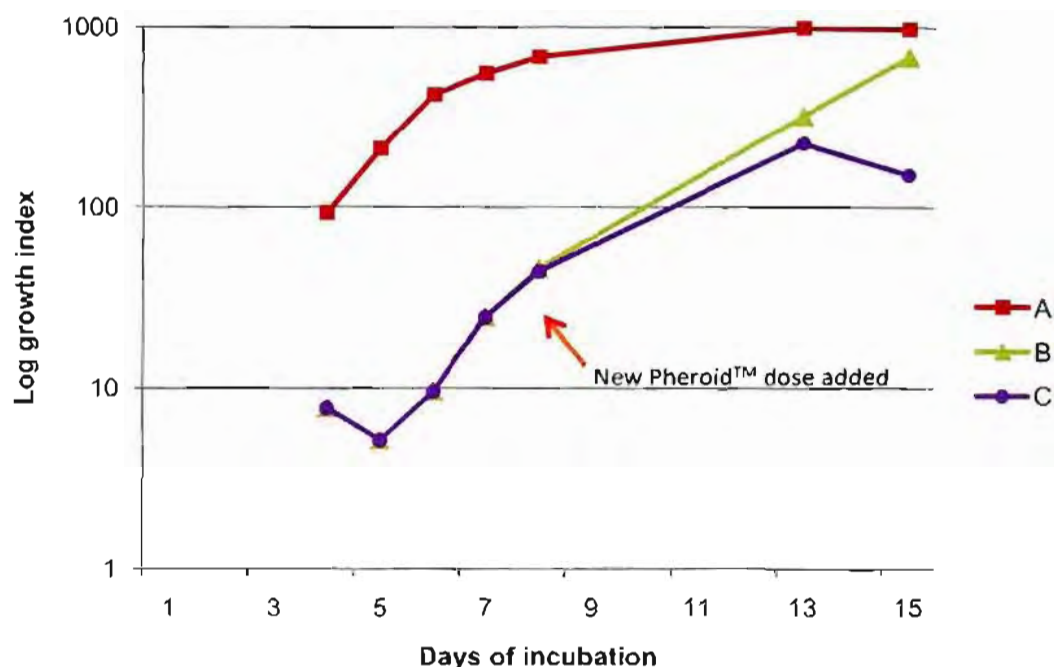


Figure 5.14: Impact of Pheroid™ on *M.tb.* growth. *M.tuberculosis* H37 RV was treated with Pheroid™ (1:40 dilution) in two parallel cultures (B and C). Both cultures were simultaneously inoculated from a single primary culture. One of the cultures (C) was treated with a supplementary dose of Pheroid™ on day 7 of incubation and both cultures (B and C) were incubated to day 12. An untreated culture (A), inoculated from the same stock as B and C, was included to monitor culture growth.

Subcomponents of Pheroid™ were evaluated to establish the origin of the inhibitory effect observed. The nitrous oxide-saturated essential fatty acid fraction (1.95% of the total Pheroid™ preparation) showed the same inhibitory effect described above, as did the complete formulation, whereas the nitrous oxide gassed water fraction (92.8% of the whole suspension) showed no effect on growth compared to the control (results not shown). These results may be interpreted as meaning that:

- (a) nitrous oxide plays no role in the mycobacterial growth inhibition, or
- (b) the nitrous oxide in the water is diluted upon addition to the bacterial growth medium, since a number of salts cause dissipation of the gas from the water phase, or
- (c) the fatty acid fraction itself interferes with the bacterial growth, or
- (d) sufficient nitrous oxide is entrapped within the fatty acid phase and nitric oxide groups are available to result in growth inhibition as discussed, or
- (e) probably a combination of more than one of these possibilities.

5.3.3.3 Efficacy of Pheroid™ entrapped tuberculosis drugs in drug sensitive strains

Although Pheroid™ itself may contribute to bacteriostatic or bactericidal effects, the anti-tuberculosis drugs are necessary for effective treatment. The efficacy of the Pheroid™-entrapped drugs against various *Mycobacterium tuberculosis* drug sensitive and multi drug resistant strains was investigated. The effect of entrapment of the drugs on *M.tb.* growth is shown in figure 5.15.

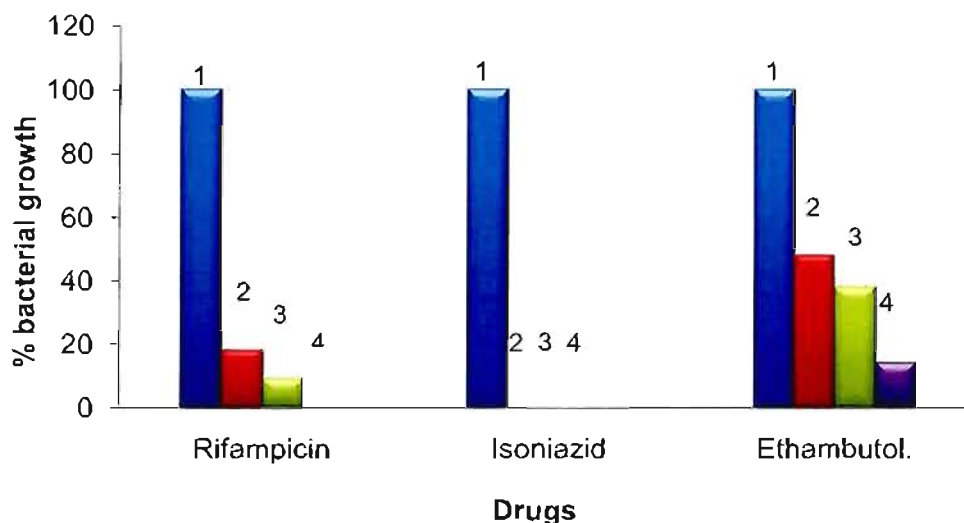


Figure 5.15: Percentage growth of *M.tuberculosis* H37Rv in BACTEC in the presence of Pheroid™ and various concentrations of antituberculosis drugs. Growth was calculated relative to the control which was a *M.tb.* culture treated with a 1:40 dilution of Pheroid™. This Pheroid™ was allocated a value of 100% growth (column 1 in all cases above). Drug efficacy was determined in the presence of a 1:40 dilution of Pheroid™ in all above cases and the comparative bacterial growth was calculated from an average of three measurements of radioactivity each. RMP concentrations were: column 2=0.03125µg/ml, column 3=0.0625µg/ml, column 4=0.125µg/ml (approx. MIC). INH concentrations were: column 2=0.005µg/ml, column 3=0.01µg/ml, column 4=0.02µg/ml (approx. MIC). EMB concentrations were: column 2=0.125µg/ml, column 3=0.25µg/ml and column 4=0.5µg/ml.

RMP in Pheroid™ kills the bacilli of the drug sensitive strain H37Rv to a much greater extent than RMP alone at concentrations below 2µg/ml. Microscopy illustrated delivery of RMP by the Pheroid™ into the interior of the bacillus, and furthermore suggest that the RMP does not stay entrapped in the bacillus, but is released from the vesicle to the internal environment of the bacilli for biological activity (figure 5.12). The concentration of the Pheroid™ used in the drug challenge studies was too low to have a significant bactericidal effect. Figure 5.16 below shows

the effect of Pheroid™ on H37Rv cultures in the presence and absence of anti-tuberculosis drugs at a point where the growth indices of controls were +/- 500, which was then taken as 100% growth. The most marked effect was observed with a combination of INH and Pheroid™, which showed 0% bacterial growth at 0.25x and 0.5x MIC for INH compared to nearly 80% bacterial growth in the absence of Pheroid™. At 0.25x MIC, Pheroid™ combined with RMP allowed 20% bacterial growth in the presence and 45% in the absence of Pheroid™, and EMB allowed 50% bacterial growth in the presence of compared to 100% bacterial growth in the absence of Pheroid™.

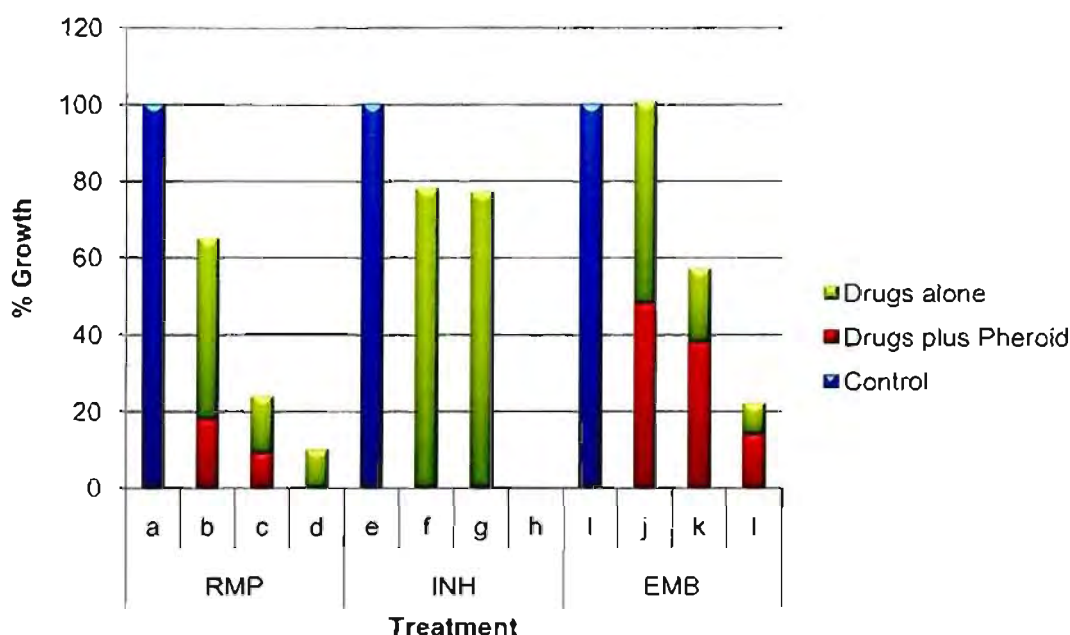


Figure 5.16: The impact of the presence of the Pheroid™ and various concentrations of antituberculosis drugs on the in vitro growth of *M.tb* in the BACTEC system. Growth was calculated relative to the control which was *M.tb* culture treated with a 1:40 dilution of Pheroid™ alone and was allocated a value of 100% growth (columns a, e and i). Drug efficacy was determined in the presence of a 1:40 dilution of Pheroid™ in all above cases. RMP concentrations were: column b=0.03125µg/ml, column c=0.0625µg/ml, column d=0.125µg/ml (approx. MIC). INH concentrations were: column f=0.005µg/ml, column g=0.01µg/ml, column h=0.02µg/ml (approx. MIC) and EMB concentrations were: column j=0.125µg/ml, column k=0.25µg/ml and column l=0.5µg/ml.

Time to culture positivity is a well-accepted indication of bacterial load and is widely used in BACTEC drug efficacy assessment in *M.tuberculosis* (Baka *et al.*, 2007). Figure 5.17 shows the time-to-positive growth results of *M.tuberculosis* H37Rv treated with anti-tuberculosis drugs in the presence and absence of Pheroid™. The growth-inhibitory effect of Pheroid™ alone was

taken into account when the combined effect of anti-tuberculosis drugs and Pheroid™ was determined. The values depicted in the graph represent a single concentration for each drug. Each drug concentration was also lower ($0.25\text{--}0.125 \times \text{MIC}$) than the minimal inhibitory concentration (MIC) for that drug.

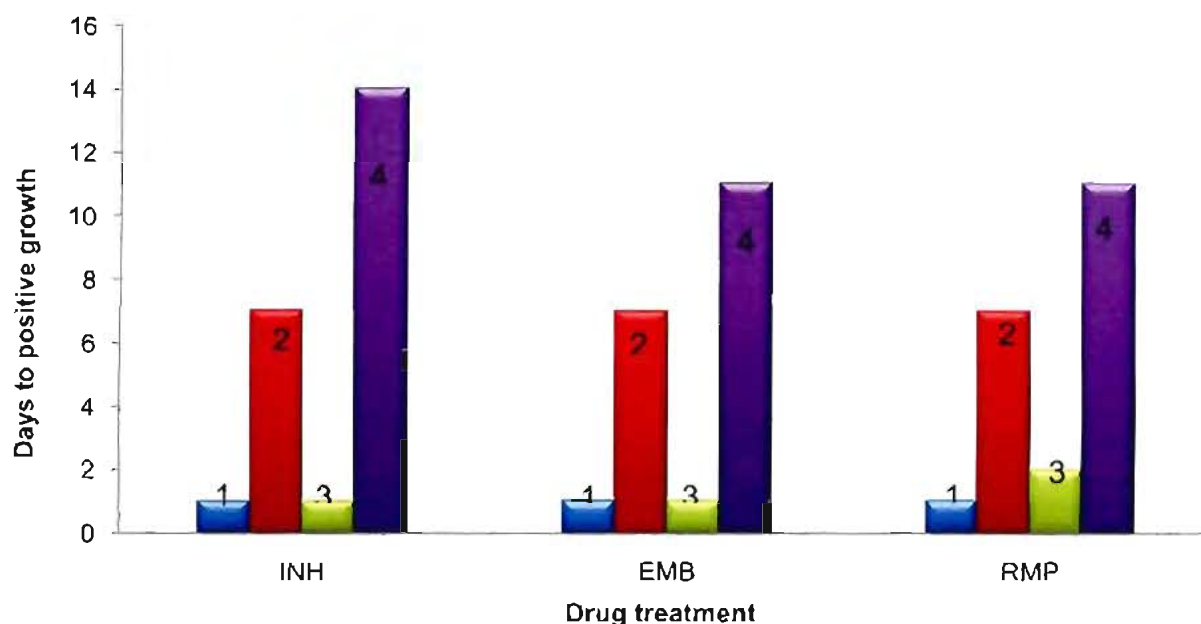


Figure 5.17: Time to positive growth of *M.tb. H37Rv* cultures after the administration of various treatments. Columns 1 are untreated controls, columns 2 are cultures treated with Pheroid™ only, columns 3 are cultures treated with drugs only while columns 4 represent those cultures treated with Pheroid™-entrapped drugs. Cultures treated with $0.005\mu\text{g/ml}$ INH ($\text{INH}, 0.25 \times \text{MIC}$) became BACTEC culture positive within 24 hours of incubation (one doubling time is approximately 22hours). The same time to culture positivity was observed for EMB ($0.25\mu\text{g/ml}=0.25 \times \text{MIC}$). Controls in all cases (Figure 5.15; columns 1) became culture positive within the first 24 hour incubation period. In the presence of a 1:40 dilution of Pheroid™, cultures took 6 days longer to become culture positive (Figure 5.15 columns 2). Combining the Pheroid™ with the various drugs extended the time before cultures became BACTEC positive by a further 7 days in the case of INH and by 3 days for EMB and RMP.

