A Poly (Lactic Co Glycolic) Acid Based Dry Powder Inhaler for the Treatment of Pulmonary Tuberculosis

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A Poly (Lactic Co Glycolic) Acid Based Dry Powder Inhaler
for the Treatment of Pulmonary Tuberculosis

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A Thesis in the Field of Biotechnology
for the Degree of Master of Liberal Arts in Extension Studies

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Abstract

The goal of this paper is to investigate the feasibility of a novel Poly (Lactic Co Glycolic) Acid (PLGA) based Dry Powder Inhaler in the treatment of pulmonary tuberculosis (TB). First line tuberculosis drugs are highly effective in treating TB, but the lengthy treatment course and side effects associated with their use impacts negatively on treatment compliance. The advantages of using a Dry Powder Inhaler containing a fixed dose combination of first line antitubercular drugs in a PLGA carrier are investigated. Issues relating to the adequacy of current World Health Organization dosing recommendations are discussed and a set of clinical trials is proposed to assess the safety and efficacy of the Dry Powder Inhaler.
Dedication

This thesis is dedicated to my father, Giampiero Bartolucci, and to the memory of his father, Filiberto Bartolucci (1906–1935) who died from tuberculosis at the age of 29.
Acknowledgements

I would like to acknowledge the invaluable advice and guidance I have received from Dr. Steven Denkin and Dr. Donald Kirsch.
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Chapter I

Introduction

Tuberculosis (TB) is one of the most widespread infectious diseases in the world. Approximately one third of the world’s population is latently infected with *Mycobacterium tuberculosis* and approximately ten percent of infected individuals go on to develop active tuberculosis infection (Smith, 2011). Although increased availability of diagnostic testing and greater public awareness over the last quarter century has led to a reduction in TB related mortality, tuberculosis still infects and kills more people than any other bacterial infection, and is now the leading cause of death worldwide from a single infectious agent (Pai, 2017). In 2015 alone 10.4 million people became infected with TB, and 1.8 million died from the disease. This is a high mortality rate, given that an effective treatment for TB already exists.

Treatment of Tuberculosis

The standard drug regimen for TB has remained unchanged since the 1950s (Smith, 2011). The internationally accepted first line treatment for TB is a lengthy course of four antibiotics: isoniazid (INH) rifampicin (RIF) pyrazinamide (PZA) and ethambutol (EMB) (World Health Organization, 2014). During the standard course of treatment, all four drugs are administered orally seven times a week for two months, after which RIF and INH alone are administered three times a week for an additional four months (Smith, 2011; Toit, Pillay, & Danckwerts, 2006). Although this drug combination
is effective at treating pulmonary TB, the lengthy course of treatment, high pill burden, and toxic side effects have traditionally resulted in low patient compliance. Failure to complete the full course of treatment has led to the spread of multiple drug resistant TB (MDR-TB) (Figure 1), TB that is resistant to RIF and INH \textit{in vitro}, and extensively drug resistant TB, (XDR-TB) (Figure 2), which is resistant to RIF and INH \textit{in vitro}, and resistant to any of the second line quinolones and at least one second line injectable drug (World Health Organization, 2014). XDR-TB is estimated to affect approximately 500,000 people worldwide, though the actual number is likely significantly higher due to limited surveillance of XDR TB in poor and developing nations (Wang et al., 2014). The financial burden associated with both MDR-TB and XDR TB is extremely high: it is estimated that the cost to treat one case of MDR-TB in developed countries can reach 60,000 USD, while the cost of treating one case of XDR-TB can exceed 160,000 USD (Dambrosio et al., 2015). Moreover, XDR TB is generally considered untreatable and is almost always fatal (Andrews, 2008). The emergence of MDR-TB and XDR-TB has resulted in the placement of \textit{M. tuberculosis} on the National Institute of Allergy and Infectious Diseases (NIAID) list of Biodefense and Emerging Infectious Disease Threat Agents, underscoring the urgent need for new treatment modalities to combat its spread.
Figure 1. Percentage of new tuberculosis (TB) cases with multidrug-resistant TB. Figures are based on the most recent year for which data have been reported, which varies among countries (World Health Organization, 2014).

There are currently few promising first line TB drugs in development. When the FDA approved Bedaquiline as a second line drug for MDR-TB in 2012, it was the first tuberculosis drug the FDA had approved in over 40 years, highlighting the dearth of new TB drugs over the last several decades. Moreover, due to the high mortality rate associated with Bedaquiline, its use is indicated only in individuals who have no other treatment options available (Mahajan, 2013). More recently, Delamanid, a second line TB drug, was approved for use in Europe, Japan and South Korea, but it has not yet received FDA approval for marketing in the United States. The preexisting second line...
TB drugs Capreomycin, Kanamycin and Amikacin are significantly more toxic than their first line counterparts and are associated with severe side effects that range from hearing loss, depression, neuropathy and coma. They are also less effective at clearing TB than first line drugs, which requires that they be used for a greater length of time, in some cases up to 20 months (Dambrosio et al., 2015).

Figure 2. Map showing increased use of second line drugs for MDR-TB and XDR-TB (World Health Organization, 2015).

Drug chemotherapy remains the standard treatment for TB in part because vaccines have not proven successful against the spread of the disease. The Bacillus Calmette Guerin
(BCG) vaccine has been administered to over three billion people since becoming available in 1921. However, while it confers immunity against meningitis and miliary tuberculosis in children, it has proven largely ineffective against the spread of pulmonary TB, which is the most common and deadly form of the disease (Rodrigues, Diwan, & Wheeler, 1993). There are currently few promising vaccines in development. Preliminary data from the recent Phase II clinical trial of the MVA85A TB vaccine has been disappointing and trials for another vaccine, RBCG30, are currently ongoing, however it is unlikely that either vaccine, if proven viable, will be available for many years to come (Hoft et al., 2008; Tameris et al., 2013).

The lack of an effective vaccine and the paucity of new chemotherapies is cause for concern given the high mortality rate associated with TB. The spread of MDR-TB and XDR-TB throughout the developing world has led to a steady increase in the number of individuals infected with drug resistant strains of TB, instead of with the more easily treatable wild type strain (Andrews, 2008). The global community desperately needs a treatment for TB which is safe, effective, affordable, and which will ensure a high degree of patient compliance.

Although the lack of new TB chemotherapies represents a serious problem, it is mitigated to some extent by the fact that an effective treatment already exists. Increasing patient compliance with the existing first line drug therapy lies at the heart of combatting the spread of TB. Patient compliance with the existing TB treatment regimen is poor because of the large pill burden required to achieve minimum plasma drug concentrations (up to eight pills at a time), the length of the course of treatment, which can last up to six months, and the side effects with which anti tubercular drugs are associated, including
severe nausea, hives, jaundice and hepatitis (Shafiq, Kondel, & Malhotra, 2013; Sosnik, 2010; Toit et al., 2006). It is estimated that 480,000 individuals acquire multi drug resistant TB every year, of which an estimated 200,000 people die annually (World Health Organization, 2016). The continued overuse and misuse of antibiotics in the developing world has compounded the issue by compromising the effectiveness of the current treatment regimen (O’Neill, 2016). Future research must therefore focus on developing improved delivery methods which reduce the toxicity of antitubercular drugs (ATDs).

In this regard, nanotechnology based drug delivery systems represent the most promising treatment modality for TB in the 21st century and beyond. Nanotechnology based drug formulations offer several distinct advantages over traditional forms of drug administration, including greater bioavailability, enhanced solubility, sustained drug release, fewer side effects, greater drug internalization by target cells and more rapid onset of therapeutic action (Danhier et al., 2012; Mansour & Wu, 2009; Pandey, 2003; Sharma, Pandey, Sharma & Khuller, 2004; Smith, 2011). Furthermore, nanotechnology based drug formulations allow drugs to be targeted to specific tissues, which increases therapeutic efficacy and avoids the so called ‘first pass metabolism’ which reduces drug bioavailability (Hoppentocht, Hagedoorn, Frijlink, & Boer, 2014; Mehanna, Mohyeldin, & Elgindy, 2014).

**Background**

Tuberculosis is an ancient affliction which has plagued mankind for millennia. Descriptions of tuberculosis are found in the medical text *Huang Chi Nei Ching*, written in the third millennium BC, and in second century BC cuneiform tablets of the
Babylonian King Hammurabi. Tuberculosis was known in Ancient Greece as ‘phthisis’ from the Greek for ‘phthiein’ meaning to waste away (Frith, 2014). Galen referred to the disease as an ‘ulceration of the lungs, chest or throat, accompanied by coughs, low fevers, and wasting of the body because of pus’ (Pease, 1940). Hippocrates, in his work Of the Epidemics describes phthisis as “a weakness of the lung” accompanied by fever and cough, and as almost always fatal (Frith, 2014). Similarly, Aretaeus of Cappadocia wrote of tuberculosis in the first century AD: “If from an abscess in the lung or a settled cough or spitting of blood, pus should develop within and the patient should spit it out, the disease is called pye or phthisis”. References to tuberculosis also exist in ancient literature: in his epic poem The Odyssey Homer describes “a grievous consumption which took the soul from the body and caused a person to lie in sickness … a long time wasting away” (Pease, 1940).

Although descriptions of tuberculosis and the wasting illness with which it is associated can be found throughout much of recorded history, it was not until the late 17th century that the Dutch physician Sylvius de la Boe, in his work Opera Medica (1679), described the characteristic ‘tubercles’ in the lungs of the afflicted, which he termed ‘tuberculaglandulosa’ (Frith, 2014). Shortly thereafter, in 1689, the English physician Richard Morton described ‘tubercles’ that he observed in the lungs of patients suffering from phthisis. Phthisis was endemic in Europe in the latter part of the 18th and early 19th century, chiefly afflicting the lower classes and causing widespread mortality and suffering. The widespread suffering and mortality wrought by phthisis is reflected in the popular culture of the time, where it was known colloquially as “The White Death”, “The Great White Plague”, “The King’s Evil” and “The Graveyard Cough”. John Bunyan, in
his work *The Pilgrim’s Progress* refers to tuberculosis as “The Captain of all these Men of Death”. However, it was not until Robert Koch identified the Tubercle bacillus as the causative agent of tuberculosis in 1882 that the disease came to be known as tuberculosis and in the 20th century simply as TB (Frith, 2014).

**The Origins of *M. tuberculosis***

The origins of *M. tuberculosis* are not entirely clear, but the common ancestor of the *M. tuberculosis* Complex (C) of which *M. tuberculosis* is a member, is believed to have originated approximately 40,000 years ago in East Africa (Wirth, 2008). From the *M. prototuberculosis* progenitor two distinct clades are believed to have arisen between 20,000-30,000 years ago, one of which spread among human populations. This clade subsequently spread to animal populations with the domestication of cattle and goat that took place in Africa and later in Europe approximately 13,000 years ago, as nomadic societies began to be replaced with domesticated livestock and crops. This theory is supported by studies utilizing PCR analysis to confirm the presence of *M. tuberculosis* in human long bones found in a 9000 year old settlement in the Eastern Mediterranean (Hershkovitz et al., 2008). The pervasiveness of *M. tuberculosis* in human populations throughout recorded history has led some to speculate that it has probably killed more people throughout history than any other microbial pathogen (Frith, 2014).

**Characteristics of *M. tuberculosis***

*M. tuberculosis* is a non motile rod shaped member of the Mycobacteriaceae family of bacteria. Because the bacterium resists conventional staining methods, acid fast stains are used to detect it instead (Fukunaga, Murakami, Gondo, Sugi, & Ishihara, 2002). A defining feature of *M. tuberculosis* is that it possesses a highly complex, lipid rich
envelope with a highly unusual architecture consisting of a capsule, a plasma membrane and an inner cell wall (Brennan & Nikaido, 1995; Daffe & Draper, 1998). The capsule constitutes the outermost layer and consists of polysaccharide and protein with trace amounts of lipid, while the plasma membrane consists of the phosphatidic acid derivatives phosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol and it is not believed to contribute to the pathogenicity of *M. tuberculosis* (Brennan & Nikaido, 1995; Daffe & Draper, 1998). The Corynebacterium-Mycobacterium-Nocardia (CMN) branch of Mycobacteria, of which *M. tuberculosis* is a member, possess a uniquely structured cell wall referred to as a chemotype IV cell wall. (Brennan & Nikaido, 1995). The cell wall of *M. tuberculosis* is unique in that it contains N-glycolylated muramic acid residues which are believed to strengthen the peptidoglycan structure through increased hydrogen bonding, and are likely responsible for the strength and impermeability of the cell wall (Brennan & Nikaido, 1995; Chatterjee, 1997). The outer cell wall of *M. tuberculosis* also contains a unique polysaccharide called arabinogalactan (AG), covalently attached to mycolic acids C=70-90 in length, forming what has been termed the mycolylarabinogalactan-peptidoglycan complex, or mAGP (Brennan & Nikaido, 1995; Chatterjee, 1997; Rajni, Rao, & Meena, 2011). The mAGP contains covalently linked mycolic acids between 70 and 90 carbons atoms in length assembled into tetramycolylpentarabinofuranosyl clusters (Chatterjee, 1997). The combination of the N-glycolylated peptidoglycan layer and tetramycolylpentarabinofuranosyl clusters creates a barrier that acts as a hydrophobic protective layer around the bacterium. The highly complex cell wall renders the envelope impermeable to small polar molecules and
protects the bacteria from desiccation, alkali, chemical disinfectants and therapeutic agents including many antibiotics (Daffe & Draper, 1998).
Chapter II

Existing Inhalation Therapies

This section investigates the advantages and limitations of existing inhalation therapies, and how existing technology can be utilized to develop an inhalation delivery system for antitubercular drugs.

