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STRATEGIES TO COMBAT DRUG-RESISTANT TUBERCULOSIS: INHALABLE MICROPARTICLES FORMULATED WITH ANTI-TUBERCULAR ANTIBIOTICS AND EFFLUX PUMP INHIBITORS

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Strategies to Combat Drug-Resistant Tuberculosis: Inhalable Microparticles Formulated with Anti-tubercular Antibiotics and Efflux Pump Inhibitors

BY

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THESIS

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DEDICATION

I dedicate this work in loving memory of my father, Wien Miller, my grandmother, Unis Kinoshita, my grandfather, George Kinoshita, and my aunt, Krystal Kinoshita. I could not have become half of the man I am today without these people acting constantly as role models, mentors, and friends whom fostered hardworking ideals and gave me the absolutely best upbringing anyone could ask for. I think of these people every day of my life, and I hope that I have made them proud with the work I have accomplished and the person I have become.

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STRATEGIES TO COMBAT DRUG-RESISTANT TUBERCULOSIS: INHALABLE MICROPARTICLES FORMULATED WITH ANTI-TUBERCULAR ANTIBIOTICS AND EFFLUX PUMP INHIBITORS

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ABSTRACT

Mycobacterium tuberculosis (*Mtb*) is an intracellular pathogen which can remain dormant within the human body for years (latent tuberculosis), and once active requires extensive multidrug therapies for treatment. Long-term antibiotic(s) exposure have led to the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains which require novel treatment approaches to combat *Mtb* resistance. *Mtb* may also utilize efflux pumps to expel antibiotics and promote bacterial survival, potentially mediating drug tolerance, which has been hypothesized to contribute to antibiotic resistance. Efflux pump inhibitors (EPIs) such as verapamil (VER) may potentiate effects of first-line anti-tubercular drugs (ATDs) such as rifampicin (RIF).

The goal of this project is to formulate and characterize a microparticulate inhalational delivery system consisting of a sustained released (SR) EPI and immediate release (IR) RIF. This formulation can be beneficial by (1) allowing for higher bactericidal concentrations at the site of infection when compared to systemic delivery, (2) improved bactericidal killing with efflux pump inhibition,

potentially leading to shorter treatment durations, and (3) combating mechanisms that can contribute to the selection of drug-resistant *Mtb.*

We *hypothesize* that formulating the VER (the model EPI) into a SR system through the integration of the polymer Eudragit E PO (EPO) in combination with IR RIF through the integration of mannitol and leucine (ML) will potentiate the anti-tubercular activity of our formulation against *Mtb*, compared to traditional systemic delivery with no EPI. The slow release of VER will potentially prolong the inhibition of efflux pumps; further, concentration-dependent RIF will be immediately released thus allowing high intra-bacterial concentrations of the ATD. This novel formulation exploits properties of the EPO polymer, which is soluble at pH below 5.0, such that once the microparticles are taken up by the alveolar macrophage (in which *Mtb* resides), the entire payload will be immediately released. Such a formulation could improve the sterilizing activity of RIF which could decrease the total amount of ATD required and potentially shorten treatment durations.

Microparticles (MPs) with VER and RIF were formulated using a Buchi 290 laboratory scale mini-spray dryer in aqueous solvents. Briefly, MPs of IR-RIF, IR-VER, and SR-VER were prepared. These powders were further mixed homogenously to produce the formulations of interest (IR-RIF/IR-VER and IR-RIF/SR-VER). MPs were characterized for volumetric and aerodynamic particle sizing using Malvern Mastersizer 3000 with an attached dry powder disperser (Aerosizer), and a Next Generation Impactor (NGI, Copley Scientific, UK), respectively. For all powders (individual and mixed), we were able to achieve a volumetric diameter (D-50) in the size range of 1.77 - 2.63 μm. Further, we were

able to achieve a mass median aerodynamic diameter (MMAD) in the size range 2.45 – 4.95 μm for the above powders. With regard to the mixed powders, the IR-RIF/IR-VER formulation achieved a RIF:VER ratio of 2.33:1 (wt/wt). The IR-RIF/SR-VER formulation achieved a RIF:VER ratio of 2.58:1 (wt/wt). For the IR-RIF/IR-VER mixed powder both drugs showed approximately 70% drug release at 4 hours. With the IR-RIF/SR-VER mixed powder, however, the RIF component achieved only 60% drug release at 4 hours where the VER component was able to achieve approximately 80% release. For both formulations a release of nearly 100% was achieved for both the EPI and ATD.