The significance of the differences between the drug treatment groups versus the untreated or control pharmacokinetic parameters was determined for the test and control groups by using the student's t-test (parametric) and the ANOVA (analysis of variance). The p-value can be defined as a measure of the probability, i.e. how strong the evidence is that the null hypothesis is correct. The null hypothesis generally represents the hypothesis of no change or no effect. Usually, the hypothesis is rejected if the p-value is less than 0.05. A small p-value is evidence

against the null hypothesis while a large p-value means little or no evidence exists against the null hypothesis. The probability (p-value) that the different treatments were not effective is shown in Table 5.4. The drug concentrations used ranged from 0.25 -1 times the minimal inhibitory concentration (MIC) of each of the drugs evaluated.

Table 5.4: Significance of the difference of drug treatments on <i>M.tb.</i> growth		
Drug	Efficacy of drugs entrapped in Pheroid™	Efficacy of drugs not entrapped in Pheroid™
RMP	p = 0.02	p = 0.134
INH	p = 0.0001	p = 0.8
EMB	p = 0.00247	p = 0.16

The probability that the treatment does not cause any change from the baseline (i.e. the null hypothesis) has to be rejected in the case of treatment with Pheroid™-entrapped drugs. According to the statistical probability the null hypothesis has to be accepted for un-entrapped drugs, i.e. this treatment did not have a statistically significant effect on mycobacterial growth.

5.3.3.4 Efficacy of Pheroid™ entrapped tuberculosis drugs in drug resistant strains

For an investigation into the effect of INH entrapment into vesicles, the viability of drug resistant strain TV25 was evaluated in the presence of INH/Pheroid™ combinations in BACTEC. Delta Growth Index (dGI) was calculated for each combination. Pheroid™ was filter sterilized through a 0.22µm syringe filter (Millipore). Filtration was slow and difficult due to the physical properties of the Pheroid™. Pheroid™ was diluted so that the dilutions with an inhibitory effect on the viability of *M.tb.* had nearly no effect. INH was reconstituted with Pheroid™ dilutions in two ways: First, dilutions of Pheroid™ in growth medium of 1:2, 1:8, and 1:32 were made, and INH was added to the dilutions to give a final concentration of 0.1µg INH/ml Pheroid™ dilution per BACTEC vial (4ml content). Secondly, INH was reconstituted with undiluted Pheroid™ to give a final concentration of 0.1µg INH per ml Pheroid™. The results are set out in Figure 5.18.

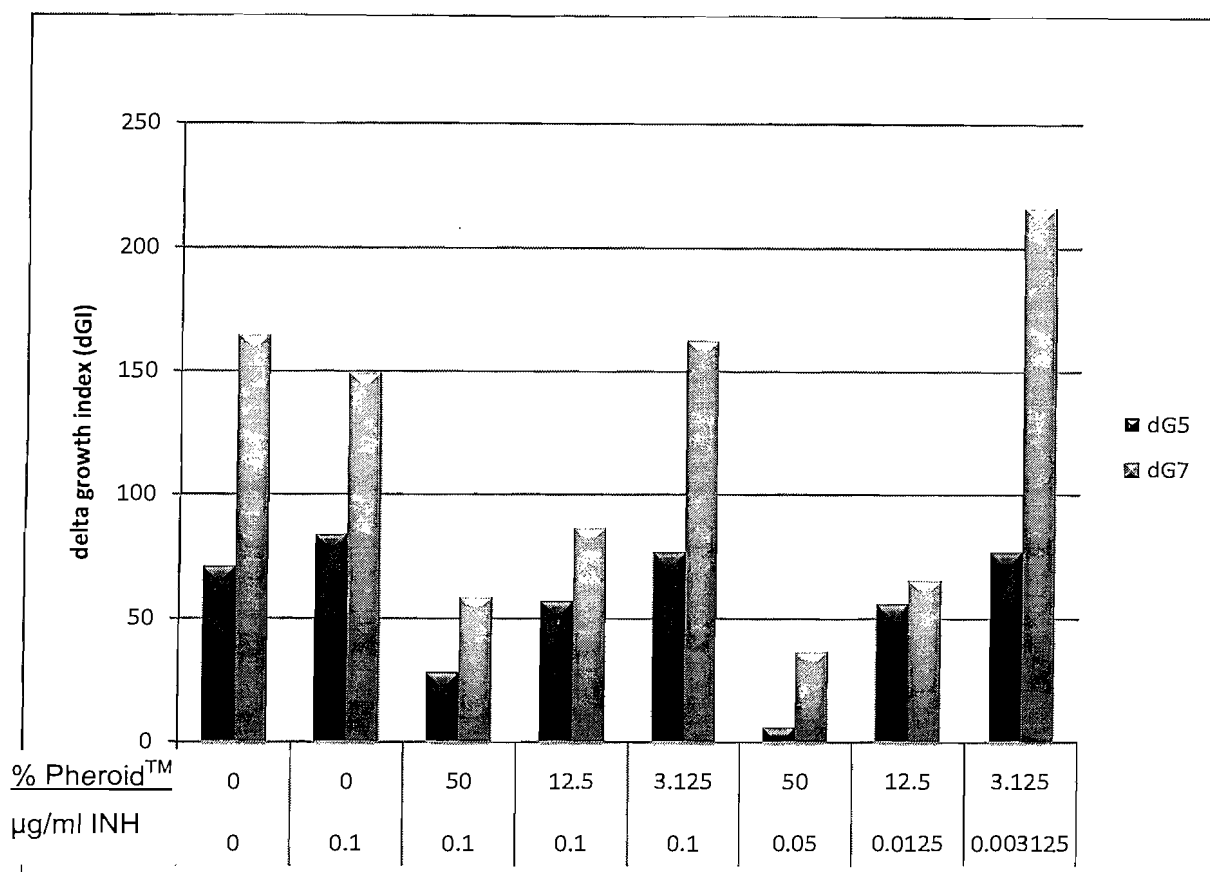


Figure 5.18: Growth responses of *M.tb.* MDR strain T25 after being challenged with INH in the absence and presence of PheroidTM-entrapment. The x-axis reflects the specific combination of PheroidTM and drug used. The results show that after 6 and 8 days incubation in BACTEC (dG5 and dG7 respectively), the dGI for *M.Tb.* strain TV25 was still below 10 in the presence of PheroidTM with entrapped INH at a 1:2 dilution, whereas the sample subjected to the formulation in which the 1:2 dilution of PheroidTM was made prior to INH dilution already showed strong positive growth with a dGI = 28 at dG5 and >50 at dG7. INH alone showed no inhibitory effect at this concentration and the result thus represented an increased inhibition of 78% compared to the effect of PheroidTM dilution prior to INH addition. The effect of PheroidTM alone was 50% relative to the control for strain TV25. In addition, the effect of PheroidTM combined with INH, followed by dilution was 91% relative to the control, giving an additional effectiveness of 41.5% of INH entrapped in PheroidTM.

Because post-dilution of PheroidTM/INH gave a more pronounced effect, it would appear that a critical concentration of PheroidTM vehicles is required to entrap INH effectively for delivery. Furthermore, the observed effect of the PheroidTM/INH combination prior to dilution appears to be that of more effective drug delivery and not of any other effect from the PheroidTM itself, as a lower effect was observed in the PheroidTM dilution prior to INH addition. Other dilutions of PheroidTM/INH combinations showed no differences in effect. This is to be expected as dilutions

of INH went far below the effective range for *M.tb.* strain TV25. The Pheroid™ is also diluted far below its bactericidal concentration and it has been shown that, although Pheroid™ has a direct effect on *M.tb.* viability, it alone does not generate a situation that would render the bacilli more vulnerable to INH.

Ethambutol prepared in sterile deionized water and dissolved in Pheroid™ at appropriate concentrations was added to a culture of *M.tuberculosis* H37Rv known to be resistant to EMB. The GI was determined every 24 hours \pm one hour. The observations are recorded in Figure 5.19.

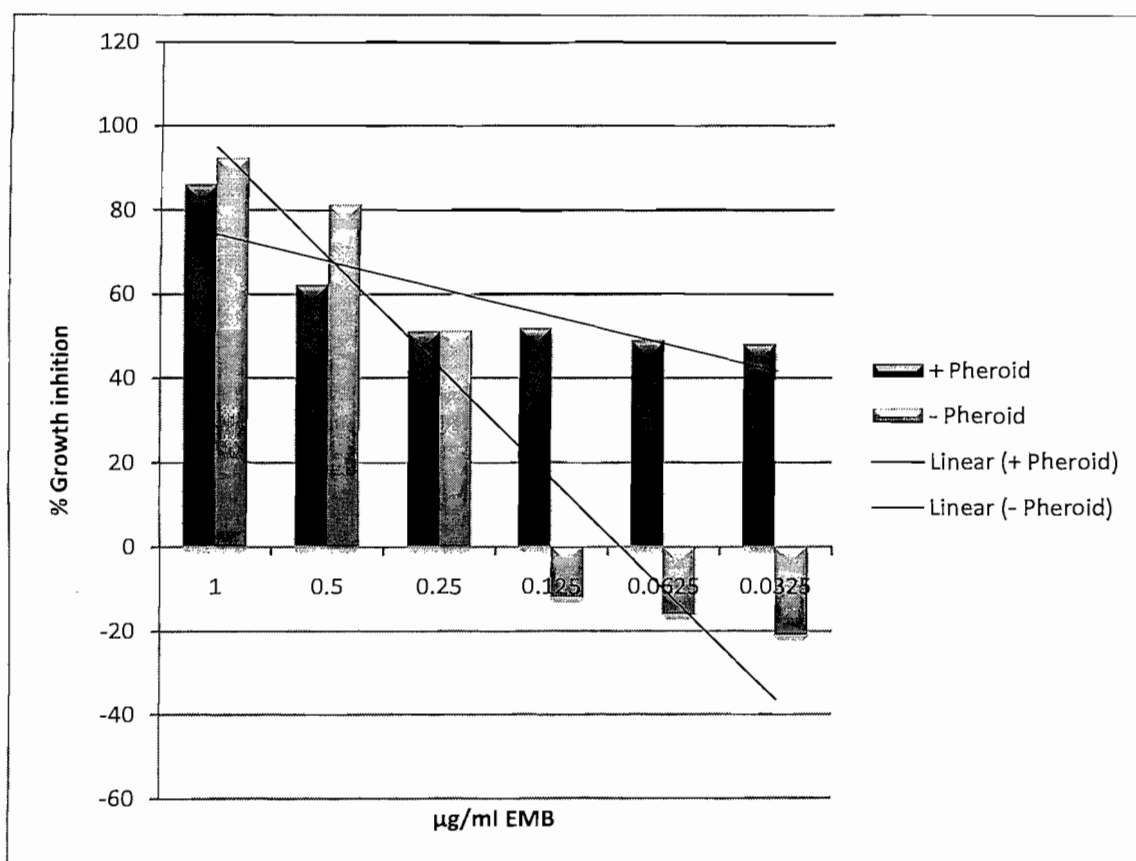


Figure 5.19: The % inhibition of growth caused by the addition of EMB at various concentrations with and without Pheroid™ entrapment. The % inhibition was calculated from growth curves obtained for each of the concentrations. A 0% inhibition was obtained when the mycobacterial growth in the absence of treatment is at GI=540 – at this point mycobacterial growth becomes stationary. All GI values were calculated relative to this control value to give % inhibition of growth inhibition. The untreated control values of each of the growth curves were normalized to make results comparable. The trend in growth inhibition of each of the treatments is shown.

These results show that the MIC for EMB in *M.tb.* H37Rv strain is around 1.0µg/ml. From concentrations as low as 0.125µg/ml EMB, entrapped in Pheroid™ still maintains a strong

inhibitory effect on mycobacterial growth compared to un-entrapped EMB where there was no growth inhibition. In fact, a slight stimulation of growth (negative values) is observed, as is usual for very low antibiotic treatments. At the higher EMB concentrations (0.25-1.0µg/ml), the impact of entrapment in Pheroid™ is much less or negligible. Penetration into *M.tb.* by Pheroid™-entrapped EMB may reach saturation at the higher concentrations - as the concentrations become lower Pheroid™-entrapped EMB is transported into the bacteria in a concentration-dependent fashion and this effect is most prominent in the concentration range relevant to therapeutic efficacy.