In 2006 the World Health Organization launched the Stop TB Strategy, with the goal of reducing the global TB burden. The mandate of the Stop TB Strategy is to enable and promote TB research, increase social mobilization and awareness of the disease, and to increase delivery of health services to poor and rural areas throughout the world. At the core of the Stop TB strategy is the DOTS (Directly Observed Treatment, Short Course) program. The DOTS program has five core mandates: (i) a political commitment to combat TB with increased long term financing at the government level (ii) increased case detection through improved bacteriology (iii) standardized treatment with patient supervision (iv) effective drug supply and management, and (v) implementation of a monitoring strategy to measure the impact of the DOTS program (World Health Organization, 2017). The DOTS program aims to increase patient compliance through directly observed drug administration. Patients in the DOTS program are directly supervised taking their TB medication by a healthcare worker or aide in a staffed clinic. By emphasizing patient compliance, the program has achieved excellent results: it is estimated that between 2000-2015, DOTS successfully treated 49 million people worldwide (World Health Organization, 2017). In addition, between 1990 and 2013 the
TB mortality rate dropped by 45%, and prevalence fell by 41% (Siroka, 2016). However, DOTS often requires patients to travel to clinics anywhere from two to six times per week, which is both time consuming and disruptive to the patient’s daily routine, and because the success of DOTS therapy is entirely dependent on the ability of TB patients to be present at a facility where they can be observed taking their medication, it may not be practical in rural areas with poor infrastructure and transportation (Munro, 2007). Other factors which discourage patients from completing their treatment regimen include long wait times at health clinics, inconvenient appointment times, health care facilities which are often dirty and crowded, and poor availability of TB medication (Munro et al., 2007). While the DOTS program offers a relatively inexpensive method of ensuring patient compliance, it is estimated that approximately one third of patients diagnosed with TB do not receive any treatment, and that the number of untreated may be as high as 90% in people with MDR-TB (World Health Organization, 2011). As long as large pools of individuals remain infected with TB and MDR-TB worldwide, it will be difficult to prevent the continued spread of the disease. There remains a need for an effective TB therapy whose success is not entirely dependent upon direct supervision and which is safe and easy to administer.

**Advantages of Inhalation Therapies**

There is a growing body of data which suggests that the respiratory route is a superior route of administration for ATDs (Mehanna, 2014, Pandey, 2003, Sung, 2009). Data from a number of animal studies has shown that inhalable preparations of ATDs significantly increases drug bioavailability, potentially offering a method of decreasing the dosing frequency and systemic toxicity associated with the traditional orally based TB
treatment regimen (Mehanna et al., 2014). By targeting the lung directly, inhalable drug formulations avoid first pass metabolism and allow for more rapid onset of therapeutic action (Hoppentocht et al., 2014). Given that approximately 80% of all TB infections are pulmonary in nature, inhalable drug formulations are especially well suited for delivery of ATDs (Mehanna et al., 2014). Sung et al. have already demonstrated the safety and tolerability of a powder form of Capreomycin in a guinea pig model of TB. Sung et al found that levels of a powder form of Nitroimidazopyran, (PA-824) a novel TB drug consisting of 75% PA-824, 20% L-leucine, and 5% DPPC (dipalmitoylphosphatidylcholine) were detectable systemically up to 24 hours after intratracheal insufflation, while drugs administered orally were not detectable systemically after 12 hours. Moreover, tissue concentrations of PA-824 remained detectable in the guinea pig lung for up to 32 hours post insufflation. Delivery of drugs via the pulmonary route therefore appears to confer the advantage of increasing drug concentration at the site of primary infection (Sung et al., 2009). The development of an inhalable TB drug delivery system would mark an important step forward in the fight to eradicate tuberculosis worldwide.

**Therapeutic Aerosols**

The use of therapeutic aerosols dates back several thousand years (Anderson, 2005; Stein & Thiel, 2017). The first use of a therapeutic aerosol in recorded history is found in the Theban Ebers papyrus, an Egyptian medical scroll dating from c 1554 BC, which describes the practice of smoking black henbane (*Hyoscyamus niger*), likely as a treatment for colic (Stein & Thiel, 2017). Similar accounts of smoking alkaloid containing plants like Ma-Huang 麻黄 (ephedra stem) are found in ancient Chinese
medical texts, and accounts of opium use for medicinal and ritualistic purposes in China date back many thousands of years (Anderson, 2005; Stein & Thiel, 2017). The seventeenth and eighteenth centuries witnessed the development of a variety of inhalation devices, though most were of dubious efficacy. The mid 19th century saw the development of the first nebulizer by Sales-Girons which utilized a pump and atomizer to create a mist which was inhaled through a small face mask (Anderson, 2005; Garcia-Contreras et al., 2015). The first steam based nebulizer followed in 1864, and in 1930 the first electric nebulizer, the Pneumostat, was developed for use in the American market (Anderson, 2005). Although first line ATDs are sufficiently water soluble to be administered via nebulizer, nebulizers are not well suited as a vehicle for TB drug administration for several reasons, including lengthy treatment time, the need for bulky and noisy compressors, complexity of operation, and lack of portability which confines their use to clinical and home settings (Muralidharan et al., 2015). Moreover, nebulizers are readily susceptible to contamination with bacteria like *Pseudomonas aeruginosa* (Zhou, Tang, Leung, Chan, & Chan, 2014). For all of the above reasons, nebulizers will not be considered as a potential vehicle for delivery of ATDs here.

**Pressurized Metered Dose Inhalers (pMDIs)**

The first modern inhalation devices can be traced back to George Maison, an engineer for Riker Labs who sought to develop a small dispenser to treat his daughter’s asthma. In 1950 he began work on two prototype pressurized metered dose inhalers (pMDIs) which he named the Medihaler Iso and Medihaler Epi. Maison’s metered dose inhalers were simple devices consisting of a canister, a mouthpiece, an actuator and a release valve. The devices were designed so that after each use, a small chamber filled
with a Freon propellant-drug mixture in liquid form. When the canister was pressed for activation, a precise amount of aerosol containing isoproterenol or epinephrine in an ethanol base was released (Garcia-Contreras et al., 2015; Stein & Thiel, 2017). Upon being marketed in 1956, the Medihaler Epi proved highly effective in controlling asthma and COPD (Myrdal, Sheth, & Stein, 2014). Modern pMDIs utilize a hydrofluoroalkane (HFA) propellant or compressed air in lieu of chlorofluorocarbons (CFCs), and a variety of different materials are now used for the canister component, including glass, stainless steel or aluminum. Depending on particle size, pMDI drug formulations may exist as a solution or a suspension in a single propellant or a mixture of propellants, and many use ethanol to solubilize the drug (Garcia-Contreras et al., 2015; Myrdal et al., 2014). Newer models feature indicators which let the user know once a dose has been dispensed, and many include a counter that displays the total number of doses remaining, but apart from these minor modifications, their basic design has remained largely unchanged since their introduction in the 1950s. The chief advantages of pMDIs include short drug onset, little to no preparation prior to dosing, minimal risk of contamination, and consistency between doses (Chrystyn & Price, 2009). Their small size, portability and relative ease of use have made pMDIs the most frequently prescribed devices in the world (Myrdal et al., 2014; Virchow, 2008). Use of pMDIs has grown to include treatment for a range of diseases, including chronic obstructive pulmonary disease (COPD), emphysema, and chronic bronchitis; more recently they have been used as a delivery platform for proteins, plasmid DNA and live attenuated viruses (Garcia-Contreras et al., 2015).
PMDIs for TB Drugs

Despite their role as the standard of care for asthma, pMDIs are not suitable as a vehicle for inhalable TB drugs, in large part because organic compounds have generally poor solubility in HFA propellants. This limits the types of compounds which can be used in pMDIs to those with favorable solubility profiles in propellants like HFA 134a and HFA 227 (Garcia-Contreras et al., 2015; Hoye, 2008; Zhou et al., 2014). In addition, widely used co-solvents like ethanol impact negatively on aerosol performance by increasing the droplet size in the aerosol spray, reducing the amount of drug which reaches the lung (Myrdal et al., 2014; Newman & Busse, 2002). In addition, propellant volume limits the amount of drug that can be loaded into the canister (Zhou et al., 2014). As a result, the development of combination drug formulations for pMDIs has proven to be major challenge for industry. Currently, the Foster® inhaler, which administers a combination of beclomethasone and formoterol fumarate, is the only multi drug pMDI on the market (Zhou et al., 2014). The design limitations associated with pMDIs underscores the need for an alternative inhaler design for tuberculosis drugs.

Dry Powder Inhalers (DPIs)

The years immediately following the Montreal Protocol, which mandated the use of non CFC propellants in pMDIs, witnessed an increase in the use of non propellant based inhalation devices like DPIs (Myrdal et al., 2004). Like pressurized metered dose inhalers, DPIs are small, handheld devices consisting of a mouthpiece and either a small chamber or reservoir which contains the drug or drugs to be delivered. The key difference between pMDIs and DPIs is that DPIs utilize a dry powder form of drug instead of a liquid drug mixture. As a result, DPI formulations are chemically more stable, can
accommodate more than one drug, and, unlike pMDIs, do not generate aerosol more quickly than the patient can inhale (Garcia-Contreras et al., 2015). DPIs also differ from PMDIs in that they are essentially ‘passive’ inhalation devices, in which the drug is inhaled directly out of the device by the user (Islam & Gladki, 2008; Stein & Thiel, 2017). This feature of some DPIs poses its own unique set of challenges, but DPIs also possess numerous characteristics which make them highly suited for use as ATD delivery devices.

Types of DPIs

DPIs can be classified in several different ways, but perhaps the most widely used method of differentiation is based on the number of doses the DPI can accommodate (Garcia-Contreras, 2015). In this regard, there are three basic types of DPI.

Unit dose DPIs. The first, known as a unit dose inhaler, utilizes a small gelatin packet or foil blister which contains the drug or drug formulation to be inhaled. The foil blister is sold separately from the device and is inserted into a small chamber prior to use. Upon loading, the blister is pierced, either by twisting the base of the device or by simply closing the chamber. The powder is then released from the blister and is ready for inhalation. After use, the spent blister pack is removed from the device and discarded. The Spinhaler®, marketed by Fison in the 1970s, is perhaps the best known example of this type of ‘capsule based’ device (Atkins, 2005). One of the biggest disadvantages of unit dose DPIs is that they can be difficult to load and are therefore they are not well suited for use during an asthma attack, nor are they ideal for those lacking in manual dexterity, including the very old and the very young (Garcia-Contreras et al., 2015). The Diskhaler®, a fluticasone propionate delivery system marketed by Glaxo SmithKline in
the 1980s, sought to improve upon the Diskhaler by featuring a small multi dose disk. (Figure 4) However, like the Spinhaler, the Diskhaler was perceived as cumbersome and difficult to use and has since been discontinued (Atkins, 2005). Despite these drawbacks, numerous iterations of this type of unit dose DPI have been successfully marketed over the last several years. The Spiriva HandiHaler® marketed by Boehringer Ingelheim is perhaps the best known example of a unit dose DPI still in wide use (Atkins, 2005). (Figure 3)

_Multidose reservoir DPIs._ The second type of DPI is referred to as a multi unit reservoir DPI. Multi dose reservoir DPIs feature a small reservoir containing the drug to be delivered. Prior to use, the device is primed by turning the base of the device, which loads a dose of the drug into a small chamber for inhalation. The first such example of a multi dose inhaler was the Turbuhaler®, developed and marketed by the AB Draco company in 1988. While the Turbuhaler has been adopted for wide use in Europe, issues related to variability in the drug delivery rate has limited wider acceptance in the United States, where it is marketed as the Pulmicort Turbuhaler® (Atkins, 2005). Other examples of this type of DPI include the Clickhaler® manufactured by Innovata Biomed and the Easyhaler® manufactured by Orion Pharmaceuticals. A key disadvantage to reservoir based DPIs is that dry powders tend to aggregate in the reservoir, reducing their ability to disperse properly, and they require specialized storage condition to prevent aggregation and contamination from moisture (Chrystyn & Price, 2009; Grant, Walker, Hamilton, & Garrill, 2015).