The *in vitro* cytotoxicity of the IR-RIF/IR-VER formulation at 1 milligram (of dry powder) per milliliter after 24 hours of exposure led to only 14.47% $\pm$ 0.29% THP-1 cellular viability. Lower concentrations achieved higher cell viability, with approximately 45% for the 500 micrograms per milliliter, and over 99% cellular viability at concentrations of 250 micrograms per milliliter or less. IR-RIF/SR-VER formulation was around 30% cell viability for all concentrations evaluated. The *in vitro* bacterial (*Mycobacterium smegmatis*) killing of the IR-RIF/IR-VER formulation achieved higher killing of the mycobacterium at lower drug concentrations when compared to that of the IR-RIF/SR-VER formulation.

The above data demonstrated the feasibility of spray drying to develop an inhalable dry powder formulation with properties suitable for delivery to the deep lung (based on aerodynamic size), macrophage uptake (based on volumetric size), toxicity studies (in THP-1 cells) and mycobacterial killing (using *M. smegmatis* as a surrogate mycobacteria). The data also suggest that our IR formulation was less

toxic to macrophages and more effective in mycobacterial killing at lower concentrations than our SR formulation. The drug release studies, however, provided inconclusive results for both the formulations.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

Introduction

Tuberculosis (TB) is an airborne pathogen that infects ~10.4 million people and results in 1.8 million deaths annually; this makes TB the leading cause of death from a single infectious agent, ranking above HIV/AIDS (WHO, 2017). TB can remain dormant within the human body for years without showing any symptoms; however once active, treatment requires complex and extensive multidrug therapies administered over 6 to 9 months (CDC, 2014b). This long-drawn and demanding treatment has led to the emergence of drug-resistant *Mycobacterium tuberculosis* (*Mtb*); future therapies may require a different approach to combat the further selection and advancement of these dangerous multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of TB (CDC, 2014a). Identifying the mechanisms by which these organisms acquire resistance is critical to combat drug resistance. This will allow first-line therapies to remain viable, thus extending the longevity of the limited antibiotics that provide favorable outcomes in TB treatment. One such mechanism has been identified that *Mtb* employs to avoid death. *Mtb* utilizes efflux pumps to remove intracellular drug and thus promote bacterial survival, mediating variable levels of drug tolerance ((Gupta et al., 2013), (Adams et al., 2011)). Researchers have successfully repurposed drugs such as verapamil (VER) and thioridazine (TRZ) and have shown them to be effective efflux pump inhibitors (EPIs), thus combating drug tolerance mechanisms of TB as well as potentiate the effects of first-line treatment options such as rifampin (RIF) and isoniazid (INH) ((Gupta et al., 2013), (Vibe et al., 2016)). Various ways of incorporating these combination drugs (EPIs and anti-TB drugs) into a single

formulation and determining the best route of administration and optimal drug release profile for each antibiotic remains to be resolved.