The RMP resistant strains used displayed increased sensitivity when RMP was delivered to the bacilli via Pheroid™. The application of Pheroid™/RMP combinations to the RMP resistant strain TV79 resulted in an inhibition of the same order as was observed with the RMP resistant patient isolates – growth was inhibited by Pheroid™ entrapped RMP at a concentration of 0.075µg /ml (Figure 5.20 and Table 5.5).

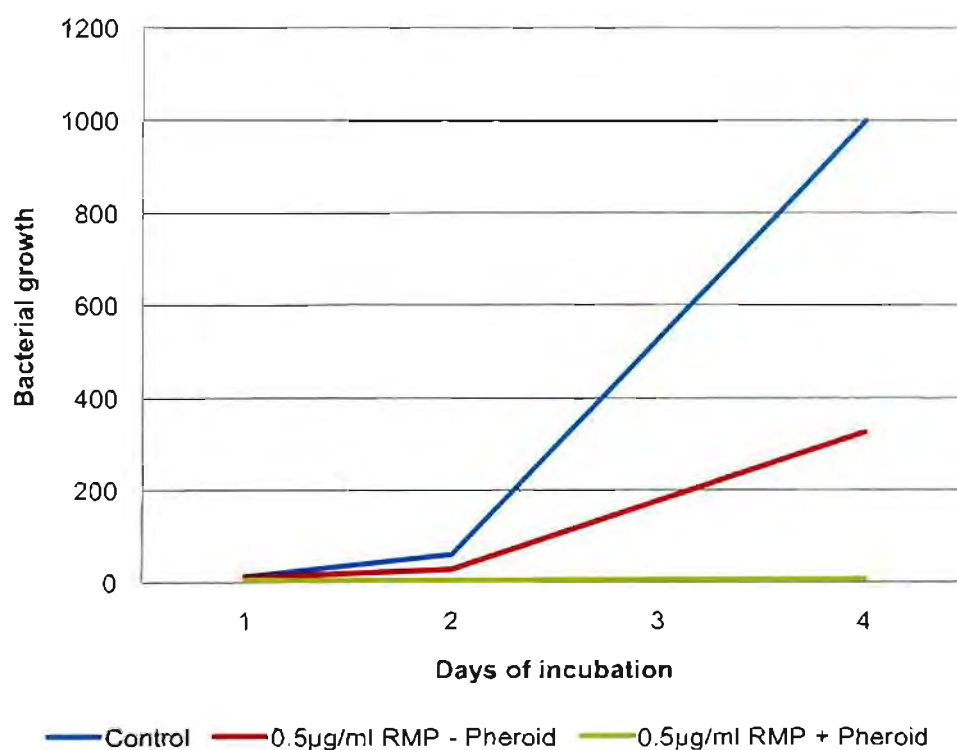


Figure 5.20: Growth response of *M.Tb.* strain TV79 treated with 0.5 µg/ml RMP and RMP entrapped in Pheroid™ at the same concentration. This strain is resistant to INH, streptomycin, and rifampicin.

Table 5.5: Comparative growth responses of MDR *M.tb.* strain TV79 after treatment with RMP in the absence and presence of Pheroid™

	Control	0.5µg/ml RMP		1µg/ml		2µg/ml	
		- Pheroid	+ Pheroid	- Pheroid	+ Pheroid	- Pheroid	+ Pheroid
Day1	13	11	5	10	3	9	4
Day 2	60	28	4	17	1	12	0
Day 4	999	325	7	132	1	56	0

These results show that RMP sensitivity of an MDR *M.tb.* strain cultured from a patient isolate is increased with complete inhibition of growth when the drug is delivered to the bacilli via Pheroid™.

All BCG strains are resistant to pyrazinamide (Morbidity and Mortality Weekly Report, 1996). The effect of entrapment of this antibiotic in Pheroid™ and its delivery in that form to BCG was investigated. In this investigation, performed mainly by confocal laser scanning microscopy (CLSM), use was made of a live/dead fluorescent stain known as *BacLight* (see above). General viability of the BCG's was determined by the observed green/red ratio of the bacteria. The general viability of the BCG's before the addition of antibiotics was between 85 – 95% and the viability after a two-hour incubation of the applicable dosage of free PZA was 68 – 72%. Figure 5.21 shows the effect of 0.25µg/ml of free and Pheroid™-entrapped PZA. The bacilli were mostly labelled red, and were therefore dead, after treatment with Pheroid™-entrapped PZA. No granuloma-type clumps or single bacteria were observed. Such clumps and single bacteria, all coloured green, were seen only in the samples treated with the same quantity of PZA in medium alone. The results indicate that bacterial resistance to PZA may at least partially be overcome by entrapment of the antibiotic in Pheroid™.

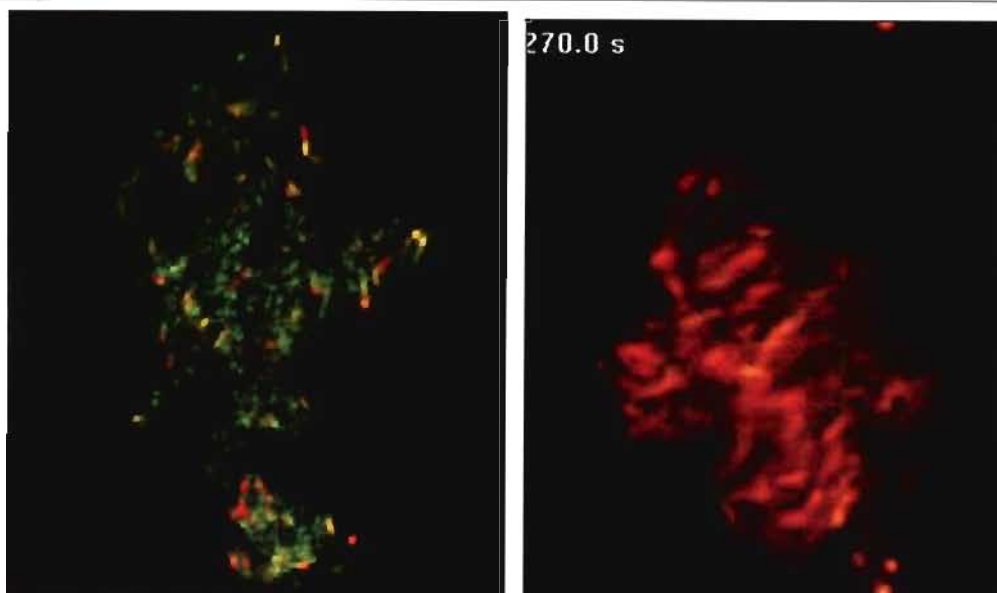


Figure 5.21: Micrographs of the growth response of BCG after treatment with PZA in the absence (left) and presence (right) of Pheroid™. BCG was pre-labelled with live/dead BacLight viability stain and observed by CLSM to determine the effect of the PZA challenge with and without Pheroid™ on bacterial viability. Incubation with free PZA resulted in the appearance of single live bacteria with a few granuloma-type clumps, which gradually secrete single live bacteria. The single live bacteria were mobile. Entrapment of PZA in the Pheroid™ vesicles led to a 65-75% decrease in BCG viability within a two-hour incubation. No moving BCG was observed (results not shown).

5.3.3.5 BCG-macrophage infection studies

The entrapment of INH, EMB, PZA and RMP in the Pheroid™ led to inhibition of bacterial growth of isolates from patients infected with both drug-sensitive and multidrug resistant strains. To determine whether entrapment of an antibiotic by Pheroid™ vesicles can be used to overcome resistance of intracellular bacteria, BCG growth in human macrophages in culture after a challenge with PZA with and without Pheroid™ was investigated. The human THP1 macrophage cell line (ATCC reference TIB-202™) was cultured in RPMI 1640 with L-glutamine cell culture medium. Foetal Bovine Serum and phosphate buffered solution (Gibco BRL) were added according to general cell culture methods. To optimize growth conditions, the impact of a number of parameters on the proliferation of THP1 at various Pheroid™ concentrations was pre-determined. Methyl paraben was used as positive control for growth inhibition in the Pheroid™ formulations. Note that in these positive controls the paraben were added to the Pheroid™ before it was diluted to a final concentration of 0.5%. The paraben was therefore similarly diluted. At the same time, the optimal dilution of Pheroid™ for the infection study was determined (figure 5.22).

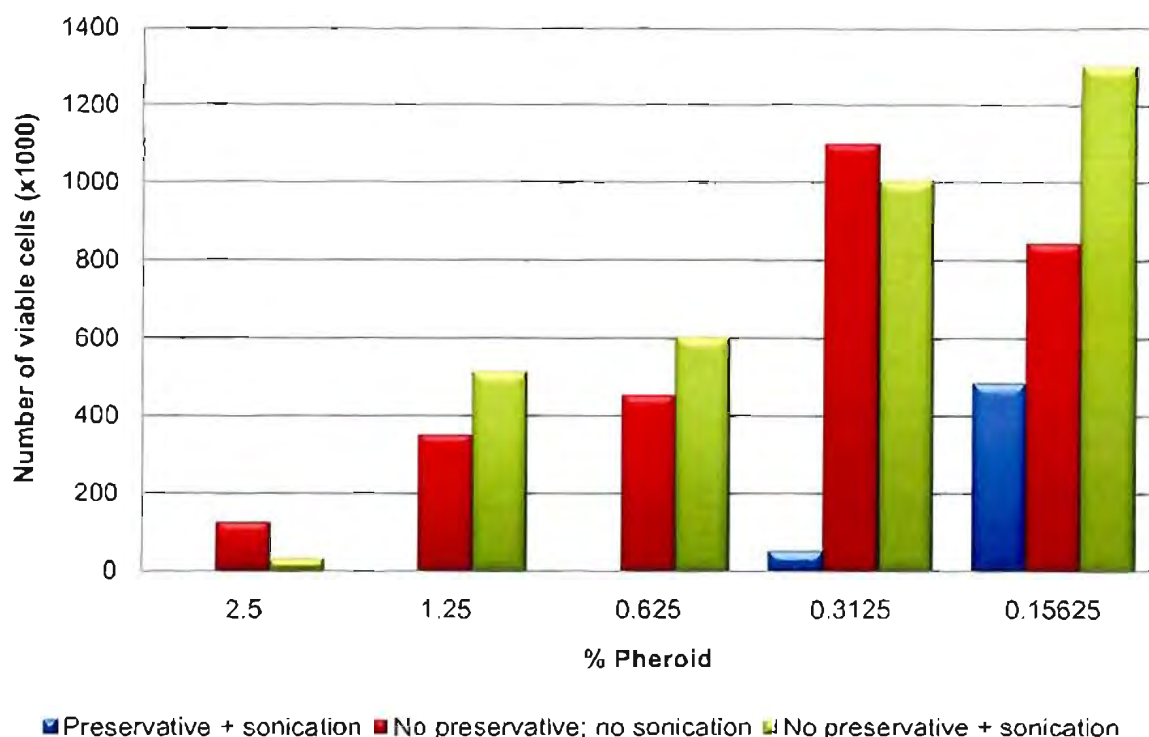


Figure 5.22: The growth of THP1 viable cells after incubation of the cells for 4 days in the presence of various dilutions of Pheroid, as reflected on the X-axis by the % Pheroid. The preservatives methyl paraben at a concentration of 0.5% caused severe growth inhibition at normal concentrations (blue bars). In the absence of preservatives the doubling time at Pheroid™ dilutions of 1: 640 (0.15625%) compare well with normal growth (red bars). Sonication of the Pheroid™ dilutions increased growth further (green bars).

High concentrations of Pheroid™ in cultures lead to the formation of an oily layer on the surface of the culture media. This layer probably prevents proper gas exchange during culturing. Sonication of the dilutions is thought to inhibit the oily layer formation. THP1 cultured at a Pheroid™ dilution of 1:640 (0.156% Pheroid™) in the absence of preservatives showed normal growth patterns. The dilution of the Pheroid™ in the drug treatment was 1:666 (0.15%) Pheroid™. The Pheroid™ itself is therefore not expected to influence cell or bacterial growth.

THP1 macrophage cells were infected with pre-treated labelled BCG bacteria and then challenged with drug treatments. Treatment consisted of a concentration of 0.075 µg/ml of free and Pheroid™ entrapped PZA. As illustrated in the CLSM micrograph figure 5.18, the viability of BCG's after infection in macrophages reflects that the intracellular bacilli in the macrophages treated with Pheroid™ containing PZA is effectively killed by the antibiotic, even though the bacteria are generally recognised to be resistant to the antibiotic used.