_Multi-unit dose DPIs._ Multi unit dose DPIs are designed to accommodate multiple discretely packaged drug doses of the drug to be inhaled. The Advair Diskus®
inhaler, approved by the FDA in 2000, can be considered the first true multi dose DPI. (Figure 5) The Diskus inhaler addressed many of the problems inherent with earlier versions of DPIs by utilizing a high capacity cartridge which held 60 drug doses, enough for one month’s supply of asthma medication. It was also the first multidrug DPI, delivering a formulation of fluticasone propionate and salmeterol. Its consistent performance and widespread patient acceptance have made the Diskus inhaler the industry standard among multi dose asthma inhalers (Atkins, 2005). The Foster Nexthaler®, (manufactured by Chiesi Farmaceutici, S.p.A.), a combination DPI that administers beclometasone dipropionate anhydrous and formeterol fumarate dihydrate, is another example of a well designed inhaler. (Figure 6) A notable feature of the Nexthaler is that it makes a loud clicking sound once a dose has been dispensed, which reduces the likelihood of inadvertent double dosing (Voshaar et al., 2014).

### Active and Passive DPIs

**Passive DPIs.** DPIs may also be categorized on the basis of whether they are active or passive. As noted previously, passive DPIs rely upon the patient’s inspiratory flow to disperse the drug. The main drawback of passive DPIs is that variability in the strength and consistency of inspiration among patients due to age, concomitant illnesses or disease progression leads to inconsistent dosing (Muralidharan, Malapit, Mallory, Hayes, & Mansour, 2015).

**Active DPIs.** Active DPIs address the problem of dosing inconsistency by incorporating small mechanisms which use compressed air or electrical vibration to deaggregate the powder, while others use small electrical strips to superheat the drug into a vapor which can then be inhaled. Although active DPIs represent a significant
improvement over passive DPIs, their use has so far been limited by their higher cost and larger size (Muralidharan et al., 2015; Zhou et al., 2014). However, because of their ability to ensure dosing consistency, active DPIs may be viewed as superior to passive DPIs. The Microdose® (Merck) and the Staccato® (Alxeza) are examples of currently marketed active DPIs.

**DPI Design Considerations**

Despite recent technological advances in DPI design, there is no ideal DPI inhaler for all respiratory illnesses. Instead, the efficacy of any inhaler depends on the physicochemical characteristics of the particles to be inhaled and the design characteristics of the device itself (Geller, 2005; Islam & Gladki, 2008). Four key factors in particular determine the dose that a patient receives when using a DPI. These are 1) the properties of the drug formulation (including powder flow, particle size and interactions between the drug and the carrier) 2) the performance characteristics of the DPI itself, including aerosol generation and ease of use 3) the patient’s inhalation technique and 4) the patient’s inspiratory flow rate (Islam & Gladki, 2008). Because all of these factors impact on dosing, drug efficacy varies widely across different types of DPIs. (Geller, 2005). Keeping in mind the factors responsible for the variability in drug efficacy among DPIs, it is helpful to consider those design characteristics which are most desirable in an inhaler designed for the delivery of ATDs.
Chapter III

Proposed Treatment Strategies

The characteristics of the proposed inhaler are outlined in this section. The way in which dry powder formulations can be optimized during the manufacturing processes are investigated, and specific characteristics of the proposed inhaler are discussed in detail.

A DPI for the administration of ATDs should at a minimum possess the following characteristics: it should be small, reliable, lightweight and easily transportable. It must be capable of accommodating a fixed dose combination of the four first line TB drugs (rifampicin, isoniazid, pyrazinamide and ethambutol), and must be capable of delivering a fixed dose combination of drugs consistently. The inhaler should be an active, multi unit dose model, similar to the NEXThaler, able to accommodate approximately one month’s supply of ATDs, and it must be capable of maintaining dose consistency over a range of inspiratory flow rates, ensuring that the very young, the elderly, and those with compromised lung function will be able to use the device effectively (Islam & Gladki, 2008). In addition, the DPI must be capable of maintaining drug stability over time to prevent contamination from moisture and microorganisms. Perhaps most importantly, the DPI must feature a feedback system (such as an LED indicator light, or a mechanism which makes an audible noise) to indicate when a dose has been successfully dispensed. Studies have shown that patient adherence to a given inhaler can be greatly increased through the use of feedback mechanisms which let the user know once a dose has been successfully dispensed (Papi, 2011). In a randomized crossover comparison trial, Voshaar
et al found that the NEXThaler was associated with significantly fewer operator errors and was rated as being much easier to use than both the Diskus and Turbuhaler, in part because it provided an audible confirmation of dose delivery (368).

As a corollary, one of the most important features of a DPI for administration of ATDs is ease of operation. Although pMDIs and DPIs are some of the most widely used devices in the world, it is estimated that up to 50% of patients do not use their inhalers correctly, resulting in increased hospitalizations and emergency room visits, greater reliance on systemically administered steroids, and reduced disease control (Garcia-Contreras et al., 2015; Islam & Gladki, 2008; Peloquin, 2001; Voshaar et al., 2014). Improper inhaler use often results from lack of training and inadequate information from manufacturers on proper handling and operation (Voshaar et al., 2014). Ease of operation and adequate instruction are therefore essential for ensuring that patients will continue to use a given DPI over the long term.

Nanoparticles and TB Treatment

Just as the design of an inhaler is directly related to its suitability for treating a given disease, the properties of the drug formulation also play an important role in the clinical outcome. Dry powders can be manufactured in a variety of ways, depending on the desired characteristics of the drug particle. Commonly used techniques include Crystallization, Supercritical Fluid Technology (SFT), Pressure Swing Granularization (PSG) and Spray Drying. Crystallization is a relatively simple and commonly used process in which crystals are grown out of a drug solution. Approximately 90% of all pharmaceutical drugs, including tablets, aerosols, suspensions and capsules, contain drug in crystalline particulate form. While crystallization yields product with very high purity,
it is also resource and time intensive, inefficient, and yields little control over the final physical form of the product (Shekunov & York, 2000). Supercritical Fluid Technology (SFT), which encompasses Rapid Expansion of Supercritical Fluid Solution (RESS), Gas Antisolvent Recrystallization (GAS), Solution Enhanced Dispersion by SCF (SEDS) and Precipitation from Gas Saturated Solutions (PGSS), is a complex form of particle engineering which employs changes in pressure and temperature to generate particles with the desired characteristics. Pressure Swing Granularization (PSG), another method of particle manufacture, uses a large machine called a Pressure Swing Granulator to generate extremely fine drug particles with excellent dispersion characteristics (Shekunov & York, 2000). However, SFT and PSG are highly specialized processes which are not readily scalable and due to their complexity have yet to see widespread commercial use (Shekunov & York, 2000). In contrast, Spray Drying generates a dry powder by exposing a mist of liquid drug mixture to a hot gas. When the mist encounters the gas, the liquid evaporates, leaving a dry powder. Solvent composition, concentration and dry rate can all be adjusted during the Spray Drying process to generate particles with specific size, density and shape (Hickey, 2016). Spray Drying is well suited to manufacturing particles for use in DPIs because particles manufactured in this way are more spherical in shape than those generated by milling, more homogeneic in size, and contain a high fraction of particles < 5 µm in size, which can penetrate deep into the lung. Spray Drying is also cost effective and easily scalable (Hickey, 2016). Examples of dry powder inhalers which use powders manufactured via Spray Drying include the Tobi® (Novartis) and Aridol® (Pharmaxis) (Islam & Gladki, 2008; Parumasivam, 2016).
**Characteristics of Inhalable Dry Powders**

Particle size is the most important parameter with respect to efficacy of inhalable drug formulations (Kaialy & Nokhodchi, 2015). A commonly used expression of particle size is the Fine Particle Fraction (FPF), the proportion of particles in a given formulation between 500 nm and 5 µm. The FPF is often equated with the fraction of inhalable drug that is pharmacologically active because particles in this size range are capable of reaching the lower respiratory tract upon inhalation (Kaialy & Nokhodchi, 2015). Therefore, in designing a powder formulation of ATDs, the manufacturing process should aim to maximize the fraction of particles in this range. Chan et al (2013) have successfully employed the Spray Drying technique to generate dry powder formulations of ATDs in a PLGA carrier with particle diameter of 4.14 ± 0.04 µm and FPF of 53.3%. Using High Performance Liquid Chromatography (HPLC) for particle quantification, Chan et al were able to demonstrate that individual drug particles in the spray dried powder consisted of (Pyrizinamide: rifampicin: isoniazid) in the WHO recommended ratio of 5:2:1, indicating that the drugs are uniformly distributed throughout the powder (288). The techniques employed by Chan and others (Hickey & O’Hara, 2000; Ungaro, Angelo, Miro, Rotonda, & Quaglia, 2012) demonstrates that the technology to manufacture dry powder formulations of TB drugs with optimal characteristics for inhalation already exists. Testing the efficacy of similar drug powders in humans is the logical next step forward in designing a more effective TB drug delivery system.

**Poly (Lactic Co Glycolic) Acid (PLGA)**

Another key determinant of drug efficacy is the choice of drug carrier. In this regard, polymeric nanoparticles less than 1 µm in size are ideal for use in drug transport.
Polymeric nanoparticles serve three important functions: they serve as a vehicle to transport drugs, they protect the drug from premature degradation, and they control drug release (Shekunov & York, 2000). Polymeric nanoparticles can be composed of a wide variety of materials, including poly ε caprolactone (PCL), poly(lactic) acid (PLA), chitosan, gelatin and poly (lactic-co-glycolic acid) or PLGA. PLGA is a synthetic biodegradable polymer composed of lactic acid and glycolic acid monomers (Danhier et al., 2012). PLGA nanoparticles are ideally suited as carriers for drug delivery due to their biocompatibility, high mechanical strength and biodegradability (Astete & Sabliov, 2006). The Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved PLGA for use as a carrier in a number of drugs, including Eligard, Zoldex, Profact Depot and Ozurdex (Patil, 2016). Because PLGA is capable of carrying a wide variety of macromolecules it has quickly become one of the most widely utilized polymers for drug delivery (Ahmad, 2011, Danhier, 2012).

**Manufacture of PLGA**

Although there are several methods of manufacturing PLGA nanoparticles, the manufacturing process can be divided into two broad classes: top down and bottom up (Astete & Sabliov, 2006). In the bottom up approach, PLGA is synthesized from its constituent monomers, which are linked together via ester bonds. Emulsion, Microemulsion Polymerization and Precipitation Polymerization are all examples of this approach. In the top down approach nanoparticles are synthesized from the preexisting PLGA polymer and then sonicated to the desired particle diameter. Emulsion diffusion, emulsion evaporation and solvent displacement are all examples of this approach, which is also the most commonly employed method (Astete & Sabliov, 2006; Danhier et al.,...
In the top down approach, PLGA and the compounds or drugs of interest are dissolved in an organic solvent such as dichloromethane to create a polymer solution. The oil water (O/W) emulsion is prepared by adding water together with a surfactant such as polysorbate 60, which is then added back to the polymer solution. The polymer solution and emulsion is then sonicated or homogenized to create nanoparticles (Astete & Sabliov, 2006; Danhier et al., 2012). The process may be varied to effect changes in key physical parameters of the resulting PLGA, including size distribution, morphology, hydrophobicity and hydrophilicity (Astete & Sabliov, 2006).

Once administered, drug loaded PLGA nanoparticles are taken up by cells through pinocytosis or through clathrin mediated endocytosis (Cheng, 2007). Inside the cell, drug release occurs through three mechanisms: desorption of surface bound drug, diffusion of the drug through the polymer matrix, or through a combination of erosion and diffusion through the nanoparticle matrix (Danhier et al., 2012). What happens to the bound drug once it is released into the cytosol varies in large part with charge. Positively charged nanoparticles are less readily internalized into membrane bound vesicles with a low pH, while negatively charged nanoparticles more readily cross membrane barriers, which tend to be positively charged (Danhier et al., 2012). During the manufacturing process PLGA nanoparticles can be endowed with a positive charge through conjugation with Poly Ethylene Glycol (PEG), or neutrally charged using the linear polysaccharide chitosan. Because M. tuberculosis resides in macrophage phagosomes, positively charged PLGA nanoparticles may be better suited to deliver drugs to cell phagosomes within the cell, which are negatively charged; however the degree to which nanoparticle charge
plays a role in drug efficacy as manifested in antimicrobial activity is unclear (Yeung, 2009).

Although aerosolized PLGA nanoformulations of ATDs have proven highly effective at clearing active pulmonary TB infection in guinea pig, rat and murine models of TB, they have not yet been tested in humans (Pandey, 2003; Sharma, 2004). Pandey et al have demonstrated that nebulized isoniazid (10 mg/kg), rifampicin (12mg/kg), and pyrazinamide (25 mg/kg) encapsulated in PLGA nanoparticles administered to guinea pigs infected with \textit{M. tuberculosis} via insufflation significantly increased the half life and bioavailability of all three drugs compared to the standard oral form (982). Remarkably, five nebulized doses of ATDs administered for 3-4 minutes over a 45 day period was sufficient to clear guinea pig lungs of tubercle bacilli, whereas 46 daily doses were required when the ATDs were administered orally (Pandey, 2003). Moreover, a single nebulized dose of these three ATDs resulted in blood plasma levels of isoniazid and pyrazinamide at or above Minimum Inhibitory Concentrations (MICs) for up to 192 hours, and blood plasma levels at or above the rifampicin MIC of up to 144 hours. By comparison, plasma levels of isoniazid, pyrazinamide or rifampicin were not detectable beyond 12-24 hours when the free form of the drugs was administered orally. Furthermore, caudal lobe lung homogenates from guinea pigs dosed via nebulizer showed no detectable colony forming units (cfus) after day 46, whereas 4.92 cfus ± 0.39 log\textsubscript{10} were observed in caudal lobe lung homogenates of untreated animals (Pandey, 2003). These results demonstrate that PLGA is capable of facilitating the sustained release of ATDs over a period of several days, suggesting that a PLGA based nanoformulation of ATDs could allow for significantly reduced dosing frequency in humans. Testing is
human subjects is required to assess whether a PLGA based formulation of ATDs will allow for reduced dosing frequency compared to the standard dosing regimen.

Pandey et al also investigated the efficacy of orally administered PLGA encapsulated rifampicin, isoniazid and pyrazinamide in clearing TB infection in a murine model of TB. Following oral administration of rifampicin (12 mg/kg), isoniazid (10 mg/kg), and pyrazinamide (25 mg/kg), plasma MICs of rifampicin were observed up to 96 hours post administration, while MICs for INH and PZA were detectable for up to 216 hours, whereas plasma drug levels of free drugs administered orally were not detectable beyond 12-24 hours (Pandey, 2003). This confirms the ability of PLGA to sustain blood concentrations at or above the MICs for these three ATDs for significantly longer periods of time than free drug in both the guinea pig and murine model of TB.

Sharma et al achieved similar results using lectin functionalized PLGA nanoparticles in a guinea pig model of tuberculosis. Guinea pigs administered isoniazid (10 mg/kg), rifampicin (12 mg/kg) and pyrazinamide (25 mg/kg) encapsulated in PLGA nanoparticles delivered via insufflation resulted in rifampicin plasma levels at or above MICs for up to 144 hours post administration, while MIC levels of isoniazid and pyrazinamide were observed up to 216 hours; levels which are comparable to those observed by Pandey et al. (Sharma, 2004; Pandey, 2003). Finally, no colony forming units (cfus) were detectable in guinea pig spleen and lung homogenates following three doses of rifampicin, isoniazid, and pyrazinamide encapsulated in lectin functionalized PLGA, confirming that PLGA based formulations of ATDs confer antimicrobial activity comparable to standard formulations of free drug administered orally. Together, the research by Pandey and Sharma demonstrates that ATDs encapsulated in PLGA
nanoparticles are capable of sustaining blood concentrations of ATDs for significantly longer periods of time than free drugs administered orally, and confirms that ATDs in a PLGA carrier are as effective at clearing *M. tuberculosis* from lung tissue as the oral form of free drugs (Sharma, 2004; Pandey, 2003).
Current Tuberculosis Chemotherapy

This section investigates the shortcomings of the current TB regimen, and proposes changes to current dosing and administration standards to increase the efficacy of antitubercular drugs.

The bactericidal activity of first line ATDs is directly correlated with their peak drug concentrations in blood plasma (Gumbo, Angulo-Barturen, & Ferrer-Bazaga, 2015; Peloquin, 2001). This stands in contrast to so called ‘time dependent’ antibiotics like penicillin, whose bactericidal effect is maximized by increasing the duration of drug exposure (Peloquin, 2001). The parameters most closely associated with clinical outcomes for TB drugs are the AUC24/MIC ratio and the Cmax/MIC ratio (Gumbo et al., 2015). Maximizing Cmax/MIC in particular enhances bacterial killing and is believed to limit the emergence of resistant subpopulations (Gumbo et al., 2015; Peloquin, 2001).

However, one of the drawbacks associated with use of traditional PK and PD parameters is that they were developed to characterize the effects of antibacterial compounds on the growth dynamics of rapidly growing bacteria (Gumbo et al., 2015; Peloquin, 2001). Because M. tuberculosis is a slow growing bacterium, and because the growth dynamics of M. tuberculosis are more difficult to reproduce in vitro, there is less data from in vitro studies on M. tuberculosis than for fast growing bacteria. This issue is compounded by the fact that M. tuberculosis displays a somewhat unusual growth cycle, characterized by an initial rapid phase followed by a slow phase, in which the bacilli enter into a poorly
defined metabolic state known as the latent state, characterized by downregulated metabolic processes and increased resistance to antibacterial compounds. The manner in which the latent state impacts the bactericidal effect of first line antibiotic compounds remains poorly understood (Alnimr, 2015; Peloquin, 2013).

Rifampicin

Rifampicin is a macrolide antibiotic derived from the natural products of *S. mediterranei*. Macrolide antibiotics are characterized by the presence of a chromophoric naphthohydroquinolone group spanned by a long aliphatic bridge and an acetyl group at carbon C25 (Gumbo et al., 2015). The standard dose of rifampicin for treatment naïve adults is 600 mg daily. Rifampicin has a half life of between 2-5 hours, a MIC of 0.05-0.50 µg/mL and a Cmax of 8-20 µg/mL. The presence of food in the stomach lowers Cmax by as much as 36% and therefore current dosing guidelines recommend administration on an empty stomach (Gumbo, 2011). Following oral administration approximately 85% of rifampicin remains bound to protein, which may account for the reduced efficacy often observed at currently recommended doses. Approximately 24% of RIF is excreted unmetabolized in the urine. Rifampicin exerts its antimicrobial effects by binding to the beta subunit of DNA dependent RNA polymerase (rpoB) to form a complex which inhibits microbial RNA synthesis.

Pyrazinamide

Pyrazinamide is a synthetic analogue of nicotinamide with an oral bioavailability of >90%. It is metabolized by microsomal deamidase to pyrazinoic acid (POA) and hydroxylated to 5 hydroxy POA before being excreted by the kidneys. The clearance rate of PZA increases with patient mass and significantly higher doses than the currently
recommended dose of 15-30 mg/kg a day are likely required for optimal AUC/MIC in patients with high weight (Gumbo et al., 2015; Pasipanodya et al., 2013). Doses of 30, 40 and 60 mg/kg do not result in statistically significant incidence of hepatotoxicity or arthropathy when administered over a period of two months, and doses in the 60 mg/kg range has proven safe in numerous prospective controlled clinical studies (Gumbo et al., 2015). Renal impairment significantly reduces pyrazinamide clearance and therefore should be avoided entirely in patients with kidney disease.

Pyrazinamide is activated under acidic conditions which exist at the peripheries of necrotic tubercular cavities in the lung where inflammatory cells of the immune system produce lactic acid. (Gumbo, 2011). In this environment, pyrazinamide is deaminated to pyrazinoic acid (POA) which is pumped into the extracellular matrix by an efflux pump in the cell wall of *M. tuberculosis*. POA is then protonated to POAH, a lipid soluble form of POA which reenters the bacillus through the nicotinamide transport pathway (NTP) (Sarathy, Dartois, & Lee, 2012). pyrazinamide is therefore the only TB drug which enters the *M. tuberculosis* bacillus in an ATP dependent fashion. The way in which pyrazinamide exerts its bactericidal effect is not well understood, but may involve reduction of intracellular pH, disruption of transport across the cell membrane, or inhibition of the enzyme fatty acid synthase type I which disrupts mycolic acid synthesis (Gumbo, 2011).

**Isoniazid**

Isoniazid is synthesized from ethyl isonicotinate and hydrazine. Its antitubercular properties were not fully realized until 1945, after nicotinamide was discovered to have antimycobacterial properties. The bioavailability of orally administered isoniazid is >90%
based on a 300 mg dose. Isoniazid is metabolized by arylamine N-acetyltransferase type 2 (NAT) and is excreted in the urine within 24 hours as acetylisoniazid and isonicotinic acid (Gumbo et al., 2015). Isoniazid has a MIC of 0.025-0.05 mg/L. Isoniazid enters *M. tuberculosis* through passive diffusion. Once inside, it is activated by the catalase-peroxidase enzyme KatG. Activation by KatG leads to the production of an NAD (nicotinamide adenine dinucleotide) isomer which interferes with enoyl acyl carrier protein reductase (InhA) and Beta ketoacyl acyl carrier protein synthase (KasA), two enzymes essential for synthesis of the mycobacterial cell wall. Isoniazid also acts as a potent inhibitor of nucleic acid synthesis (Gumbo, 2011).

**Ethambutol**

Ethambutol is a chemically synthesized bacteriostatic antibiotic discovered in 1961. Ethambutol has an oral bioavailability of approximately 80%, a half life of 3 hours and a MIC of 0.5-2 mg/L. Ethambutol is oxidized by alcohol dehydrogenase to an aldehyde, which is oxidized to dicarboxylic acid by aldehyde dehydrogenase. Approximately 80% of ethambutol is excreted in urine unmetabolized. Ethambutol is typically administered daily at 15 mg/kg, but can be administered twice a week at 25 mg/kg to optimize microbial killing activity (Gumbo, 2011). Ethambutol inhibits mycobacterial cell wall synthesis by interfering with the transfer of arabinose into arabinoglycan.

**The DOTS Program**

The World Health Organization DOTS program is universally recognized as the standard of care for treatment naïve and multi drug resistant tuberculosis. An estimated 93% of the world’s population has access to DOTS (Dowdy & Chaisson, 2009) and since
1995 the program has successfully treated over 41 million people. DOTS also serves as a global network through which new cases of TB are identified and tracked (World Health Organization, 2015). Although some have questioned DOTS’ efficacy, noting that the rates of relapse and mortality are comparable to those obtained with self administration of ATDs, (Karumbi & Garner, 2015; Munro et al., 2007), DOTS is associated with greater overall treatment completion rates, higher sputum smear conversion rates during treatment and a greater number of patients cured (Nahid et al., 2016). For these reasons DOTS remains the standard of care in public TB programs throughout the developed and developing world.

The DOTS program for treatment naïve pulmonary TB consists of two months of an intensive phase during which rifampicin, isoniazid, ethambutol and pyrazinamide are administered seven days a week (for a total of 56 combined doses) or five days a week (for a total of 40 combined doses). The first phase is followed by a four month continuation phase during which isoniazid and rifampicin are administered for seven days a week (for a total of 126 doses) or five days a week (for a total of 90 doses). In cases where more frequent dosing may be difficult to maintain (i.e. in patients with limited access to transportation), continuation phase dosing may be decreased to two or three days a week without impacting negatively on clinical outcome (Nahid et al., 2016). The Centers for Disease Control (CDC) and WHO publish comprehensive first line drug dosing guidelines for adults and children. The dosing guidelines recommended by the WHO and CDC for first line TB drugs calculated according to body weight (mg/kg) are as follows:
Table 1

*WHO and CDC Recommended Doses of First Line TB Drugs*

<table>
<thead>
<tr>
<th>Drug</th>
<th>WHO</th>
<th>CDC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rifampicin</strong></td>
<td>10 mg/kg (range of 8-12 mg/kg, up to maximum 600 mg/kg daily dose)</td>
<td>10 mg/kg (up to 600 mg daily dose)</td>
</tr>
<tr>
<td><strong>Isoniazid</strong></td>
<td>5 mg/kg (range of 4-6 mg/kg, up to maximum 300 mg/kg daily dose)</td>
<td>5 mg/kg (up to 300 mg daily dose)</td>
</tr>
<tr>
<td>a<strong>Pyrazinamide</strong></td>
<td>25 mg/kg (range of 20-30 mg/kg) Maximum dose not provided</td>
<td>Age 40-55 (18.2-25 mg/kg up to 1000 mg daily dose)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Age 56-75 (20-26.8 mg/kg up to 1500 mg daily dose)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Age 76-90 (22.2-26.3 mg/kg up to 2000 mg daily dose)</td>
</tr>
<tr>
<td>b<strong>Ethambutol</strong></td>
<td>15 mg/kg (15-20 mg/kg) Maximum daily dose not provided</td>
<td>Age 40-55 14.5-20 mg/kg (up to 800 mg daily dose)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Age 56-75 16-21.4 mg/kg (up to 1200 mg daily dose)</td>
</tr>
</tbody>
</table>
Age 76-90 17.8 -21.1 mg/kg (up to 1600 mg daily dose)

a. Dose can be adjusted based on whether the regimen is administered daily, twice weekly or three times weekly
b. Dose can be adjusted based on whether the regimen is administered daily, twice weekly or three times weekly

Limitations of the Current Dosing Regimen

Despite the widespread adoption of DOTS as the universal standard of care, compliance remains a significant barrier due to the high pill burden and side effects of first line TB drugs. In addition, monotherapy, in which only one TB drug is administered to patients, is common in many parts of the world, owing to logistical issues faced by local clinics as a result of having to order drugs from several different manufacturers (Blomberg, 2001). Monotherapy is a particularly pernicious practice since it can very quickly lead to drug resistance (Blomberg, 2001). To address the issues associated with monotherapy, the past several years have witnessed greater use of fixed dose combination (FDC) formulations of ATDs.