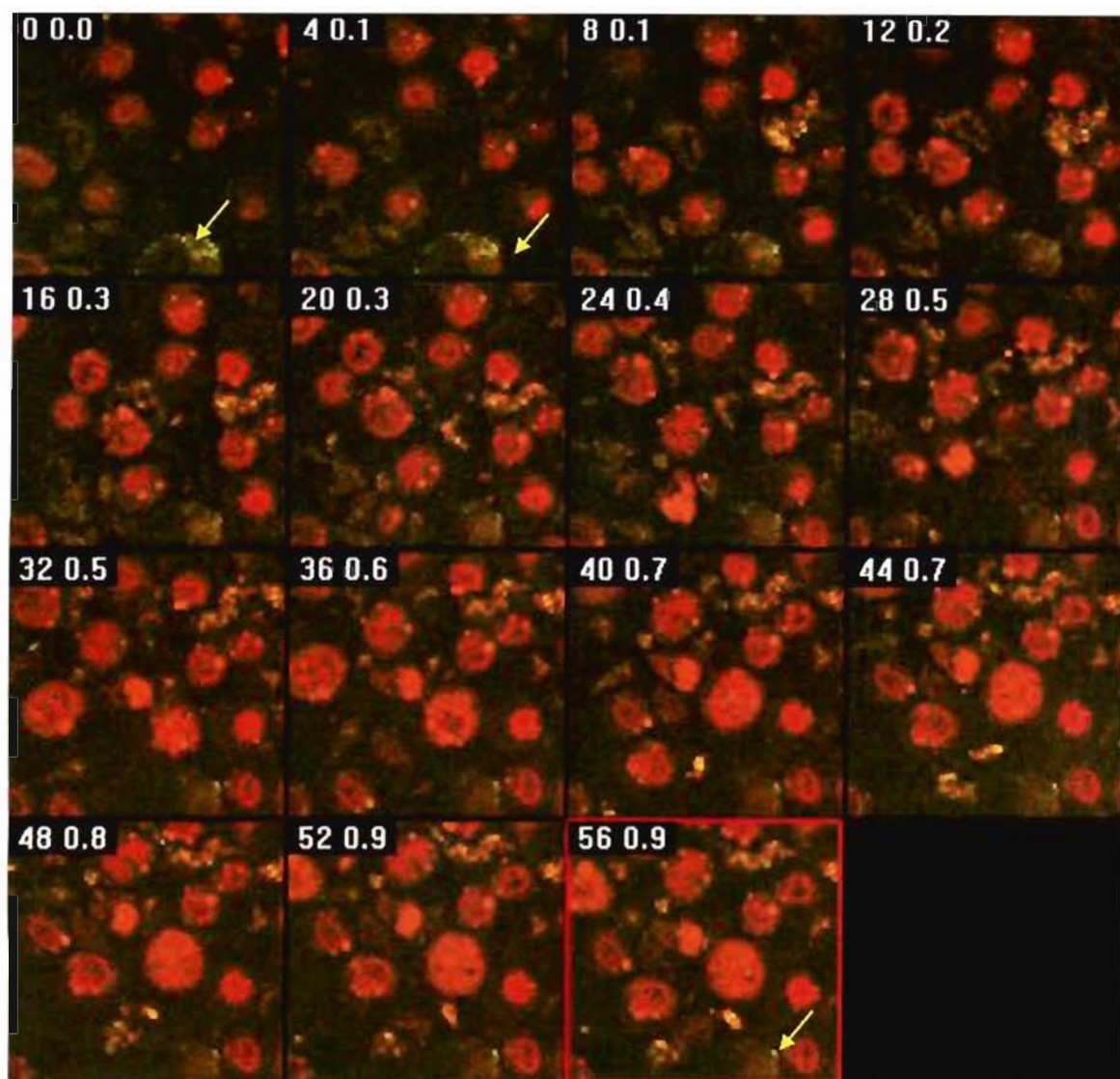


Figure 5.23: Confocal micrographs of one of the infected cell preparations were captured every 4 minutes in a time series after the initial 30 minute incubation and are shown as individual micrographs in the grid above with the white legend in each micrograph reflecting the time in minutes (first two figures in each legend) and seconds (last two figures in each legend). This time series shows the response over time of intracellular BCG infecting THP1 macrophages after treatment with 0.075 μ g/ml Pheroid-entrapped PZA. BCG bacilli were pre-stained with the BAClight viability stain according to manufacturer's instructions. Macrophages were infected, incubated for half an hour with treatment and a time series was then captured on a PCM2000 confocal system, using excitation by argon and He/Ne lasers at 488nm and 543nm. The individual bacilli are visible as indicated by the yellow arrow. The greenish bacilli are still alive and quite a few are present in the first micrograph. With time the number of green bacilli inside the macrophages decreases while the number of red bacilli increases. The nuclei of the macrophages are labelled the with a cell permeant nuclear stain to allow visualization.

5.3.4 Development and investigation of a Pheroid™-based tuberculosis treatment

5.3.4.1 Development and manufacture of a non-aqueous pro-Pheroid™ dosage form for tuberculosis treatment

5.3.4.1.1 Dosage determination for clinical studies

The pro-Pheroid™ system unlocks the potential of Pheroid™ technology for oral dosage form development. The system is based upon the intrinsic property of hydrated membrane lipids to form vesicles and/or other lipid aggregates on dilution with a water phase. Manipulation of the basic Pheroid™ formulation led to the development of a pro-delivery system (see Chapter 3). This technology is especially important in the case of the so-called unstable or labile drugs, such as rifampicin.

The formulatory practicalities of the sheer amount of anti-tuberculosis active ingredients within a daily dose for an adult of normal weight dictated to a large extent the dosage form development. The DOTS prescribed daily dosage of the four actives at the time this study started is set out in Table 5.6. The prescribed daily dosage has now been increased to 750mg for RMP and some slight increase in the dosage of INH is recommended. For the purposes of this study however, the dosages that will be regarded as that of the reference treatment regime is the dosages listed in Table 5.6.

Table 5.6: Daily and weekly dosage of tuberculosis drugs									
RHZE dosage in mg/day					RHZE dosage in mg/week				
RMP	INH	EMB	PZA	Total	RMP	INH	EMB	PZA	Total
600	300	1500	1000	3400	3000	1500	7500	5000	17000

As can be seen from the above table, 3400mg has to be formulated into a daily dosage. Since the pro-Pheroid™ is a liquid dosage form, and the taste of both the pro-Pheroid and the 4 drugs is unacceptable for public use, the dosage forms were limited to capsules. The dry weight dosage of the 4 drugs, as well as the required volume of pro-Pheroid™ had to be fitted into capsules. A number of gelatine-based capsule types, such as liquid capsules (Li-caps® from Capsugel), vegetable material based capsules (Vegicaps® from Capsugel) and soft gelatine capsules (Pharma Natura) were considered for this study. In the initial single case studies, Li-caps®, a gift from Capsugel (Belgium), were used. No plant or manufacturer in South Africa is geared to the filling and sealing of these capsules with the specified drugs. Capsules thus had

to be filled and sealed manually. This procedure was preferred at this early part of the study, since the dosage of the drugs entrapped in the pro-Pheroid™ needed to be determined. For ethical and safety reasons, the preliminary dosage determination was performed in a single volunteer. For this purpose, the delivery of the prescribed anti-TB drugs by way of the pro-Pheroid™ delivery system to 1 healthy volunteer in an open crossover study design was investigated. The volunteer was a Caucasian female in good clinical health, i.e. blood chemistry, full blood count and liver function tests of the subject were within the normal range, who used no concomitant medication. The single subject study was performed under Section 21 approval from the Medicines Control Council and under the direct supervision of the Medical Director and the responsible pharmacist of MeyerZall Laboratories, Dr. J.C. Venter and A Kruger respectively. The comparative bioavailability of RMP administered in the form of Rifafour-e200 and the same four drugs entrapped in pro-Pheroid™ at the same concentration was determined. RMP was selected as it seems to be the most problematic of the 4 APIs used. Pharmacokinetic parameters of this drug delivered by the test formulation at full and at reduced doses were compared to those achieved when the same drugs were administered in Rifafour-e200, the reference product of established quality and in the standard treatment doses at that time. The volunteer was monitored daily during the study. The investigation was performed in preparation for a Phase 1 clinical trial.

For the phase 1 study, the treatment was based on the *South African Standard Treatment Guidelines and Essential Drugs List (2002)*. The drug treatments were supplied by MeyerZall Laboratories (Pty) Ltd: The trial material was formulated and manufactured by the author as described above, subject to quality control. After entrapment of the relevant drugs in the pro-Pheroid™, the formulation was encapsulated in soft gelatine capsules. Manufacturing complied with Good Laboratory and Manufacturing Practice (GLP and GMP) Guidelines. All drug treatments were orally administered. The study was conducted over two periods of 4 days each, interrupted by a two-week washout period. Typically, 5 Rifafour-e200 tablets were taken daily for four consecutive days, followed by sample collection on day 4 (profile day). After a washout period of 2 weeks, the pro-Pheroid™ entrapped drugs were taken in the same manner. Dosing was followed by blood sample collection and plasma preparation in order to determine the comparative pharmacodynamic profiles of RMP and INH. The study was single blind for laboratory procedures.

During day 4 (blood profile day) of each period, blood samples (10ml each) were collected for plasma analysis in heparinized tubes at the following intervals after administration of the drugs: 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 6 hours, 9 hours. Blood was also collected for liver function determinations, lipid profile analysis and full blood cell counts and chemistry. Plasma was extracted by centrifugation within 15 minutes of collection and stored at - 80°C. An

accredited pathology laboratory (PathCare) assayed full blood count and chemistry, liver functions and lipid profiles. Blood samples were collected and handled in accordance with Good Clinical Procedures (GCP) Guidelines. Plasma level assays of the actives were performed in the conventional manner and as described (Smith *et al.*, 1999). The concentrations of RMP and INH and their active metabolites were determined by high performance liquid chromatography (HPLC) after their simultaneous extraction from plasma by the Dept of Pharmacology, University of Cape Town, South Africa.

Both the RMP and INH plasma levels obtained after administration of equal dosages are higher for the test than reference drugs and are shown in figures 5.24 and 5.25 and also in table 5.7. Evaluations of the plasma levels obtained for the pro-Pheroid drug formulations at the different doses indicated that plasma levels corresponding to that of Rifafour-e200 were obtained when the dosage of the RMP was less than 60% but more than 40% of that of Rifafour-e200.

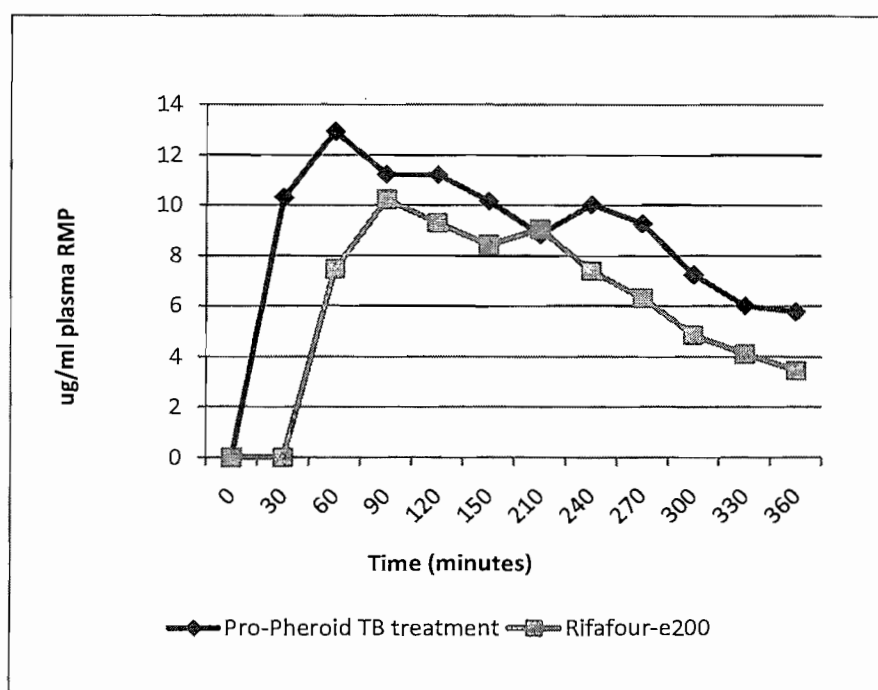


Figure 5.24: The concentration/time plasma curves of RMP in the reference and test pro-PheroidTM formulation at equal dosages. The comparative bioavailability was determined from these drug plasma profiles of the volunteer, who took the respective treatments for 4 consecutive days, after which plasma was collected at the times indicated. The treatments were separated by a wash out period of 2 weeks. The volunteer took the medication in the morning before a meal with 200ml of tea, as food has been shown to influence absorption. Meals were standardized and supplied a couple of hours after first administration of the medication.

Time(min)	Pro-Pheroid™ formulation(μ g RMP/ml plasma)	Rifafour- e200 (μ gRMP/ml plasma)	Time (minutes)	Pro-Pheroid™ formulation(μ g INH/ml plasma)	Rifafour- e200 (μ gINH/ml plasma)
0	0	0	0	0	0
30	10.31	0	30	2.29	1.9
60	12.93	7.46	60	3.81	2.3
90	11.23	10.23			
120	11.22	9.3	120	3.63	2.45
150	10.16	8.43	180	3.08	1.7
210	8.82	9.03			
240	10.03	7.39	240	1.97	2.5
370	9.27	6.32			
300	7.22	4.84	300	1.61	0
330	6	4.1			
360	5.77	3.45	360	1.46	0

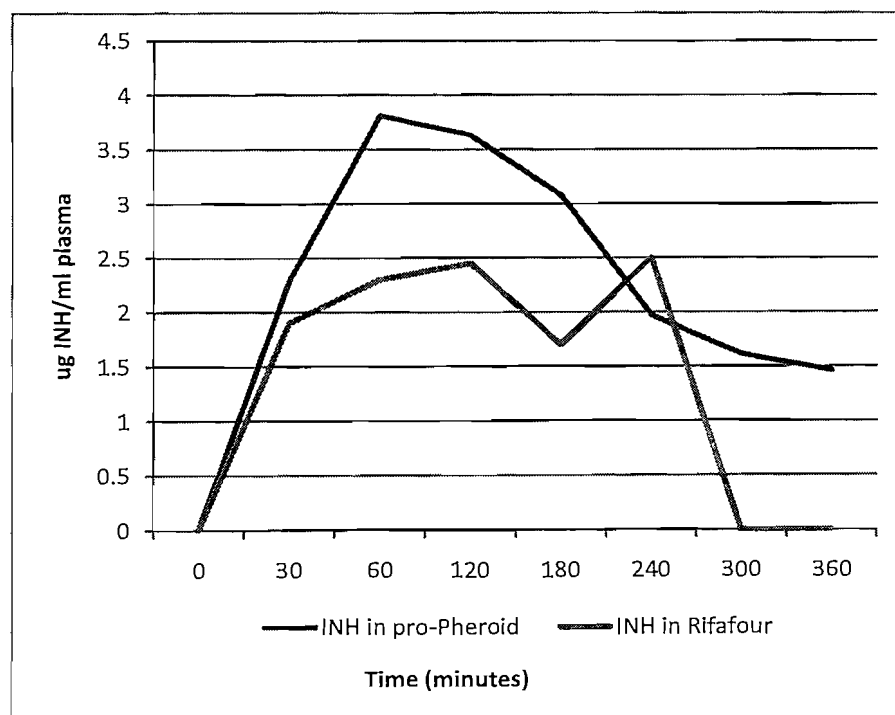


Figure 5.25: The concentration/time plasma curves of INH in the reference and test pro-Pheroid™ formulation at equal dosages. The comparative bioavailability was determined from these drug plasma profiles of the volunteer, who took the respective treatments for 4

consecutive days as described, after which plasma was collected at the times indicated. The treatments were separated by a wash out period of 2 weeks.