Fixed dose combination (FDC) tablets, which contain the recommended dose of all four first line TB drugs in a single tablet, are superior to individual drug doses for several reasons. Most notably, FDCs reduce the bill burden from 9-16 pills a day to 3-4 per day. In addition, FDC tablets eliminate confusion as to the correct number and type of pills patients need to take, and they prevent the practice of ‘selective ingestion’, wherein patients preferentially take only some of their pills. Fixed dose combinations also eliminate the common practice of crushing or breaking pills into smaller pieces to make them easier to swallow, or to achieve the proper dosing where smaller dosages are not
available. WHO has recently implemented a liquid fixed dose combination regimen for children to address these issues (Blomberg, 2001; Hitt & Hart, 2011). Clinical trials conducted between 2003-2008 in Asia, Africa and Latin America using fixed dose combinations have demonstrated noninferiority of the four drug regimen compared to the standard dosing, and have established that both regimens share similar toxicity profiles (Acocella, 1988; Hitt & Hart, 2011; Lienhardt, 2011). In an acknowledgement of the advantages of FDCs, the World Health Organization has published a conditional recommendation that FDC tablets be used whenever possible (Blomberg, 2001). A Fixed Dose Combination dry powder inhaler is the logical next step towards shortening treatment time and increasing patient compliance.

**Pharmacological Issues Associated with First Line TB Drugs**

Despite the overall effectiveness of first line tuberculosis drugs in treating TB, significant challenges remain with respect to optimizing therapeutic dosing. The reality is that not all patients who complete the DOTS program are cured of TB; many relapse or go on to develop multiple drug resistant TB (Magis-Escurra, 2012). The reasons for treatment failure are multi factorial and complex. Between-patient pharmacodynamic variability, disease progression, concomitant illnesses like HIV and Type II diabetes mellitus, malabsorption of drugs from the gut due to malnutrition, hepatic or renal dysfunction, and genetic variability have all been cited as factors which adversely affect treatment outcome (Magis-Escurra, 2012). Furthermore, levels of ATDs well below therapeutic blood plasma concentrations are common (Alcorn, 2006, Magis-Escurra, 2012; Peloquin, 2003). Low plasma concentrations of rifampicin and isoniazid in particular have been observed in a surprising number of studies (Magis-Escurra, 2012).
Patients with Type II diabetes mellitus and HIV in particular are frequently found to have sub optimal blood concentrations of isoniazid and rifampicin (Alcorn, 2006; Reynolds & Heysell, 2014). Nijland et al found that levels of rifampicin in TB patients with Type II diabetes are significantly lower than in non diabetic patients, possibly due to reduced secretion of gastric hydrochloric acid in diabetic patients which impacts on absorption of rifampicin from the gut. This poses a significant problem since it is estimated that by 2025 approximately 75% of those with Type II diabetes will live in countries with the highest rates of TB, including India and South Africa (Nijland et al., 2006).

Sub therapeutic levels of isoniazid may also be due to high clearance variability between patients depending on whether they are ‘fast’ or ‘slow’ acetylators (Gumbo, 2011; Tostmann, 2008). Specifically, fast acetylators exhibit substantially shorter mean elimination half times compared to slow acetylators. This variation is believed to be related to variation in the NAT2 gene, which is thought to be defective in slow acetylators. Slow acetylators are at greater risk of adverse effects including neuropathy and neurological toxicities, dizziness, ataxia, paraesthesia and toxic encephalopathy. Although genetic testing for acetylator type is essential for therapeutic dose monitoring, questions remains as to whether genotyping is financially feasible, particularly in third world countries with limited resources (Kinzig-Schippers et al., 2005).

Wide variations in rifampicin plasma levels have also been commonly observed in a number of treatment populations. Weiner et al found significant variations in blood plasma levels of rifampicin among TB patients from Africa, North America and Spain due to polymorphisms in the drug influx and efflux transporter genes SLC01B1 c.463CA and MDR1 (4196). The SLC01B1 c.463CA gene codes for anion-transporting
polypeptides, while the MDR1 gene codes for the P-glycoprotein, a multidrug resistance protein complex which pumps drugs out of cells (Weiner, 2010). Weiner found that the AUC24 for rifampicin was 36% lower in patients with the transporter gene (29.8 micrograms hour/mL) than among those without (46.7 micrograms hour/mL). Patients with the SLC01B1c.463CA polymorphism also had 42% lower rifampicin blood concentration levels (25.6 microgram/mL versus 44.4 microgram/mL), 34% lower Cmax levels, and a 63% greater clearance rate than those without the SLC01B1c.463CA polymorphism (4197). The results obtained by Weiner et al clearly demonstrate the extent to which genetic polymorphisms impact blood plasma levels of rifampicin among TB patients. An inhalable formulation of TB drugs would increase therapeutic efficacy by avoiding first pass metabolism which reduces drug bioavailability (Hoppentocht, 2014; Mehanna, 2014).

The degree to which suboptimal levels of ATDs contribute to treatment failure is unclear. However, increased awareness over the last several years regarding sub optimal blood plasma concentrations of rifampicin and isoniazid has led to a growing consensus among many clinicians and researchers that current recommended dosages are inadequate. (Gumbo et al., 2015; Magis-Escurra, 2012; Milstein et al., 2016; Nijland et al., 2006; Peloquin, 2001). In recent years, Therapeutic Dose Monitoring (TDM) has emerged in response to the wide variability in drug plasma levels observed across populations of TB patients.

**Therapeutic Dose Monitoring (TDM)**

TDM relies upon careful monitoring of blood concentrations to assess sub therapeutic drug levels and to adjust dose accordingly. TDM has come increasingly to be
used in cases of relapse and delayed response and has proven especially helpful for complex or difficult cases that may involve multiple concomitant illnesses or advanced tuberculosis (Magis-Escurra, 2012). However, while TDM may be effective at treating more complex cases, it is a specialized form of care; the medical staff and advanced laboratory and testing facilities required are costly and often unavailable in the third world. TDM should therefore not be seen as a solution for the large number of patients who fail to successfully sputum convert or relapse. Instead, one could argue that the increased reliance on TDM in recent years is a reflection of underlying shortcomings in the current dosing regimen. Although the extent to which increased reliance upon TDM reflects inadequacies in the standard dosing regimen is difficult to assess, the prevalence of sub therapeutic plasma concentrations of isoniazid and rifampicin suggests that the current dosing regimen requires careful reevaluation.

The Historical Basis for Current rifampicin Dosing Levels

The history of rifampicin is particularly illustrative of the degree to which current ATD dosing guidelines appear to be based on political and economic considerations as opposed to scientific data. rifampicin came to prominence in the mid 1960s following several clinical trials which showed it to be effective against \textit{M. tuberculosis} (Ingen, 2011). Early pharmacokinetic studies on rifampicin established that a single 600 mg daily dose yielded a serum concentration of 7.0 micrograms/mL approximately 90 minutes after ingestion, and a serum concentration of 8.80 -12.0 microgram/mL two hours post administration (Ingen et al., 2011). Subsequent studies established that a 900 mg daily dose yielded mean serum concentrations of 16.2 micrograms/mL 3 hours post administration, although Furesz et al observed serum concentrations as high as 20.87 ±
3.25 micrograms/mL 2 hours after a 750 mg dose (Ingen et al., 2011). Because the blood plasma levels observed with the 600 mg dose were significantly higher than the rifampicin MIC of approximately 0.2 microgram/mL 24 hours post administration, the authors of early studies on rifampicin pharmacokinetics concluded that the 600 mg dose was sufficient for patients to successfully sputum convert (Ingen et al., 2011). However, none of the early studies on rifampicin incorporated a dose escalation component to investigate the potential for increased therapeutic efficacy of rifampicin at higher doses (Peloquin, 2001). This issue was compounded by the fact that the one study which did investigate the potential increases in efficacy at higher rifampicin levels did not include safety data. This study, conducted by Kreis and Pretet, achieved a 94% sputum conversion rate after two months, and nearly a 100% sputum conversion after only 3 months using a regimen of 900 mg isoniazid, 1200 mg rifampicin and 1000 mg Streptomycin, with all patients remaining sputum negative after one year (Peloquin, 2001). However, because the Kreis and Pretet study included only limited safety and toxicity data, it did little to alleviate concerns over the safety of using rifampicin at higher doses (Peloquin, 2001; Ingen et al., 2011).

Moreover, when rifampicin was first approved there appears to have been an overemphasis on minimizing its potential toxicity (Milstein et al., 2016). One of the few studies which investigated use of high dose rifampicin, conducted by Poole, used 1200 mg of rifampicin twice weekly in conjunction with 900 mg isoniazid but was terminated due to a high incidence of pyrexia, thrombocytopenia and formation of anti rifampicin antibodies (Poole, Stradling, & Worlledge, 1971). While the Poole study was widely cited as evidence of rifampicin toxicity at higher doses, it should be noted that all 3 cases
of thrombocytopenia (n= 3, 6%) were in patients assigned the 1200 mg dose for between seven and nine months, a far longer duration than what would normally be used in a standard treatment regimen. Moreover, all cases of antibody development (n= 24, 49%) occurred well after the six month mark. The adverse events experienced by patients in the Poole study may have led to the perception that rifampicin was unsafe at doses above 600 mg, and as such this may have discouraged further investigations into the potential benefits of high dose rifampicin in the treatment of TB.

A third key determinant of the 600 mg rifampicin dose appears to have been economic. When rifampicin was first introduced it was prohibitively expensive (Ingen et al., 2011). In an attempt to reduce treatment costs, the British Medical Research Council (BMRC) encouraged drug regimens which used isoniazid and pyrazinamide in place of rifampicin. As a result, the first early short course therapy trials conducted by BRMC in East Africa in the 1970s sought to minimize rifampicin use, encouraging the use of cheaper and less effective medications over rifampicin. (Fox, Ellard, & Mitchison, 1999). However, the concern over pricing which existed in the 1970s is no longer warranted; the current cost of one complete treatment course of a fixed dose combination of TB drugs is $14-$18 when purchased through the Stop TB Partnership Global Drug Facility (Stop TB Partnership, 2017).

There is a need for greater data regarding rifampicin toxicity. The current dose of 600 mg appears to have been determined by a combination of cost considerations, lack of clarity as to what constituted an optimal therapeutic dose and unwarranted fears of toxicity at doses above 600 mg (Milstein et al., 2016). Despite the encouraging results observed in the Kreis and Pretet study, remarkably little research has been conducted on
the possible benefits of higher dose rifampicin on shortening treatment duration. Current rifampicin dosing guidelines merit a full reevaluation to establish whether higher doses may allow for a reduced treatment times.
Proposed Experiments

Research by Pandey, Sharma and others suggests that PLGA modulates drug release so that therapeutic drug plasma levels are maintained for longer periods of time than when free drugs are administered orally (Pandey, 2003; Sharma et al, 2004; Suarez, 2001). This suggests that a PLGA based formulation of ATDs could allow for significantly reduced dosing in humans. Testing a fixed dosed PLGA based formulation of first line drugs in guinea pigs will provide data on dose optimization and will provide an opportunity to assess the safety and tolerability of the novel powder formulation. Preclinical testing will serve three purposes: 1) to assess the pharmacology of the oral control versus the inhaled formulation 2) to compare the toxicology of the oral control versus the inhaled formulation and 3) to assess the efficacy of the oral regimen versus the inhaled formulation.