For the phase 1 trial (see below), a 60% dosage was decided on and Table 5.9 shows the comparable amount of the four APIs.

Table 5.9: Dosage schedule of the 4 APIs for test and reference products								
Dosage	RHZE mg/day				RHZE mg/week			
	RMP	INH	EMB	PZA	RMP	INH	EMB	PZA
Reference product	600	300	1500	1000	3000	1500	7500	5000
Pro-Pheroid™ Test product	400	200	1000	660	2000	1000	5000	3300

The softgel capsules come in various sizes. Since the amount of PZA plus pro-Pheroid™ amounted to more than 1000mg per day, two capsules of 750mg each (size 00) were dedicated to that drug. The other 3 drugs in addition to the pro-Pheroid™ amounted to more or less 2500mg, which was divided into 3 capsules of 1050mg each (size 000). Capsule are generally filled to around 75% of their capacity. The formulations were named Pyrifitol, using an anagram of the names of the four drugs. The treatment containing the PZA with pro-Pheroid™ was named Pyrifitol P, while the combination of RMP, INH, EMB and pro-Pheroid™ was designated Pyrifitol C. Manual filling of the capsules were not acceptable and the pro-Pheroid™-based formulations were then encapsulated in soft gel capsules by a third party manufacturer.

The manufacturing process for producing these two formulations is described below, with reference to the manufacture of both Pyrifitol P and Pyrifitol C.

5.3.4.1.2 **Manufacture of Pyrifitol P and Pyrifitol C**

Pyrifitol P and C were produced according to the following protocol as initially described in Chapter 3. The production of each of the formulations is described below.

Pyrifitol P was made up according to the following protocol:

- Step 1:** Weigh off the PZA (5,00Kg) and reduce the particle size to less than 140µm by grinding. Particle sizes were determined by CLSM.
- Step 2:** Weigh off and add together the Poloxyl hydrogenated castor oil (1,15Kg), Vitamin F ethyl ester (2,35Kg), dl-α-tocopherol (150,0g) and polyethylene glycol 400 (1,295Kg) into mixing vessel 2 and heat to approximately 40°C until all the oil has melted.

- Step 3:** Sparge the nitrous oxide through the oil mixture for 12 hours at 2 Kpa in the stainless steel pressure vessel in the manner described.
- Step 4:** Transfer the gassed mixture to mixing vessel 1 and heat to approximately 70°C.
- Step 5:** Weigh off and add the preservative methyl paraben (50,00g) and the anti-oxidant butylated hydroxytoluene (5,00g) to mixing vessel 1, while continuously mixing to ensure that each solid is dissolved before adding the next while still maintaining the temperature at 70°C.
- Step 6:** Remove from the heat and allow to cool down to approximately 40°C.
- Step 7:** Add the Pyrazinamide (5,00 Kg) stepwise while continuously mixing.
- Step 8:** Sparge nitrous oxide through the mixture obtained in step 7 at 2kPa for 48 hours.

Pyrrifol C was made up according to the following protocol:

- Step 1:** Weigh off the 1,467Kg RMP, 733,00g INH and 2,20Kg EMB and reduce the particle size to less than 140µm. Ensure that the raw material is protected from exposure to light at all times.
- Step 2:** Weigh off and add together the 1,33Kg Poloxyl hydrogenated castor oil, 3,11Kg Vitamin F ethyl ester and 1,30Kg polyethylene glycol 400 into mixing vessel 2 and heat to approximately 40°C until all the oil has melted.
- Step 3:** Sparge the nitrous oxide through the oil mixture for 24 hours at 2 kPa in the stainless steel pressure vessel in the manner as described above.
- Step 4:** Transfer to mixing vessel 1 and continue to mix.
- Step 5:** Weigh off and add 50,00g methyl paraben, 10,00g ascorbyl palmitate and the 5,00g butylated hydroxytoluene to mixing vessel 1, while continuously mixing to ensure that each solid is dissolved before adding the next.
- Step 6:** Remove from the heat and allow to cool down to approximately 40°C.
- Step 7:** Check the pH and add potassium hydroxide while continuously mixing until the pH reads 7.
- Step 8:** Add the 1,467Kg RMP, 733,00g INH and 2,20Kg EMB, respectively, each stepwise allowing mixing after each addition.
- Step 9:** Sparge with nitrous oxide at 2 kPa for 48 hours.

The Pyrrifol P and C formulations so obtained were encapsulated in soft gelatine capsules by a third party manufacturer (Pharma Natura) in the manner well known in the pharmaceutical trade. The manufactured oral capsules were used in analytical, stability and bioavailability studies as described below. These capsules were then supplied as trial medication and were labelled in accordance with Good Laboratory and Manufacturing Practice (GLP and GMP) Guidelines for the labelling of study medication as set out in Table 8 below. The Pyrrifol P (green) and Pyrrifol C capsules are shown in figure 5.26.



Figure 5.26: Pyrifitol P (green) and Pyrifitol C (red) soft gelatine capsules containing the four tuberculosis drugs. The daily dosage for an adult was 2 Pyrifitol P and 3 Pyrifitol C capsules.

5.3.4.2 Analysis of manufactured capsules

5.3.4.2.1 Capsule disintegration, vesicle formation and entrapment efficiency

The rate of release from capsules was measured by the disintegration method described in the United States Pharmacopoeia of 2000. Briefly, one capsule was dissolved in 9ml of 0.1N HCl and shaken for 10 minutes, after which the solution was subjected to CLSM analysis. This process was repeated 9 times. The time to disintegration could be visually determined for capsules containing RMP, since the appearance of the brick-red colour is typical of RMP. The time it took for a Pyrifitol C capsule to disintegrate was on average around 7.5 minutes. Dissolution studies showed repeated disintegration of the capsules after 11 minutes (\pm 30 seconds) in acid conditions mimicking those of the stomach. CLSM showed the formation of delivery vesicles after exposure of the pro-PheroidTM anti-TB capsules to the acidic fluids (figure 5.27).

CLSM was used to investigate the entrapment efficiency of the pro-PheroidTM formulations in Pyrifitol P and C. Entrapment efficiencies approaching 100% is aimed for. The formulation characteristics, presence of PheroidTM as well as interaction of pharmacological active ingredients were confirmed by CLSM technology.

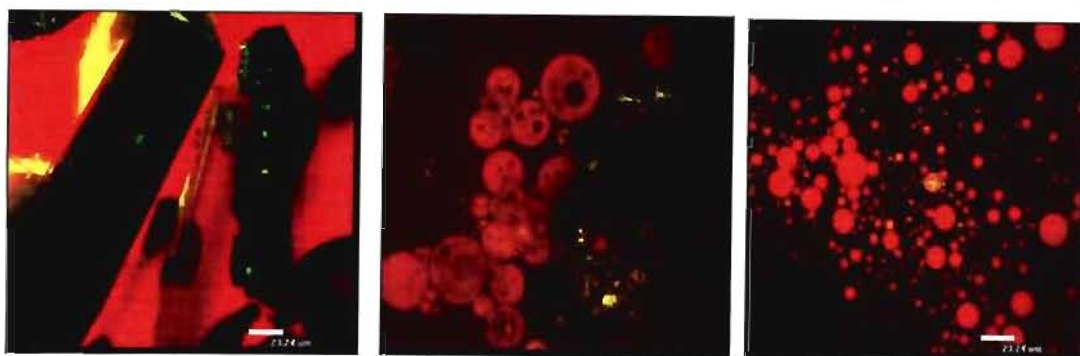


Figure 5.27: A confocal micrograph series of the process of entrapment. On the left, the dark PZA crystals with green autofluorescence are visible on a background of Nile red labelled oil phase of the pro-Pheroid™. Mixing with 0.1N HCl to mimic the gastric environment led to the disappearance of the crystals and the formation of sponge-like vesicles with some green material entrapped and some still outside of the vesicle 10 minutes after mixing (micrograph in the middle). After 30 minutes, no un-entrapped green material is visible and the vesicle size is much smaller (right).

5.3.4.2.2 Stability analysis of the active metabolites

The concentrations of RMP, INH, EMB and PZA and their active metabolites were determined by high performance liquid chromatography (HPLC) after their simultaneous extraction from the formulations stored at the various conditions for stability analysis according to generally accepted procedures in the industry.

The materials used included INH, RMP, PZA and pyrazinoic acid; HPLC–grade acetonitrile, methanol and trifluoroacetic acid (TFA) and C18 Bondelut extraction columns, 200mg, 3 ml 40 microns. The concentrations of RMP were determined using a mobile phase of 80% acetonitrile in 0.1% trifluoroacetic acid. A reversed phase C8 analytical column (Spherisorb, 250 X 4.6 mm ID, 5μm) linked to a C8 precolumn, with flow rate at 2.0 ml per minute and detection at 270 nm was used. For the determination of INH and PZA, the mobile phase was 3% acetonitrile in 0.06% TFA. A reversed phase C8 analytical column (Spherisorb, 150 x 4.6 mm ID, 5μm) linked to a C8 pre-column with flow rate at 1.5 ml per minute and detection at 254 nm was used. A stock standard solution of RMP (0.5mg/ml), PZA (0.5mg/ml), INH (0.5mg/ml) and pyrazinoic acid (0.5mg/ml) was prepared in methanol. Relative retention times were established by spiking and comparing peak area ratio of RMP. All stock solutions are kept at 4°C and protected from light.

Acceptance criteria: A validated analytical method meets the following criteria and these criteria had to be complied with in the execution of the stability analysis:

- Precision and accuracy: The between batch CVs for low, medium and high concentrations should be <15%, and 20% for the LOQ QC.
- Sensitivity: The lowest standard should be accepted as the LOQ if the %CV is <20%.
- Specificity: The responses of interfering peaks at the retention time of the analyte should be less than 20% of the response of an LOQ standard.
- Stability: Stock solution stability should meet the criteria specified in the SOP.

As for the specificity, analyses of blank samples of the appropriate biological matrix were tested for endogenous interferences in the reference standard region for RMP, INH and PZA. Calibration graphs (peak areas vs. concentration) were constructed for RMP and INH in the range 0.1-20µg/ml and for PZA in the range 0.1-60µg/ml. INH, RMP, PZA and pyrazinoic acid analysis were performed in triplicate.

Intra- and inter-assay coefficients of variation were determined. Five replicate samples of four concentrations were run through the procedure with exactly controlled volumes, as described for the extraction of the samples. To verify recovery/quality control, precision and accuracy, the peak areas obtained for the extracted samples were compared to those of fresh standards of the analytes in mobile phase with respect to the volumes handled during extraction. C18 Bondelut extraction cartridges were washed sequentially with 2 x 2 ml of methanol, 2 x 2 ml of water and 2ml of 0.05 M potassium phosphate, pH 4.5 (phosphate buffer) prior to application of the sample to the columns. The columns were washed with 1 ml of phosphate buffer to be discarded, and the drugs eluted with 0.5 ml of acetonitrile, followed by 0.5 ml of methanol after which these elutes were pooled. 60µl of the pooled eluates were injected immediately onto the HPLC column to assay for RMP. 0.5ml of the combined eluates were dried by vacuum centrifugation and taken up in 0.5ml of 3% acetonitrile in 0.06% TFA. 60µl of this were injected onto the autosampler of the HPLC to assay for INH and PZA, which were detected together on the same column.