Material and Methods

The study population will consist of Dunkin Hartley guinea pigs grouped by weight so that each group consists of animals with similar weight prior to being infected with *M. tuberculosis* (Hickey, 2008). Guinea pigs will be inoculated with a low dose (10-20 bacilli) of the *M. tuberculosis* H37Rv strain in a whole body Glas-Col chamber. The H37Rv strain will be chosen for its well documented history and worldwide distribution (Kubica, 1972). Successful inoculation will be confirmed using Systemic flow cytometric
evaluation of T cell influx in guinea pig lung, lymph node, spleen and blood using the protocol outlined by Ordway et al, wherein MIL4neg CD4+ and MIL4neg8+ antibodies are used to distinguish T cell influx from the influx of other immune cells (1821). MIL4negCD4+ and MIL4negCD8+ cell influx will be compared to uninfected controls at Day 5, 15, 20 and 30 to confirm successful inoculation. Animals that fail to demonstrate a robust CD4 T cell response after 72 hours will be removed without replacement. Drugs administered orally will be dissolved in isotonic saline prior to administration. The PLGA based formulation will be manufactured using the Spray Drying technique described by O’Hara and Hickey: isoniazid, rifampicin, pyrazinamide and ethambutol will be dissolved in a 0.5% (w/v) polymer solution and then spray dried using inlet air temperature of 60° Celsius, outlet temperature 40° Celsius, 16.7 mL/min feed rate, air pressure 0.45 MPa and air flow rate of 700 NL/h. Particle diameter will be assessed using Scanning Electron Micrography; drug loading will be determined using reverse phase High Performance Liquid Chromatography (HPLC) (Hickey & O’Hara, 2000). Bacterial burden in the guinea pig lung typically plateaus after four weeks; drug administration will therefore not be initiated until four weeks post inoculation (Garcia-Contreras et al., 2015).

**Pharmacology Testing**

Pharmacology testing will be conducted using 120 guinea pigs divided into five groups of 24 animals. Previous studies have demonstrated that guinea pigs can be treated with the same oral doses of rifampicin (10 mg/kg), isoniazid (5 mg/kg) and pyrazinamide (25 mg/kg) used in humans. However, there is uncertainty as to whether this method of dosing in guinea pigs is sufficient due to the unique physiological characteristics of the
guinea pig gut (Hickey, 2016). Therefore, dosing will be determined using the Animal Equivalent Dose (AED) calculation after Nair & Jacob (29):

\[
\text{AED (mg / kg)} = \text{Human does (mg / kg)} \times \text{Km ratio}
\]

Three groups of guinea pigs will receive one of three doses of the PLGA based formulation, one group will receive the AED standard oral dose (RIF 46.5 mg/kg, ISO 23 mg/kg, PZA 115 mg/kg and ETH 69 mg/kg), and one untreated group will serve as a control. Blood draws will be conducted at 1, 2, 3, 4, 8 and 12 hours post administration and every 12 hours thereafter for the first 72 hours. Drug plasma levels measured using High Performance Liquid Chromatography (HPLC) will be used to calculate area under the curve (AUC), total body clearance (CL) and elimination rate constants (K). The goal of the pharmacology testing will be to establish the optimal dose and dosing schedule of the new PLGA based formulation. Minimum target drug plasma levels at 12 hours will be 0.05 µg/mL (RIF), 0.1 µg/mL (ISO), 0.025 µg/mL (PZA) and 4.0 µg/mL (ETH). Because the experimental protocol will be using higher doses of RIF and ISO, it is expected that maximum drug plasma levels at 12 hours will match or exceed those observed by Pandey et al (RIF 0.85 µg/mL, ISO 1.0 µg/mL and Pyrazinamide 12.5 µg/mL). Studies investigating the pharmacokinetics of antitubercular drugs in the guinea pig model of tuberculosis have traditionally excluded the use of ethambutol because it does not contribute to the sterilizing activity of the short course regimen in guinea pigs (Dickinson & Mitchison, 1976). Therefore, pharmacology testing will be used to determine peak ETH levels following dosing with the PLGA based formulations. Pharmacology testing will also aim to establish whether the inhaled formulations match the blood plasma levels of the standard oral regimen and will serve to establish the inhaler dosing and dosing
frequency required to achieve the minimum inhibitory concentrations for each drug. Although Pandey et al determined that as few as five doses of rifampicin, isoniazid and pyrazinamide were sufficient to clear the guinea pig lung of TB, additional testing is required to determine whether the novel PLGA based formulation can achieve the same or similar results, and if not, dosing frequency will be adjusted accordingly. One way Analysis of Variance (ANOVA) will be used to assess differences in area under the curve (AUC), total body clearance (CL) and elimination rate constants (K) between the two groups.

**Toxicology and Determination of NOAEL**

Once the dosing level and dosing frequency have been established, acute toxicity will be assessed using an acute toxicity inhalation test for aerosol drug preparations. A group of 12 individually numbered guinea pigs will be housed in individual whole body chambers with air flow of 12–15 air changes/hour and adequate oxygen (19%/h) (Parasuraman, 2011). The guinea pigs will be exposed to the therapeutic dose of the PLGA based fixed dose formulation previously determined in efficacy testing. The duration of exposure will be defined as the time between the t95 equilibration of the chamber concentration and the t95 chamber concentration decay (Organization for Economic Cooperation and Development, 2009). Animals will be observed for 14 days post exposure. The No Observable Adverse Effect Level (NOAEL) will be calculated based on i) overt markers of toxicity and ii) surrogate markers of toxicity. Overt markers of toxicity will include (but are not limited to) tremors, salivation, coma, and convulsions. Mortality during the exposure and observation period will also be noted, and any expired animals will undergo histological and pathological examination. Surrogate
markers of toxicity will include deviations from reference range values for the following enzymes: alkaline phosphatase (ALK) 80-350 IU/L, aspartate aminotransferase (AST) 10-90 IU/L and alanine aminotransferase (ALT) 10-90 IU/L. Laboratory values which deviate from published reference values will be assessed as indicative of Drug Induced Liver Injury (DILI) (Clark, Hall, & Williams, 2014).

The third experiment will test for efficacy. Testing will be performed using 96 animals divided into four groups of 24. Three groups will be assigned one of three PLGA based fixed dose regimens administered via insufflation, and a control group will receive the fixed dose oral regimen. The control group will be orally dosed according to current WHO dosing guidelines for treatment naïve, rifampicin and isoniazid susceptible TB (RIF 10 mg/kg, ISO 5 mg/kg, PZA 25 mg/kg, ETH 15 mg/kg). The second group will be administered the PLGA based fixed dose formulation (RIF 10 mg/kg, ISO 5 mg/kg, PZA 25 mg/kg, ETH 15 mg/kg). The third and fourth groups will be administered the PLGA based formulation with doses of RIF 15 mg/kg, ISO 7.5 mg/kg, PZA 25 mg/kg, ETH 15 mg/kg and RIF 20 mg/kg, ISO 10 mg/kg, PZA 25 mg/kg, ETH 15 mg/kg, respectively. Dosing will take place over a period of six weeks. Animals receiving the PLGA based fixed dose formulations will be dosed every 72 hours; animals receiving the standard oral regimen will be dosed daily. It is anticipated that the high dose formulations will match or exceed the pharmacological parameters of the standard regimen. If this proves to be the case, the higher dose formulations should provide a bacteriologic outcome equivalent or superior to the standard oral dose. The target drug plasma levels at 12 hours will be those at or above the minimum inhibitory concentrations for each drug (0.05 µg/mL (RIF), 0.1 µg/mL (ISO), 4.0 µg/mL (ETH) and 0.025 µg/mL (PZA)). The 12 hour time
point was chosen because plasma drug levels of free RIF, ISO and PZA were not detectable in guinea pigs after 12 hours in the Pandey study when administered orally (984), and it is anticipated that drug plasma levels of all four ATDs will be detectable beyond the 12 hour time point with the novel PLGA formulation. In addition, because the inhalable drug formulation targets lung tissue directly, it is anticipated that drug levels in lung tissue will be higher in the experimental group. Two guinea pigs per week from each group will therefore be sacrificed to measure differences in drug levels in lung homogenate between the groups. Since it is anticipated that the experimental formulation will also allow for shorter treatment duration, lung homogenate from the sacrificed animals will also be tested for active *M. tuberculosis* growth for any differences in bacterial burden between the experimental and control groups. At week six, all remaining animals will be sacrificed and lung homogenates from each group will be plated to determine bacterial load. At the end of six weeks all guinea pigs will be sacrificed. The number of viable *M. tuberculosis* bacilli will be determined by plating serial dilutions of 5 mL lung homogenates on agar plates. The bacterial colonies will be counted after 4 weeks of incubation at 37°C. Differences in bacterial load among the four groups, taken as the average bacterial load in each group, and expressed as the log10 number of CFUs (± Standard Error of the Mean (SEM)), will be compared (Ordway et al., 2010). One way Analysis of Variance (ANOVA) will be used to assess the differences in bacterial load in lung homogenates among the four groups based on a significance level of 0.05.

**Phase I Trial**

There is a large body of scientific data which suggests that higher doses of rifampicin and isoniazid are more effective in treating TB (Boeree et al., 2015; Ingen et
al., 2011; Milstein et al., 2016; Peloquin, 2001). However, it remains unclear whether these two drugs are toxic at higher doses (Ingen, 2011 et al., Milstein et al., 2016). Additional research is required in order to determine whether the potential risks associated with higher doses of rifampicin and isoniazid may outweigh improvements in clinical outcome. There is currently no clinical data involving fixed dose combination PLGA based formulations in human subjects. There may be risks and unanticipated effects associated with the use of the investigational product which are not known at this time.

Materials and Methods

A Phase I, Dose-Escalation Trial will test the safety and tolerability of the PLGA based fixed dose formulation in sixty healthy adult volunteers aged 18 to 65 years. Subject participation will last approximately 4 weeks. The investigational product is a novel dry powder inhaler containing a fixed dose inhalable formulation of all four ATDs in a PLGA nanocarrier. Phase I testing will assess the safety and tolerability of a series of PLGA based drug formulations, each containing a progressively higher dose of rifampicin and isoniazid. Subjects will self administer using a dry powder inhaler under supervision of clinic staff. The target blood plasma levels will be RIF 0.05 µg/mL, ISO 0.1 µg/mL, ETH 4.0 µg/mL and PZA 0.025 µg/mL. Exclusion criteria include prior treatment for TB, or prior exposure to second or third line TB drugs; pregnant, nursing and lactating women, individuals with unresolved or uncontrolled illnesses including HIV and Type II diabetes mellitus; those undergoing chemotherapy, or currently on medications which may impair immune functioning, including interferons, immune modulators or systemic corticosteroids, and those with emphysema or similar respiratory
illnesses which significantly impair proper lung functioning. Asthma is not considered an exclusionary criterion however subjects with asthma will be subject to spirometry testing prior to enrolment to assess lung functioning.

**Study Objectives**

The primary objective will be to determine the Maximum Tolerated Dose (MTD), defined as the dose at which no more than two patients experience a dose limiting toxicity related to the investigational product. Dose limiting toxicities will be defined as adverse events assessed as a causally related to the investigational product.

**Dose Determination**

The NOAEL obtained from the preclinical trial will be used to determine the Safe Human Dose (SHD) based on the following formula:

\[
\text{Safe Human Dose} = \frac{\text{ThD} \times 70 \text{ kg}}{\text{SF}}
\]

in which ThD is the threshold dose at which no adverse effect is observed, SF is the Safety Factor (assigned a default value of 10) and 70 kg is the average weight of a male subject (Kent, 1998). The SHD will be used as the Maximum Recommended Starting Dose (MRSD) (FDA, n.d.). Subjects will self administer using a set of inhalers that have been prepared prior to the start of the trial. The DPIs will be self administered once every 72 hours under medical supervision. Blood draws will be taken at 1, 2, 4, 6, 8 and 12 hours post administration to obtain:

- Cmax (maximum observed concentration)
- Absorption
- Distribution
Pharmacology testing will provide preliminary data on the pharmacodynamics of the novel PLGA based formulation in humans and allow for the determination of the most effective dose and dosing frequency in the target population. Each set of six inhalers will contain a progressively higher dose of rifampicin and isoniazid, holding levels of pyrazinamide and ethambutol constant at 1000 mg and 800 mg respectively. Dose level increments will be based on a series of decreasing “Fibonacci” dose sequences in which the MRSD will be followed by a series of increases in rifampicin and isoniazid in steps of 100%, 67%, 50%, 40% and 33% (Rubinstein & Simon, n.d.). The dose preceding that at which two patients experience a DLT will be considered the MTD for the fixed dose combination (Rubinstein & Simon, n.d.).
Table 2

*Escalation Schema*

<table>
<thead>
<tr>
<th>Number of Subjects Experiencing DLT at Given Dose</th>
<th>Escalation Rule</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 out of 3</td>
<td>Three subjects will be entered at the next dose level</td>
</tr>
<tr>
<td>1 out of 3</td>
<td>Three more subjects will be entered at this dose level. If none of those subjects experience a DLT, three more subjects will proceed to next dose level. If one or more of this group experience a DLT then this will be considered the maximally administered dose</td>
</tr>
<tr>
<td>≥2</td>
<td>Dose Escalation will be halted and this dose level will be the MTD</td>
</tr>
</tbody>
</table>

Blood chemistry and hematology from draws taken at 1, 2, 4, 6, 8 and 12 hours post administration will be compared to published reference values. Because rifampicin, isoniazid and pyrazinamide are all metabolized by the liver and all are known or potential hepatotoxins (Tostmann, 2008), levels of liver enzymes will be monitored closely for signs of Antituberculosis Drug Induced Hepatotoxicity (ATDH) based on WHO definitions of hepatotoxicity listed in Table 3. In addition, the following values will be considered indicative of drug induced hepatotoxicity: Alanine Aminotransferase/Aspartate Aminotransferase ratio > 3 x Upper Limit of Normal,
Alanine Aminotransferase > 3 x Upper Limit of Normal and Aspartate Aminotransferase > 3 x Upper Limit of Normal (FDA, 2017; Tostmann, 2008).