5.4 Phase 1 bioavailability and safety clinical studies

A phase 1 clinical trial was undertaken. The title of the trial was: "A cross-over bioavailability study of drugs delivered by way of a delivery system in the treatment of tuberculosis used in a study with healthy volunteers". I myself was the principle scientific investigator in this trial with the following specific responsibilities:

- (i) Compilation, submission and defence of the trial protocol to and at the Medicines Control Council and South African Medical Association Research Ethical Committee for approval;
- (ii) Assurance of financial ability of trial sponsor and payment of participating parties;

- (iii) Design and manufacturing of bulk raw material for encapsulation in the capsules;
- (iv) Quality assurance of the trial capsules in conjunction with the Quality Assurance Manager (see vii below);
- (v) Identification and execution of a company able to manufacture the trial capsules;
- (vi) Identification, arrangement and establishment of trial and subject screening sites;
- (vii) Identification and canvassing of key personnel for the trial, including a principle clinical investigator, a responsible pharmacist, a quality assurance manager; internal and external trial monitors; nursing staff, a team of scientists/analysts to process the samples, manufacturing assistants and a lawyer required for contractual aspects and trial insurance.
- (viii) Identification and contracting of an applicable laboratory for the objective (blind) analysis and interpretation the data;
- (ix) Identification and contracting of a pathology laboratory for the analysis of the safety of the trial medication, including full blood chemistry, liver profiles, lipid profiles.
- (x) Design and compilation of trial documentation, including the package insert, the Subject Information Pamphlet, the Volunteer's Written Consent, the clinical trial procedures (CTPs), the coding of the subjects, the Case Report Form and Safety Procedures;
- (xi) Canvassing of the volunteers;
- (xii) Screening of the volunteers' in conjunction with the principle clinical investigator and nursing staff;
- (xiii) Training of scientists/analysts and nursing staff in trial specific procedures, such as fast and efficient plasma preparation from collected blood samples, and safety procedures;
- (xiv) Timeous transfer of samples to the analytical and pathology laboratory;
- (xv) The compilation of data from the various laboratories, monitors, centres, principle clinical investigator and Case Report Forms;
- (xvi) Compilation of trial report and submission to the Medicines Control Council.

The sponsor of the study was the company MeyerZall Laboratories (Pty) Ltd, from Sedgefield, South Africa and the protocol number was MZL 04/2000. For the trial medication, Rifafour-e200 was used as reference treatment or comparator and Pyrifitol P and Pyrifitol C as test treatment. Dr Frank Muller and Prof Joan Botes, both from George, South Africa were the principal clinical Investigator and external study monitor respectively. The trial site was the Meyer Zall Institute; Practice registration number: 9020500m Sedgefield, South Africa. Nashia Cain was the responsible pharmacist and Julia Gooden acted as internal monitor. The protocol was submitted to and approved by the South African Medicines Council and the South African

Medical Association Research Ethical Committee. The protocol described the dosage level, the specific drug treatments, the time period of the study, the parameters investigated and the combinations of drugs used. The schedule of activities during the trial is shown in table 10.

Table 5.10: Schedule of activities of Phase 1 trial								
	Screen	Daily	Day 0	Day 4	Washout	Day 0	Day 4	Exit
Informed consent	√							
Admission criteria	√							
Demography	√							
Possible tuberculosis history	√							
Prior and concomitant medication	√	√	√	√	√	√	√	
Blood profile	√			√			√	
Subject/investigator global assessment	√			√		√	√	if withdrawn before day 4
Medication dosing record		√	√	√	√	√	√	if withdrawn before day 4
Adverse events		√	√	√	√	√	√	√
Termination record								√
Investigators statement (CRF)								√

5.4.1 Objectives and endpoints of the trial

The primary objectives of this trial was to determine the bioavailability and safety of generally used anti tuberculosis treatment agents, i.e. RMP, INH, EMB and PZA, each packaged in the pro-Pheroid™ drug delivery system in the form of the capsules produced in the manner described in Chapter 3 and 5.3.4.1.2 above. A significant change from baseline of the following pharmacokinetic parameters was determined:

- (i) peak plasma concentration (C_{max}),
- (ii) the time needed to reach this concentration (T_{max}),
- (iii) exposure (the area under the plasma curve (AUC 0-9 hours), and
- (iv) coverage.

The pharmacokinetic results were compared with those of reference formulations. Bioavailability is understood to be the rate and extent to which the active substance or therapeutic moiety is absorbed and delivered from a pharmaceutical form

- into the general circulation and
- becomes available at the site of action.

As in other bioavailability studies, the kinetics of the therapeutic moiety in the general circulation was monitored in this study. The secondary objectives were:

- (i) to determine whether bioequivalence exists for drugs packaged in the delivery system by comparison to reference agents;
- (ii) to determine whether there are changes in the status of side effects caused by the actives;
- (iii) to determine the relative safety levels of the comparative products;
- (iv) to determine possible partitioning of the pro-PheroidTM delivered drugs to cells and possible cytotoxicity as a result; and
- (v) to note the possible advantages to the volunteer's well-being (i.e. malaise, bone ache, nausea etc) when using the pro-PheroidTM delivery system for the administration of anti-tuberculosis drugs.

The primary endpoint of the study was to determine the changes in bioavailability as a result of the delivery of the drugs by the delivery system when compared with that of the reference anti-tuberculosis drugs, as reflected by changes in the blood drug profiles between day 0 and day 4. The secondary endpoints included a change in the status of side effects caused by the actives, changes in patient global assessment, i.e. significant change from baseline pharmacokinetic parameters, such as peak plasma levels (C_{max}), clearance, time to peak plasma levels (T_{max}), and the subjects' acceptance of the drug delivery system and projected compliance to the treatment regime.

5.4.2 Study design

The study design was a crossover bioavailability design for the specified tuberculosis drugs and was single blind for laboratory procedures. As for the volunteer studies, the study was conducted over two periods of 4 days each, interrupted by a two week wash out period. Sixteen volunteers participated in the study. These volunteers were selected from a group of 39 applicants, based on pre-determined inclusion and exclusion criteria, during a screening process. The screen assessment evaluated an individual's suitability to participate. The screening process included blood chemistry, chest X-rays, liver functions and lipid profiles, besides the conventional clinical evaluation. All subjects had to fulfilled all screen requirements before commencing with study treatment.

5.4.3 Inclusion and exclusion criteria

Subjects had to fulfil all of the following criteria to be eligible for the study:

- (i) Subjects had to volunteer for participation in the trial.
- (ii) Males had to be 18 to 55 years of age and females had to be the same age with proven sterilization or contraception.

- (iii) Subjects had to be clinically diagnosed as healthy.
- (iv) Subjects' weights had to be within 25% of the target weight for age and gender and had to be over 50 Kg.
- (v) Blood chemistry, lipid profile, full blood count and liver function tests of subjects had to fall within the normal ranges.
- (vi) Volunteers had to be capable of understanding the informed consent and the clinical trial information leaflet.
- (vii) Subjects had to grant his or hers written informed consent before any invasive procedure was carried out.

Any one of the following warranted exclusion from the study: -

- (i) Gastric abnormalities that could influence the absorption of the medication, such as Crohns disease or ulcerative conditions.
- (ii) Active neoplasma.
- (iii) Metabolic problems such as diabetes mellitus, thyroid problems, porphyria.
- (iv) Any chronic lung disease.
- (v) Acute hepatitis B viral infection.
- (vi) HIV infection at the start of the study.
- (vii) Heart valve lesions or any heart defect with possible secondary heart failure.
- (viii) Liver enzymes GT, AST and ALT were not to be higher than the normal ranges.
- (ix) Any subject with proven serious adverse reaction to the prescribed treatment regime.

5.4.4 Study execution

The 16 healthy volunteers satisfying the inclusion and exclusion criteria evaluated during the screening process were randomized into two groups of 8 subjects each; group 1 and group 2. Randomization was blind for the study personnel and the randomization code was held by the Department of Pharmacology at the University of Cape Town. Group 1 received the currently prescribed treatment regime, i.e. Rifafour-e200 (Aventis) for 4 consecutive days, followed by a wash out period of 2 weeks. They then received Pyrifitol P and C containing the same drugs but at a 60% dose of the prescribed dosage, again for 4 consecutive days. Group 2 started with the 60% dose contained in Pyrifitol P and C, followed by the same scenario with Rifarour-e200 after a two week wash out period as illustrated below (see table 5.11 for more information about the study medication). Each of the daily dosages therefore consisted of either 5 tablets of Rifafour-e200 or 2 capsules of Pyrifitol P and 3 capsules of Pyrifitol C. Subjects took the medication in the morning before a meal with 200ml of water, as food has been shown to influence absorption. Meals were supplied one and a half hours after administration of the

medication. The dosage prescribed was that for subjects over 50 Kg in weight; one of the inclusion criteria determined that subjects had to weigh >50 Kg.

Table 5.11: Study medication content per dosage form, day and week			
Prescribed treatment: RHZE combination tablet			
Dosage	RHZE mg/tablet	RHZE mg/day	RHZE mg/week
> 50 Kg	120/60/300/200	600/300/1500/1000	3000/1500/7500/5000
Drugs administered by Pyrifitol P and Pyrifitol C			
Test treatment	Drug mg/capsule	Drug mg/day	Drug mg/week
Rifampicin; R	100mg	400mg	2000mg
Isoniazid; H	100mg	200mg	1000mg
Pyrazinamide; Z	250mg	1000mg	5000mg
Ethambutol; E	132mg	660mg	3300mg
Prescribed treatment: RHZE combination tablet			
Dosage	RHZE mg/tablet	RHZE mg/day	RHZE mg/week
> 50 Kg	120/60/300/200	600/300/1500/1000	3000/1500/7500/5000
Drugs administered by Pyrifitol P and Pyrifitol C			
Test treatment	Drug mg/capsule	Drug mg/day	Drug mg/week
Rifampicin; R	100mg	400mg	2000mg
Isoniazid; H	100mg	200mg	1000mg
Pyrazinamide; Z	250mg	1000mg	5000mg
Ethambutol; E	132mg	660mg	3300mg

5.4.5 Sample collection and preparation

During day 4 (blood profile day) of each period, blood samples (10ml each) were collected for plasma analysis in heparinized tubes at the following intervals after administration of the drugs: 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 6 hours, 9 hours. Blood was also collected for liver function determinations, lipid profile analysis and full blood cell counts and chemistry. Plasma was extracted by centrifugation within 15 minutes of collection and stored at - 80°C. An accredited pathology laboratory (PathCare) assayed full blood count and chemistry, liver functions and lipid profiles. Blood samples were collected and handled in accordance with Good Clinical Procedures (GCP) Guidelines.

5.4.6 Drug plasma concentration determination

The concentrations of RMP, INH and PZA and their active metabolites were determined by high performance liquid chromatography (HPLC) after their simultaneous extraction from plasma by

the Dept of Pharmacology, University of Cape Town, South Africa (13). The plasma levels of Ethambutol were determined by HPLC after a derivitization procedure but the procedure has not been finally validated and bioavailability results of EMB are not included.

5.4.7 *In vitro/in vivo* correlations and PK modelling

An aliquot of the prepared plasma from the participants of the trial were used to determine the MIC's of each of the drugs in the plasma. 400µl of volunteer plasma of each arm of the trial was infected with *M.tb.* and the growth indices of the bacterial growth determined using the BACTEC procedure. Using the relative MIC's obtained the projected efficacy of the drugs in the Pyrifitol capsules and in the comparator was calculated in an effort to predict the therapeutic efficacy of the test product in man before a phase 2 trial is initiated.

5.5 Results

Below a summarized list of general conditions and results are given:

- The study was approved by the South African Medicines Control Council and the South African Medical Association's Research Ethical Committee.
- All volunteers (8 males + 8 females) were above 50kg in weight and complied with all the inclusion and exclusion criteria.
- All treatments were orally administered.
- The wash-out period before the cross-over was 14 days.
- The commercial drug product (reference product) used for comparative bioavailability studies was Rifafour e-200 from Aventis.
- The test product is a fixed dose combination (4fdc) in soft gel capsules of the same 4 drugs as that in Rifafour-e200 but contained 60% of the dosage of the reference products. The dosage schedule is given in table 5.11.
- The test product was manufactured according to GMP and the execution of the trial according GCP and the Helsinki Guidelines. The safety of the treatments was assessed according to the ICH Helsinki Clinical Trial Guidelines.
- No serious adverse events occurred during the study. The study was monitored by both internal and external monitors.
- The complete study was performed within a registered private hospital facility. Volunteers were housed within this facility for the duration of the trial, and food intake was monitored. The subjects were assessed daily during the trial and continuously during the blood profile days.
- Sample analysis was blind. PathCare Laboratories was responsible for blood chemistry analysis, liver functions, lipid profiles and obvious blood cell pathologies. The WHO

Reference Centre for Tuberculosis Plasma Analysis at the University of Cape Town determined the blood drug profiles and monitored the adverse events.

- The study was repeated after a 3-month period for 4 of the 16 participants.

5.5.1 Pharmacokinetic parameters

Statistical analysis was performed by Dr. Helen McIlleron (Department of Clinical Pharmacology, University of Cape Town, South Africa) using the Stata™ 10.0 (StataCorp, Texas USA) or WinNonlin Professional™ software statistical/ data analysis program. The concentration-time data were used to determine the following pharmacokinetic (PK) parameters for each of the four APIs:

- Plasma peak concentration (C_{max}) in $\mu\text{g/ml}$
- Time to plasma peak concentration (T_{max}) in hours
- Area under the plasma concentration-time curve (AUC₀₋₉); 0 to 9 hr in $\mu\text{g.h/ml}$
- Area under the plasma concentration-time curve (AUC_{0-∞}); 0 - ∞ hr in $\mu\text{g.h/ml}$

An overlay of the mean concentration vs. time graphs (reference and test groups) is presented in figures 5.28 to 5.31 for the four active compounds. The area under the plasma concentration curve (AUC) is an indication of the comparative therapeutic value of an API, while bioavailability is understood to be the rate of, and extent to which the active substance or therapeutic moiety is absorbed through or penetrate biological barriers to access the general circulation in a pharmaceutical active form and become available at the site of action (Cynamon & DeStefano, 1999; Han and Amidon, 2000). Statistical differences between the test and reference groups were determined for each pharmacokinetic parameter using the unpaired t - test (parametric) (two-tailed p-value), the Pearson test and the Mann-Whitney non-parametric t-test (two-tailed p-value). The PK parameters of the reference and Pheroid™ groups were evaluated.

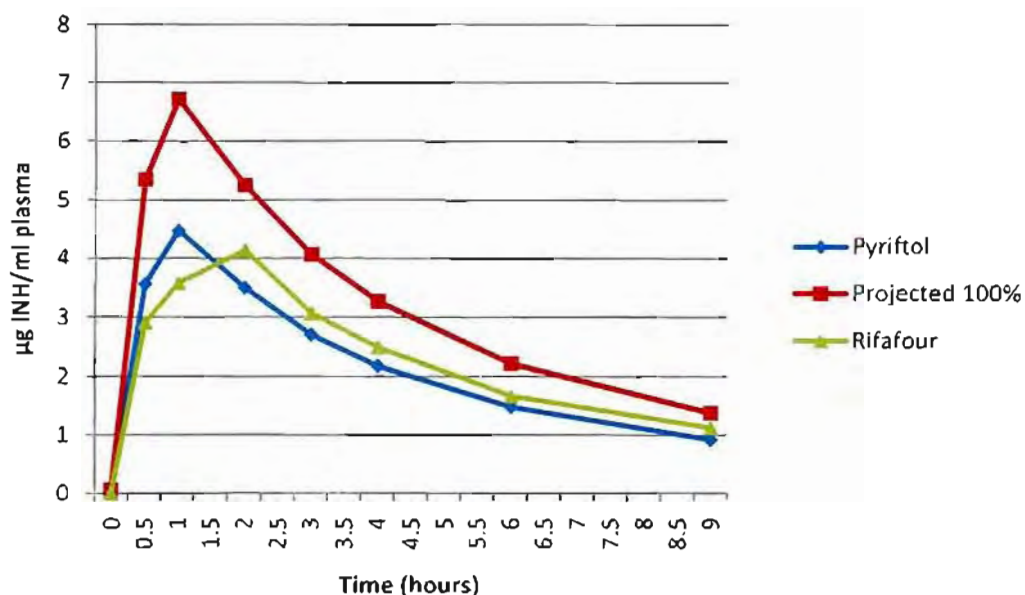


Figure 5.28: An overlay of the time versus plasma concentration of INH for the test and reference drug in a 16 subject phase 1 trial. The test product contained 60% of the dosage present in the reference product. The time/concentration curve projected for a 100% dosage of the test drug is shown as the red line.

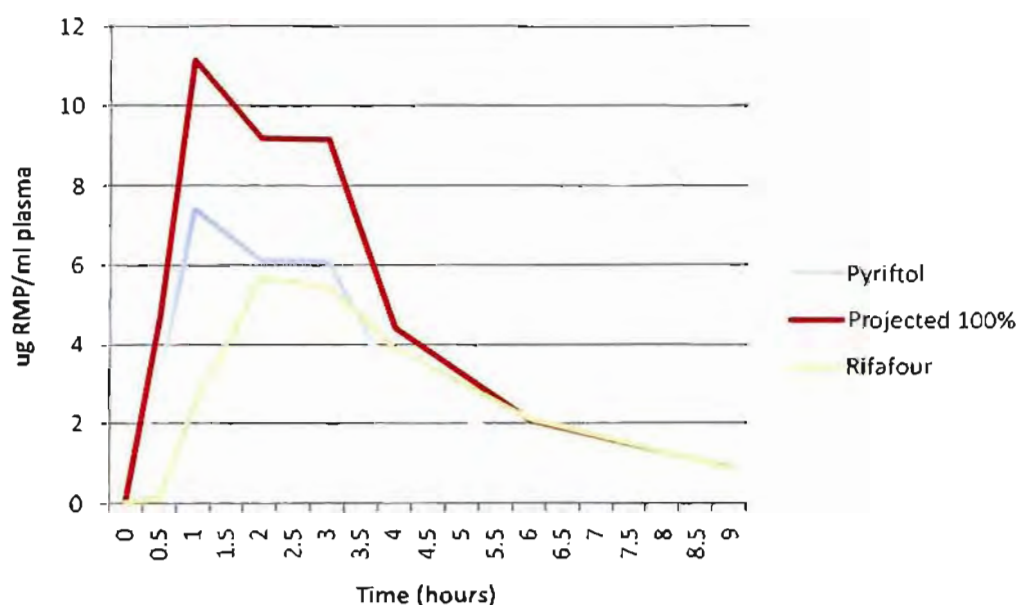


Figure 5.29: Mean plasma concentration vs. time curve for RMP of 16 healthy volunteers in a phase 1 trial. The test product contained 60% of the dosage present in the reference product. The plasma levels projected for a 100% dosage when administered by Pyrifitol is shown as the red line.

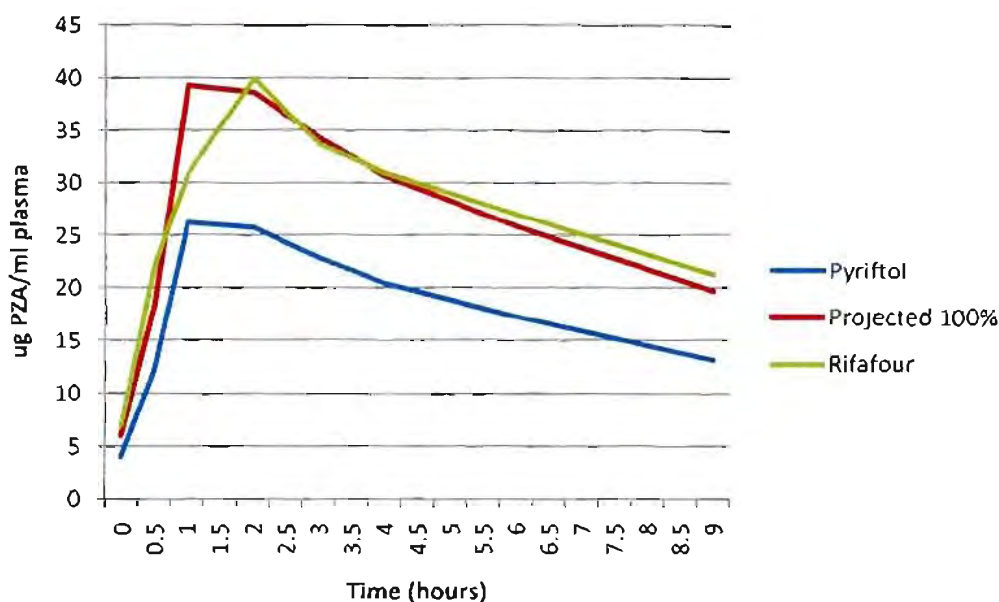


Figure 5.30: Mean plasma concentration vs. time curve for PZA of 16 healthy volunteers in a phase 1 trial. The test product contained 60% of the dosage present in the reference product. The time/concentration curve projected for a 100% dosage of the test drug is shown as the red line.

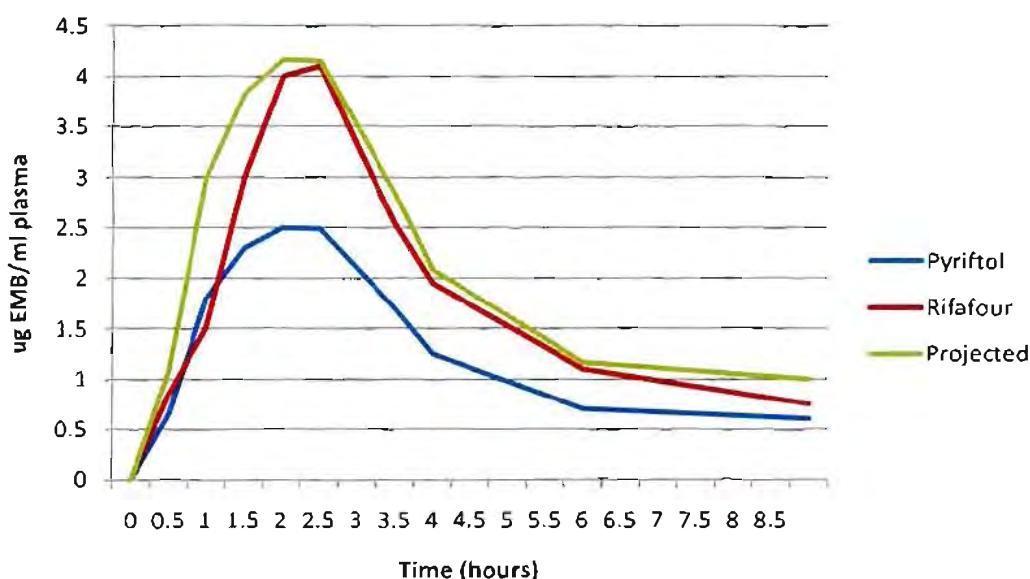


Figure 5.31: Mean plasma concentration vs. time curve for EMB of 16 healthy volunteers in a phase 1 trial. The test product contained 60% of the dosage present in the reference product. The time/concentration curve projected for a 100% dosage of the test drug is shown as the red line.

The statistical analysis indicated a significant decrease in T_{max} and a statistically significant increase in C_{max} and AUC for RMP ($p < 0.01$), despite the fact that the Pheroid™ product contained only 60% of the dosage of Rifafour-e200. The test and reference product showed

equal bioavailability (statistically verifiable) for INH, despite the Pheroid™ product containing only 60% of the INH present in Rifafour-e200. The *per weight* drug bioavailability of INH has therefore increased. The decrease in T_{max} was not significant. The bioavailabilities, as reflected by the AUC and the C_{max} , of both EMB and PZA in Pheroid™s are lower than that of Rifafour-e200. If the bioavailability of the test product was projected to a 100% dosage for PZA and EMB, the Pheroid™-based product Pyrftol was found to be bio-equivalent to Rifafour-e200. The T_{max} was again decreased by the Pheroid™ but the difference was not statistically significant. The various parameters and % difference are summarized in Table 5.12.

Table 5.12: The comparative PK parameters for the Phase 1 trial

API	PK parameter	Pyrftol	Rifafour	% enhanced	Projected 100% dose	% enhanced
INH	AUC	19.71	20.97	-6.01	29.56	40.96
	T_{max}	1.00	2.00		1.00	-50.00
	C_{max}	4.48	4.14	8.09	6.71	62.15
RMP	AUC	28.15	25.54	10.22	42.23	65.35
	T_{max}	1.00	2.00		1.00	-50.00
	C_{max}	7.42	5.68	30.63	11.13	95.95
PZA	AUC	168.8	255.40	-33.91	253.2	-0.86
	T_{max}	1.00	2.00		1.00	
	C_{max}	26.2	39.95	-34.42	39.3	-1.63
EMB	AUC	10.98	15.96	-31.20	18.3	14.66
	T_{max}	2.00	2.50		2.00	
	C_{max}	2.52	4.10	-39.02	4.17	1.63

The peak plasma concentration of RMP was determined to be 7.42 µg/ml and was reached 60 minutes (T_{max}) after oral administration of the Pyrftol, i.e. in the Pheroid™. The C_{max} obtained for the Rifafour-e200 (5.68 µg/ml) was reached 2 hours after administration. The presence of RMP in the plasma was therefore increased by at least 30% at T_{max} by the Pheroid™. Since only 60% of the normal dosage was administered in Pyrftol, the increase in plasma levels by the Pheroid™ at equal dosages was calculated to be 65% for RMP and 40% for INH above that of its comparator at equal dosages. The differences observed for EMB and PZA at T_{max} and full dosage were negligible.

The effective plasma concentrations for various TB drugs have been described (Cynamon & DeStefano, 1999; Saito *et al.*, 1989). The higher the plasma levels of these TB drugs, the greater are the therapeutic efficacies and the more effectively intracellular bacilli are killed. In many cases these high concentrations are necessary to overcome the low level of transport into

macrophages, as many antibiotics show low cell penetration, making it difficult to achieve sufficient concentrations at the infection site (Cynamon & DeStefano, 1999). The therapeutic effective concentration for RMP in plasma is described to be 7µg/ml (Cynamon & DeStefano, 1999; Saito *et al.*, 1989). Administration by Pyrifitol resulted in plasma levels of RMP of >7µg/ml, while the plasma levels of RMP reached after administration of Rifafour-e200 are probably not therapeutic, causing an exacerbation of the condition and the development of drug resistance. The shortened T_{max} is probably important in the case of RMP, an unstable drug that is degraded quickly in the harsh gastric conditions. The described therapeutic effective concentrations for each of INH and PZA are 3µg/ml and 35µg/ml plasma respectively (Cynamon & DeStefano, 1999; Saito *et al.*, 1989). Both the test and reference products reached the effective therapeutic concentration of 3µg/ml plasma for INH, although the dosage of the Pyrifitol was only 60% of the reference drug. Unfortunately, no intravenous administration was included in the protocol and the absolute bioavailability could therefore not be determined.