Table 3

<table>
<thead>
<tr>
<th>WHO definition of Hepatotoxicity</th>
<th>Grade 1 (mild) &lt; 2.5 times ULN (ALT 51–125 U/L)</th>
<th>Grade 2 (mild) 2.5–5 times ULN (ALT 126–250 U/L)</th>
<th>Grade 3 (moderate) 5–10 times ULN (ALT 251–500 U/L)</th>
<th>Grade 4 (severe) &gt; 10 times ULN (ALT &gt; 500 U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT, alanine aminotransferase; ULN, upper limit of normal, i.e. 50 U/L.</td>
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</tbody>
</table>

Definition of Hepatotoxicity According to WHO Adverse Reaction Terminology

Phase II Trial

The purpose of the Phase II trial will be to test the efficacy and safety of the PLGA based formulation in adults with active, treatment naïve pulmonary TB. The Phase II study will also provide additional data regarding dose optimization. Although the adverse effects of rifampicin and isoniazid are well documented, the relationship between dose and the number and severity of adverse events is not well documented (Boeree et al., 2015). Data from the Phase II trial will determine whether the investigational product is sufficiently safe to be studied in a large Phase III trial. A search of Clinicaltrials.gov finds numerous studies investigating high dose rifampicin to shorten treatment duration, but no studies investigating the impact of high dose rifampicin and isoniazid on treatment efficacy.
Protocol

The Phase II trial will be an open label, adaptive dose finding design to compare the clinical efficacy and short term safety of the investigational product against an active control consisting of the current treatment regimen. The adaptive dose finding design will allow for the determination of the therapeutic dose range of the proposed inhaler and limit patient exposure to unsafe or ineffective doses (Gallo et al., 2006). Patients will be assigned inhalers containing the highest well tolerated dose of rifampicin from the Phase I trial. If the higher dose rifampicin is not well tolerated, as manifested in a significantly higher number and/or severity of adverse events (AEs) compared to the standard oral regimen, the rifampicin dose will be adjusted. Interim data analyses will be conducted on a bi weekly basis to compare the overall rate of AEs deemed related to the investigational and control products. If it is determined that the rate of AEs is significantly higher in the cohort assigned the investigational product, the cohort will be assigned a new set of inhalers containing 5 mg less rifampicin while holding all doses of all ISO, ETH and PZA constant. The primary objective will be to test the efficacy of the investigational formulation. The primary outcome will be a statistically significant decrease in the amount of time required to sputum convert compared to the control group. The secondary objective will be to test the safety of the investigational product. The secondary outcome will be a statistically significant reduction in the number and severity of adverse events experienced in the experimental group compared to the control group.

It is anticipated that the increased dosages of isoniazid and rifampicin, combined with the sustained drug release characteristics of the PLGA formulation, will allow for shorter treatment duration and reduced dosing frequency. The study will test the PLGA based formulation in adults aged 18-65 with newly diagnosed, uncomplicated pulmonary...
TB. The trial will be based on the assumption that there is no difference between the investigational product and the active control, given by \( H_0 = \mu_c - \mu_d = 0 \), where \( \mu_c \) is the active control and \( \mu_d \) is the investigational product. The alternative hypothesis is that there exists a difference between the two products given by \( H_a = \mu_c - \mu_d \neq 0 \). The trial will use a 0.05 level of significance for both Type I and Type II error (\( \alpha= 0.05, \beta=0.05 \)). The total number of subjects will be determined using the formula:

\[
2N = \frac{4(Z_\alpha + Z_\beta)^2\delta^2}{\delta^2}
\]

where \( Z_\alpha \) and \( Z_\beta \) are obtained from tables of the standardized normal distribution for \( \alpha \) and \( \beta \), \( \delta \) is the standard error, and \( \delta \) represents the difference between experimental and control groups (Ng, 2015). Sputum culture will be used to confirm active TB infection prior to enrolment using BACTEC 960TB. Treatment failure and relapse at 12 months will constitute a composite endpoint (Phillips, 2016). Twelve months is considered a sufficient length of time because approximately 75% of relapse occurs within six months of conclusion of treatment and extending the follow up to 18 months would prolong the trial without providing any additional clinically meaningful data (Phillips et al., 2016). Subjects will be randomized using the SAS (Statistical Analysis Software) RANUNI feature to generate random number values from a uniform distribution; block randomization will be used to maximize the comparability of the treatment groups (Deng & Graz, n.d.). The PLGA based formulation will be manufactured using the technique described in the animal model section.

Subjects will self administer with their assigned inhalers under supervision by clinic staff. Self administration will take place over for 16 weeks, at which point sputum samples will be collected and cultured. Blood draws will be taken weekly for the first two
weeks to assess AUC24, clearance and Cmax, expressed as geometric means in data analysis. Blood samples will also be collected at 1, 2, 3, 4, 8, 12 and 24 hours post administration to collect the following PK parameters for each first line drug:

- Cmax (maximum observed concentration)
- Cmin (minimum observed concentration)
- AUC 0-24 (area under the curve for the dosing interval (0-24 hours))
- Absorption
- Distribution
- Tmax (Time to occurrence of Cmax)
- Tmin (Time of occurrence to Cmin)
- t1/2
- Clearance (K)

Pharmacology data will allow for the determination of optimal dosing and dosing frequency. In addition, previous studies investigating high dose rifampicin in human subjects have demonstrated decreased bacterial load starting 14 days after treatment, suggesting that moderate increases in rifampicin leads to steeper declines in bacterial load over time (Boeree et al., 2011). To test this, sputum samples will be collected at 2 weeks, 3 weeks, 4 weeks and every two weeks thereafter to measure bacterial load and assess the bactericidal effect of higher doses of rifampicin.

Safety will be based on the effect of dose on the number and severity of adverse events in each group. All adverse events will be assigned a severity based on the rating scale of Mild (1), Moderate (2), Severe (3) Life Threatening (4) and Death (5) according to the U.S. National Institute of Health Common Terminology Criteria for Adverse Events 4.0. Only those adverse events assessed as related to product will be included in the final data analysis. Other analyses will include the rate of discontinuation between experiment and control groups (Milstein, 2016). One way ANOVA using SAS PROC
ANOVA will be used to assess the difference in number of adverse events and the time to sputum convert between all five groups. The inhaler dose with the lowest number of adverse events and the shortest time to sputum convert will be chosen as the dose moving into Phase III.

Phase III Trial

The Phase III, randomized, open label, pivotal trial will assess the efficacy and long term safety of the PLGA based fixed dose inhaler in patients with treatment naïve pulmonary TB. To date, few studies have investigated the use of high dose rifampicin and isoniazid to shorten treatment duration, and no studies have investigated the long term safety and efficacy of a respirable PLGA based formulation of anti tubercular drugs. Phase III testing will provide data regarding the safety and efficacy of the high dose rifampicin and isoniazid PLGA based formulation of ATDs. The primary objective of the trial will be to determine efficacy of the investigational formulation. The primary outcomes will be time to sputum conversion, treatment failure and rate of relapse at 12 months (Merle et al., 2014). The secondary objectives will be safety and patient compliance. Secondary outcomes will include the occurrence of Grade 3 and 4 adverse events, death, and the rate of completion of chemotherapy per protocol (Evaluation of high dose rifampicin toxicity in pulmonary tuberculosis, 2010).

Protocol

The trial will be an open label, multinational randomized controlled superiority trial. The study will be conducted in India, South Africa and China, the three countries which have seen the largest increases in TB over the last decade and which have the highest TB burden of any countries worldwide. (TB Facts, n.d.) Inclusion criteria include
adults 18-65 with treatment naïve pulmonary TB, confirmed by sputum culture using BACTEC 960TB. HIV positive patients with CD4+ count >50 cells / µL may be enrolled, however rifampicin is a potent inducer of cytochrome P450 CYP3A4 activity. As such, patients currently taking the HIV drugs Nevirapine™, Maraviroc™ Rilvirapine™ must be willing to switch to Efavirenz™ or another HIV medication whose efficacy is not impacted by rifampicin co-administration (Centers for Disease Control, 2014; Laloo, 2009). Women of child bearing age must be willing to utilize double barrier contraception throughout the course of the trial and must be willing to submit to a pregnancy test prior to enrolment. Exclusion criteria include prior treatment for TB, prior exposure to second line or third line TB drugs, pregnant or lactating women; patients who, in the opinion of the investigator, are unlikely to survive the first six months of the trial, and patients with preexisting liver disease, cirrhosis or other hepatic disorders and HIV patients with CD4+ count <50 cells / µL, or subjects with the following toxicities at screening: absolute neutrophil count (ANC) < 1000/ mm3; aspartate aminotransferase (AST) ≥5.0 x ULN, alanine aminotransferase (ALT) ≥5.0 x ULN, total bilirubin ≥2.0 x ULN, or ≥1.50 x ULN and serum creatinine level greater than 2 X ULN (A Phase 3 Study Assessing the Safety and Efficacy of Bedaquiline Plus PA-824 Plus Linezolid in Subjects With Drug Resistant Pulmonary Tuberculosis, 2016).

Subjects will be randomized into one of two groups. The control treatment group will receive the current standard of care consisting of first line TB treatment (rifampicin 10 mg/kg, isoniazid 5 mg/kg, pyrazinamide 25 mg/kg, ethambutol 15 mg/kg) and the experimental group will receive the investigational product, the PLGA based DPI containing pyrazinamide 25 mg/kg, ethambutol 15 mg/kg and the highest doses of
rifampicin and isoniazid drugs deemed safe as determined in the Phase II trial. The trial will be based on the assumption that there is no difference in efficacy and safety between the investigational product and the active control, given by Ho = μc - μd = 0, where μc is the active control and μd is the investigational product. The alternative hypothesis is that there exists a difference in efficacy and safety between the two products given by Ha = μc - μd ≠ 0. The trial will use a 5% level of significance for both Type I and Type II error (α= 0.05, β=0.05). The total number of subjects will be determined using the formula:

\[
2N = 4(Z \alpha + Z \beta)2\delta^2
\]

Based on a projected sputum conversion rate of approximately 85% in the control group and 90% in the experimental group, α of 0.05 and power of 80% the sample size for both groups is projected to be approximately 686, for a total of 1372 subjects (ClinCalc, n.d.). Subjects will be randomized using the SAS (Statistical Analysis Software) RANUNI feature to generate random number values from a uniform distribution. Block randomization will be used to maximize the comparability of the treatment groups (Deng & Graz, n.d.).

Subjects will self-administer with their assigned inhalers once every 72 hours under supervision by clinic staff. Self-administration will take place over for 16 weeks, at which point sputum samples will be collected and cultured. Primary outcome will be time to culture conversion. Both active TB infection and culture conversion will be confirmed using BACTEC 960TB. Treatment failure and relapse at 12 months will constitute a composite endpoint (Phillips et al., 2016). Twelve months is considered a sufficient length of time because approximately 75% of relapse occurs within six months of
stopping treatment, however extending the follow up to 24 months will provide additional clinically meaningful safety data (Phillips et al., 2016). In particular, 24 month follow up will allow for the detection of Treatment Emergent Adverse Events (TEAEs), and Adverse Events of Special Interest (AESIs) not detectable in the shorter Phase II trial.

Previous studies studying high dose rifampicin have resulted in thrombocytopenia, a rare but often fatal blood disorder (Poole et al., 1971). Cases of thrombocytopenia arising from isoniazid, ethambutol and pyrazinamide are exceptionally rare; almost all cases of thrombocytopenia from TB drugs are due to rifampicin (Sharma, Sharma, & Bansal, 2013; Yakar, Yildiz, Yakar, & Kilicazlan, 2013). Although only 35 cases of rifampicin induced thrombocytopenia have been published since 1970, (Sharma, 2013) it is a potentially life threatening event. Subjects in the trial who experience thrombocytopenia, confirmed by a platelet count of < 150 × 10^3 per μL (Gauer, 2012) and a direct Coombs test will be removed from the protocol. If, after treatment with corticosteroids and a platelet transfusion, the patient again becomes thrombocytopenic upon regimen re-exposure, the event will be treated as rifampicin induced thrombocytopenia (Mori et al., 2010). All instances of rifampicin induced thrombocytopenia will be included in the Intent to Treat (ITT) data analysis.