5.5.2 *In vitro/in vivo* correlation of efficacy

PheroidTM interactions with cell membranes could lead to altered membrane characteristics, such as increased membrane fluidity or membrane destabilization, resulting in an increased transport of anti-tuberculosis drugs across the bacterial cell wall, thus lowering the gradient necessary to achieve a higher intracellular drug concentration (as observed with the PheroidTM alone). This may in fact be the reason for the negation of drug resistance observed in *in vitro* studies described in 5.3.2 above. In an effort to determine whether the PheroidTM not only increases bioavailability but also enhances efficacy of treatment *in vivo*, two different studies were performed:

- (i) The plasmas of patients infected with drug sensitive and drug resistant strains of *M.tb.* was subjected treatment by the test drug, Pyrifitol, and the reference drug, Rifafour-e200. Since the treatment is a combination treatment, the concentrations used were based on the described bioavailability of RMP and therapeutic efficacy of Rifafour-e200, which was dissolved in plasma samples with a dilution of 8700 to a concentration of 6.977µg/ml of RMP (Cynamon & DeStefano, 1999; Saito *et al.*, 1989; Goodman and Gilman's 9th Edition: 922).
- (ii) The plasmas of subjects that participated in the phase 1 trial were infected with drug sensitive and drug resistant strains and the growth of the bacilli was monitored. In this case, plasma samples similar amounts of the applicable drug as determined during the trial were selected for the study.

In order to determine the MICs of Pyrifitol and Rifafour-e200, plasma samples (400µl) from patients treated with the test and reference products were added to BACTEC vials containing *M.tuberculosis* H37Rv reference strain. The MICs determined in infected patient plasma

samples for individual drugs is designated MIC_i, while that determined for the combination of the four drugs are named MIC_c. Controls contained no antibiotics. To prevent bacterial contamination other than tuberculosis, plasma samples were incubated in BACTEC 12B medium. The results (Table 5.13 and figure 5.32) showed that *M.tuberculosis* was growth-inhibited (GI<10) by the plasma samples (400µl) from patients treated with both Pyrifitol and Rifafour-e200, although Pyrifitol treated patients only received 60% of the anti-tuberculosis drugs. Control cultures grew in the normal way with doubling times every 24 hours. The preliminary results imply that Pyrifitol, at 60% dosage inhibited *M.tuberculosis* growth to the same extent as Rifafour-e200. Table 5.13 shows the growth indices determined for drug sensitive and drug resistant *M.tb.* strains treated with plasma from subjects treated with Pyrifitol and Rifafour-e200. The subjects treated with Rifafour-e200 are abbreviated to RA and the Pyrifitol patients are indicated by PC. The numbers after the strain designation indicate dilutions of plasma. The following strains were investigated for efficacy: strain 1182 is INH resistant, clinical MDR strain 92913 is INH, RMP and EMB resistant; H37Rv is sensitive to INH, RMP and EMB. RA2-10 and PC2-10 are serial dilutions of Rifafoure-200 and Pyrifitol in 7H9 medium. Days 4-5 fell on a weekend. BACTEC was counted on day 6 for days 4-5 and those results are not included as it represented the accumulated radioactivity of days 4-6.

Table 5.13: BACTEC analysis of treated plasma							
Sample	Strain	D1	D2	D3	D7	D8	D9
1	H37RV Control	10	22	49	999	999	999
2	H37Rv RA2	7	6	3	16	18	21
3	H37Rv RA3	7	6	3	3	2	2
4	H37Rv RA4	8	7	4	3	2	2
5	H37Rv RA5	9	7	5	3	1	2
6	H37Rv RA6	9	8	5	4	2	2
7	H37Rv RA7	9	11	8	6	4	6
8	H37Rv RA8	8	16	24	263	764	999
9	H37Rv RA9	11	25	48	897	999	999
19	H37Rv RA10	9	18	27	193	499	999
11	H37Rv PC2	7	6	4	5	8	27
12	H37Rv PC3	7	6	4	4	3	3
13	H37Rv PC4	7	7	2	3	3	2
14	H37Rv PC5	7	6	6	3	0	3
15	H37Rv PC6	7	9	4	4	0	2
16	H37Rv PC7	8	10	7	4	4	5
17	H37Rv PC8	10	21	31	194	463	999
18	H37Rv PC9	9	18	39	775	999	999
19	H37Rv PC10	8	15	18	36	57	132
20	92913 Control	9	9	14	262	550	999

21	92913 RA2	8	5	3	7	19	32
22	92913 RA3	8	7	4	5	11	36
23	92913 RA4	8	7	5	9	27	105
24	92913 RA5	7	7	7	29	60	560
25	92913 RA6	8	9	11	150	320	999
26	92913 RA7	9	8	14	266	540	999
27	92913 RA8	9	10	14	266	545	999
28	92913 RA9	8	9	14	271	563	999
29	92913 RA10	10	12	15	302	620	999
30	92913 PC2	7	4	3	8	15	52
31	92913 PC3	7	4	3	6	14	108
32	92913 PC4	7	5	4	22	54	595
33	92913 PC5	8	6	6	70	199	999
34	92913 PC6	9	9	11	242	501	999
35	92913 PC7	8	8	12	276	425	999
36	92913 PC8	9	8	11	234	498	999
37	92913 PC9	8	9	14	312	660	999
38	92913 PC10	7	9	12	262	550	999
39	1182 Control	7	6	10	391	600	999
40	1182 RA2	6	4	2	6	9	26
41	1182RA3	6	4	2	2	2	3
42	1182RA4	6	4	1	1	2	3
43	1182RA5	7	3	3	0	3	1
44	1182RA6	7	5	1	1	2	3
45	1182RA7	7	4	3	16	65	695
46	1182RA8	7	4	5	144	399	999
47	1182RA9	6	5	6	201	465	999
48	1182RA10	7	5	7	311	701	999
49	1182PC2	7	3	1	6	8	25
50	1182PC3	7	4	2	1	3	3
51	1182PC4	6	3	2	1	1	2
52	1182PC5	6	4	2	2	1	2
53	1182PC6	7	4	3	1	2	2
54	1182PC7	6	5	3	8	35	523
55	1182PC8	6	5	5	123	448	999
56	1182PC9	6	5	5	210	456	999
57	1182PC10	6	5	7	276	540	999

In table 5.13 above and table 5.14 below, the bacterial growth is inversely proportional to drug efficacy. In these studies 400µl of plasma was tested in BACTEC for anti-tuberculosis activity against H37Rv reference strain and some drug resistant strains.

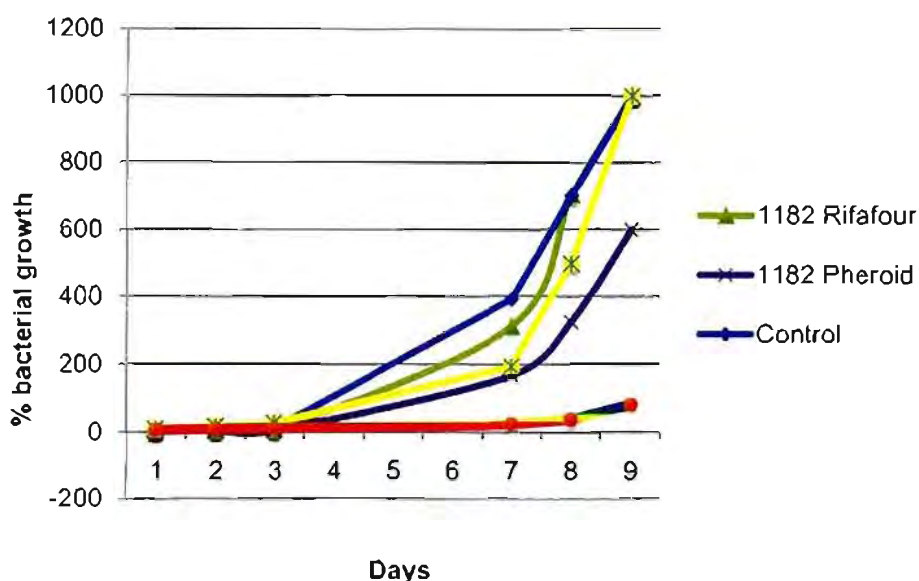


Figure 5.32: The growth of the drug sensitive reference strain H37Rv and the drug resistant strain 1182 in plasma of patients. Plasmas were treated post plasma preparation with Rifafour-e200 and Pyrftol in an effort to mimic in vivo efficacy. Bear in mind that the Pyrftol dosage is 60% of that of Rifafour-e200.

In figure 3.32, it can be seen that even at 60% of the dosage, Pyrftol inhibited bacterial growth nearly completely whereas Rifafour-e200 did not. Pyrftol inhibited drug resistant growth by about 50% at the concentration used, while treatment with Rifafour-e200 showed no growth inhibition. The bactericidal activity of the plasma following treatment with individual drugs Pyrftol and Rifafour-e200 respectively at various dilutions is given in Table 5.14. The MIC for PZA could not be determined in the BACTEC system.

	INH		RMP		ETH	
Pheroid™ entrapment	no	yes	no	yes	no	yes
Control	100	100	100	100	100	100
1.0x MICi	0	0	8	14	10	0
0.5x MICi	77	0	19	38	15	9
0.25x MICi	78	0	100	48	47	18
Drug efficacy: p-value	0.8	0.0001	0.16	0.0025	0.134	0.02

These results show the dramatic effect of Pheroid™ entrapment on INH resistance. Bacterial growth of RMP- and EMB resistant strains also showed increased inhibition by Pheroid™ entrapment but not to the same extent as INH. It has to be remembered that these results are performed in plasma, with the exclusion of the blood cell fraction. Between 5-10% of

the entrapped RMP partitioned to blood cells rather than to plasma. This partitioning should increase the effective dosage delivered to the cells but may detract from the efficacy observed above.

Therapeutic coverage or exposure can be regarded as the time period during which the effective concentration is maintained. The coverage can be determined by PK/PD modelling, in combination with MIC determinations. The MICs of Pyrifitol and Rifafour-e200 was obtained as described above. The MIC was significantly decreased for the Pheroid™ product (0.00625µg/ml) when compared with that obtained for Rifafour-e200 (0.00937µg/ml). The results of PK modelling based on these results and the bioavailability are shown in table 5.15 and illustrate the predicted increase in duration of the therapeutic window after a single dose due to the decrease in MIC.

Both the bioavailability, as reflected by AUC and peak values, and efficacy as reflected by MIC_i and MIC_c are enhanced for the Pheroid™-based Pyrifitol, when compared to Rifafour-e200. The parameters above showed the cumulative effect. The last two parameters, time above MIC_c and AUC/MIC_c are especially important. Time above MIC_c reflects the therapeutic coverage and AUC/MIC_c the comparative efficacy of the drugs. These results indicate that it should be possible to increase the interval between dosing even when using only 60% of the dosage. The results obtained for AUC/MIC_c (see table 5.15) indicate that a higher therapeutic efficacy will be achieved despite the lower dosage.

Table 5.15: Comparative pharmacokinetic parameters of Pyrifitol and Rifafour-e200					
Parameter	Treatment	RMP	INH	EMB	PZA
Clinically effective concentration	Pyrifitol (60%)	5.29µg/ml	2.31µg/ml	1.52µg/ml	23.17µg/ml
	Rifafour-e200	8.0µg/ml	3.5 µg/ml	2.3 µg/ml	35 µg/ml
Time above clinically effective concentration	Pyrifitol (60%)	2.7 hours	3.2 hours	1.8 hours	2 hours
	Rifafour-e200	0 hours	1.5 hours	1.3 hours	1.5 hours
AUC/clinically effective concentration	Pyrifitol (60%)	5.4	8.28	11.84	7.18
	Rifafour-e200	3.5	5.9	10.65	7.27
Peak/clinically effective concentration	Pyrifitol (60%)	1.77	2.15	2.05	1.27
	Rifafour-e200	0.92	1.33	2.04	1.16
MIC _i	Pyrifitol (60%)	0.125µg/ml	0.065µg/ml	1.8 µg/ml	
	Rifafour-e200	0.5µg/ml	0.20µg/ml	2 µg/ml	
Time above MIC _i	Pyrifitol (60%)	22.7 hours	23.5 hours	30 hours	
	Rifafour-e200	10.5 hours	22.8 hours	32 hours	
AUC/MIC _i	Pyrifitol (60%)	217	436	10.55	

	Rifafour-e200	54.8	138	13	
Peak/MIC _i	Pyrritol (60%)	65.44	79.3	1.73	
	Rifafour-e200	12.8	23.4	2.3	
MIC _c	Pyrritol (60%)	0.0065µg/ml	0.0065µg/ml	0.0065µg/ml	0.0065µg/ml
	Rifafour-e200	0.00937µg/ml	0.00937µg/ml	0.00937µg/ml	0.00937µg/ml
Time above MIC _c	Pyrritol (60%)	>>24 hours	>>24 hours	>>24 hours	>>24 hours
	Rifafour-e200	24 hours	24 hours	>24 hours	>24 hours
AUC/MIC _c	Pyrritol (60%)	4908	4259	3040	60320
	Rifafour-e200	2816	2844	2672	57414

5.6 Conclusion

In vitro/in vivo studies showed that entrapment of tuberculosis drugs in Pheroid™ resulted in:

- A significant increase in the drug plasma levels of the RMP despite administration of only a 60% dosage;
- A relative increase in the bioavailability of INH;
- Quicker absorption, with generally decreased T_{max} ;
- Decreased MICs;
- Increased duration of the therapeutic window;
- Reduced side effects with prospects of better compliance.

No significant side effects were found with the Pheroid™ treatments. None of the liver enzymes showed levels higher than the normal range. The S-unconjugated bilirubin, which did show levels elevated above the normal range was nearly back to normal 8 hours after drug administration, and was normal on the following day.

A Pheroid™-based anti-tuberculosis product deserves investigation. *In vitro/in vivo* correlations suggest that it may be possible to increase the interval between dosages and to decrease the dosage. It would be premature to state that the duration of treatment could be shortened but it may well be possible. Such an evaluation would be the primary objective of a phase 2 trial.

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