Non compliance with treatment regimen will be defined as two or more consecutive doses during the intensive phase, and more than six consecutive doses during the continuation phase for the control treatment group, or more than 10% of all doses over a period of 4 months for the experimental group or 6 months in the treatment control group (Merle, 2014). One way ANOVA using SAS PROC ANOVA will be used to
assess the difference in number of adverse events and the time to sputum convert between groups. Safety in the control group and the experimental group will be analyzed using ANOVA. Kaplan Meier Analysis will be used to compare the rates of hepatotoxicity reactions in patients in experimental and control groups at end of the study.
Chapter VI

Discussion

TB now kills more people annually than HIV and malaria combined. (Pai & Furin, 2017) Although the last several years have witnessed a decrease in the mortality rate, the number of people infected annually continues to rise (Siroka, 2016; World Health Organization, 2015). Inconsistent reporting of new TB cases and outdated detection methods remain a significant problem: the World Health Organization recently acknowledged that the TB epidemic is likely much worse than previously believed. WHO now estimates that fewer than 60% of new cases worldwide are reported to health authorities annually (World Health Organization, 2016). Improved reporting at the local level is therefore essential for ensuring more accurate estimates of the global TB burden; however large groups of poor, marginalized people in the developing world with little or no access to health care services pose a major challenge in this regard (World Health Organization, 2016). Case reporting in many populous countries, most notably India, remains very poor. According to WHO estimates, approximately 41% of new cases in India go undiagnosed, and approximately 54% of new cases fail to be reported to national authorities (World Health Organization, 2016). Greater coordination between governments at the local and national level is therefore essential for improving case reporting and detection so that the necessary financial resources can be allocated to regions where they are most needed.
The continued use of outdated diagnostic tests compounds existing shortcomings in case reporting at all levels (Pai, 2017; Sharma et al; 2017, Thaiss, 2012). Although the sputum smear test remains the most commonly utilized TB test, its reliability as a diagnostic tool is heavily dependent on the experience and expertise of the individual conducting the test. As a result, test sensitivity is highly variable, ranging anywhere from 32% to 97% (Thaiss, 2012). Sensitivity is also severely reduced in samples with fewer than 10,000 bacilli/mL per sputum sample, and in patients co-infected with HIV (Thaiss, 2012). Accurate data regarding TB incidence will continue to prove elusive as long as community health facilities continue to rely upon outdated diagnostic methods.

Moreover, sputum culturing can take several weeks, which increases person to person transmission, and leads many patients to decide against seeking further treatment (Millen, Uys, Hargrove, van Helden, & Williams, 2008). Although advanced diagnostic systems like the Xpert® MTB/RIF testing system - a cartridge based nucleic amplification assay which detects TB and rifampicin resistance in under two hours - have begun to see more widespread use, they constitute a small number of test systems in countries with high TB burden (Thaiss, 2012, Pai & Furin, 2017).

Although improvements in diagnostic testing will improve case reporting, additional initiatives are necessary to combat the continued spread of TB. There is now a substantial body of scientific data which suggests that current levels of rifampicin and isoniazid are well below those required for optimal therapeutic efficacy (Boeree et al., 2015; Heysell, Moore, Keller, & Houpt, 2010; Hu et al., 2015; Milstein et al., 2016; Peloquin, 2001). Additional studies are necessary to determine whether higher doses of
rifampicin and isoniazid may shorten the lengthy duration of the current treatment regimen, which is an essential first step towards improving patient compliance.

Finally, the fight against TB cannot succeed without a more effective delivery method for first line TB drugs. The current regimen will continue to be associated with low patient compliance due to the heavy pill burden and side effects associated with first line ATDs. The World Health Organization’s recommendation that fixed dose combination tablets be used whenever possible is a positive development: rates of MDR-TB are lower in countries where FDCs have been widely used for many years (Blomberg, 2001). However, any optimism surrounding this recent change must be tempered by the reality that until a delivery system for first line ATDs is developed which reduces their toxicity, patient compliance will remain at unacceptably low levels. Improving the delivery of ATDs through the use of a small handheld inhaler could help address the most pernicious aspects of the current regimen, namely the toxicity, lengthy treatment course and high pill burden. As long as first line ATDs remain associated with lengthy treatment times and serious side effects, improving patient compliance will prove difficult.

Improving patient compliance lies at the heart of combating TB. Rates of MDR-TB and XDR-TB will continue to rise unless the cure rate is increased (Sharma, 2017). Patients who fail to sputum convert following treatment are more likely to develop MDR-TB and XDR-TB, perpetuating the spread of strains that are difficult to treat (Sharma, 2017). The goal of eradicating TB therefore depends upon the ability of the global community to stop the spread of drug resistant strains of the disease. The continued spread of drug resistant strains of TB remains the single greatest threat to the continued viability of first line antitubercular medications. XDR-TB has now been identified in over
100 countries (TB Alliance, 2015). A recent study suggests that by 2040, the majority of all cases of MDR-TB and half of all new cases of XDR-TB will arise from incident exposure (Sharma, 2017). Increasing numbers of treatment naïve people are becoming infected with strains of TB that are either extremely difficult to treat or refractive to all treatment (Sharma, 2017). This trend is being accelerated by the increased prevalence of those coinfected with TB and HIV, as HIV positive patients are more likely to relapse following treatment, and more likely to develop MDR-TB and XDR-TB. The HIV epidemic is therefore fueling the spread of drug resistant strains among treatment naïve populations, particularly in Sub Saharan Africa (Thaiss, 2012). This alarming trend will continue to undermine the ability of the global community to combat the global spread of TB (Dambrosio et al., 2015; Sharma, 2017).

There is now a large body of research which suggests that a small dry powder inhaler would serve as an ideal method of administering ATDs. By avoiding first pass metabolism, a DPI would significantly reduce the side effects which have until now proven to be such an impediment to patient compliance. Second, pulmonary delivery would allow drugs to be targeted to the site of infection directly, allowing for faster onset of action and allow for greater bioavailability in lung tissue (Cheng, 2007, Hoppentocht et al., 2014). Third, the ability to self administer ATDs on a less frequent basis would confer a distinct advantage over the current regimen. However, despite the enormous promise held by this delivery method, no such inhaler is in development.

It is incumbent upon industry and government to develop a more effective delivery system to combat the TB epidemic. First line tuberculosis drugs were developed decades ago and longer enjoy patent exclusivity. As a result, their commercial potential is
limited (Gardner, 2015). Lack of financial incentive has led pharmaceutical companies to halt most research and development of new TB drugs, and thus far the majority of research being conducted by product development partnerships has met with little success. A Phase I trial for Pretomanid, one of only a handful of promising TB drugs to emerge over the last several years, was recently halted due to neurotoxicity. Trials of another drug, the nitroimidazole TBA-354, were halted in 2016 due to severe side effects, and development of AZD5847, an oxazolidinone which had been in development for several years, was halted in 2016 due to lack of efficacy (Lessem, 2017).

However, lack of patent exclusivity is not the main barrier to developing a DPI for anti-tubercular drugs. The generic drug market - which includes those drugs used in the majority of asthma and COPD inhalers - generates billions of dollars annually for the pharmaceutical industry. The majority of inhaled corticosteroids and beta agonists used in inhalers are off patent, yet the asthma inhaler market is estimated to be worth 34.3 billion USD by 2020 (Thakur, 2017). The greatest obstacle to developing a DPI for ATDs may be the fact that the vast majority of people who comprise the market for ATDs live in third world countries, where any return on investment is likely to be limited (Schwalbe, 2008, Schwalbe & Wells, 2008). The lack of financial incentive on the part of pharmaceutical companies is an unfortunate reality, but one which cannot be ignored. Despite this, there are examples of partnerships between non-profit organizations and non-governmental agencies working together to provide drugs to the poor and underserved. The Drugs for Neglected Diseases Initiative (DNDi) is one such initiative. DNDi is a non-profit outfit funded by government and philanthropic organizations which aims to develop drugs with little commercial potential. In 2013, DNDi successfully launched
ASMQ - a fixed dose combination of Artenuate and Mefloquine - for the treatment of malaria. ASMQ has no patent, costs less than 1 USD per dose, and has been administered to over 250 million times to patients in over 31 countries (Wells, Diap, & Kiechel, 2013). ASMQ is an example of an effective, low cost treatment for a life threatening illness that was developed in the absence of industry support. A similar approach may prove viable in the development of a DPI for antitubercular drugs, and such a development pathway should be explored.

The goal of the WHO End TB Strategy 2016-2035 is to limit the annual incidence of TB to fewer than 10 cases per 100,000 (Laurence, Griffiths, & Vassall, 2015). The steadily increasing prevalence of MDR-TB and XDR-TB suggests that this goal may be unrealistic without significant changes to the current treatment regimen. The question for the global community is this: how much longer will first line anti tubercular drugs retain their efficacy before treatment resistant strains become so prevalent that the current treatment regimen can no longer be relied upon to treat TB. This is not a question that can be answered with any degree of certainty. However, the continued spread of drug resistant strains of TB throughout the developed and developing world suggests that without an improved method of delivery for anti tubercular drugs, this time may arrive much sooner than anticipated.
Appendix

Figure 3. Image of Spiriva Handihaler®
Figure 4. Relenza Diskhaler®. (Photograph courtesy of GlaxoSmithKline)
Figure 5. Schematics of the Serevent Diskus® (left) and Advair Diskus® (right) powder inhalers. (Courtesy of GlaxoSmithKline)
Figure 6. Image of the NEXThaler® manufactured by Chiesi Farmaceutici SpA.
Appendix C: Toxicity Table – DMID Adult toxicity Table, November, 2007

ABBREVIATIONS: Abbreviations utilized in the Table:

ULN = Upper Limit of Normal   LLN = Lower Limit of Normal
Rx = Therapy                   Req = Required
Mod = Moderate                 IV = Intravenous
ADL = Activities of Daily Living Dec = Decreased

ESTIMATING SEVERITY GRADE
For abnormalities NOT found elsewhere in the Toxicity Tables use the scale below to estimate grade of severity:

GRADE 1  Mild     Transient or mild discomfort
          (< 48 hours); no medical intervention/therapy required
GRADE 2  Moderate Mild to moderate limitation in activity - some assistance may be needed; no or
          minimal medical intervention/therapy required
GRADE 3  Severe   Marked limitation in activity, some assistance usually required; medical
          intervention/therapy required, hospitalizations possible
GRADE 4  Life-threatening Extreme limitation in activity, significant assistance required; significant medical
          intervention/therapy required, hospitalization or hospice care probable

The grading of the laboratory AEs will be based on the DMID toxicity table.
### CHEMISTRIES (continued)

<table>
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<th>Grade 1</th>
<th>Grade 2</th>
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<th>Grade 4</th>
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</thead>
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<tr>
<td>Bilirubin (mg/dl)</td>
<td>1.1 - &lt;1.25 x ULN</td>
<td>1.25 - &lt;1.5 x ULN</td>
<td>1.5 - 1.75 x ULN</td>
<td>&gt; 1.75 x ULN</td>
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<tr>
<td>Hyperbilirubinemia (when accompanied by any increase in other liver function test)</td>
<td></td>
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<tr>
<td>Bilirubin (mg/dl)</td>
<td>1.1 - &lt;1.5 x ULN</td>
<td>1.5 - &lt;2.0 x ULN</td>
<td>2.0 - 3.0 x ULN</td>
<td>&gt; 3.0 x ULN</td>
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<tr>
<td>Hyperbilirubinemia (when other liver function are in the normal range)</td>
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<td>BUN</td>
<td>1.25 - 2.5 x ULN</td>
<td>2.6 - 5 x ULN</td>
<td>5.1 - 10 x ULN</td>
<td>&gt; 10 x ULN</td>
</tr>
<tr>
<td>Creatinine</td>
<td>1.1 - 1.5 x ULN</td>
<td>1.6 - 3.0 x ULN</td>
<td>3.1 - 6 x ULN</td>
<td>&gt; 6 x ULN or dialysis required</td>
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### ENZYMES

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<th>Grade 2</th>
<th>Grade 3</th>
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<tbody>
<tr>
<td>AST (SGOT)</td>
<td>1.1 - &lt;2.0 x ULN</td>
<td>2.0 - &lt;3.0 x ULN</td>
<td>3.0 - 8.0 x ULN</td>
<td>&gt; 8 x ULN</td>
</tr>
<tr>
<td>ALT (SGPT)</td>
<td>1.1 - &lt;2.0 x ULN</td>
<td>2.0 - &lt;3.0 x ULN</td>
<td>3.0 - 8.0 x ULN</td>
<td>&gt; 8 x ULN</td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>1.1 - &lt;2.0 x ULN</td>
<td>2.0 - &lt;3.0 x ULN</td>
<td>3.0 - 8.0 x ULN</td>
<td>&gt; 8 x ULN</td>
</tr>
</tbody>
</table>


Rubinstein, L, Simon, R. (n.d.) Phase I Clinical trial design. Biometric Research Branch, National Cancer Institute, 1–25