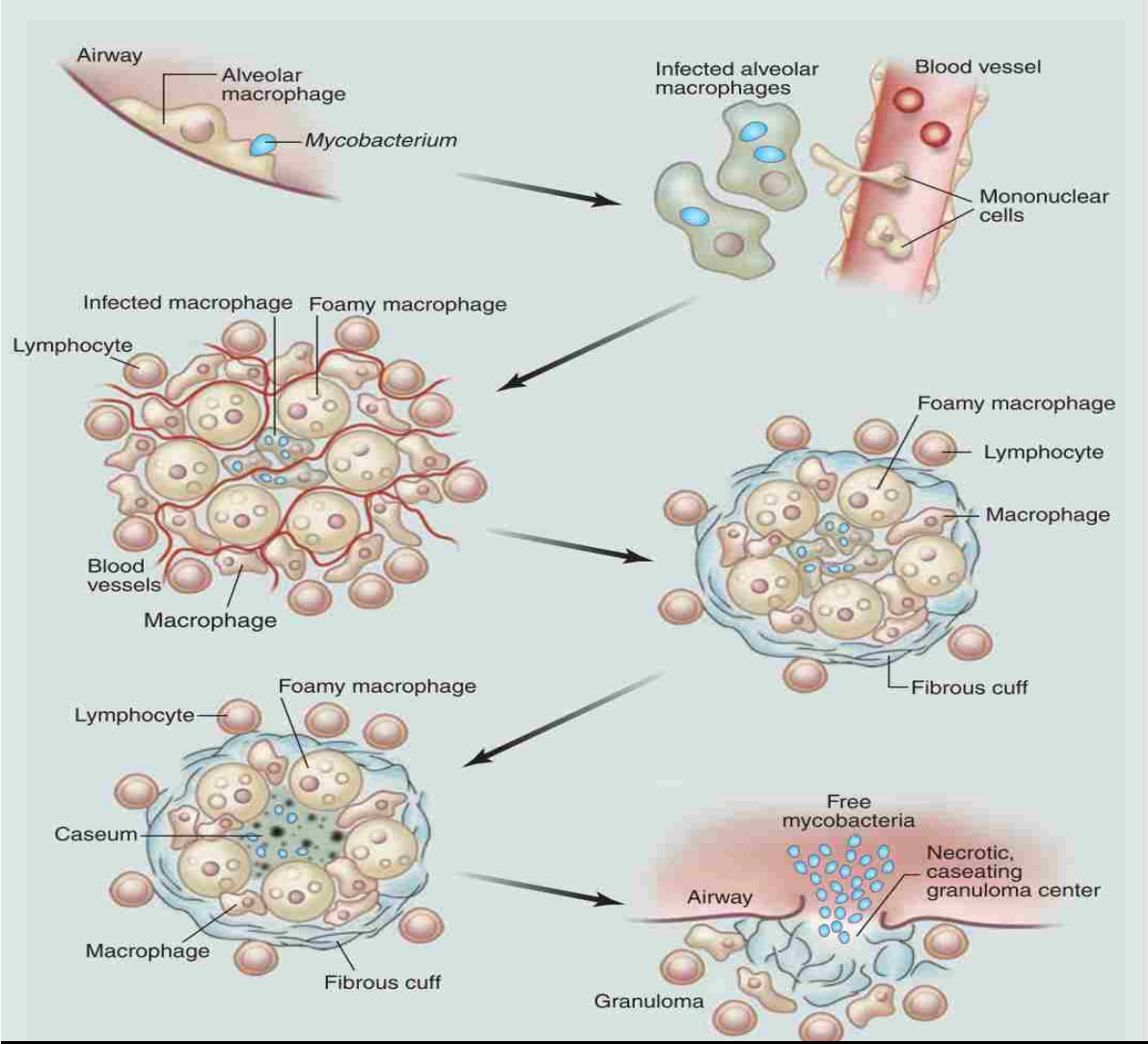
Tuberculosis Pathogenesis

TB is an infectious disease most often affecting the lungs. TB's causative pathogen is the bacterium *Mtb.* This opportunistic bacterium invades the host via airborne droplet nuclei, however other methods of host invasion occur via the gastrointestinal tract from ingestion or via cutaneous routes in compromised skin (Nicas, Nazaroff, & Hubbard, 2005). The primary source of infection, droplet nuclei, are respired from one infected host after a cough or sneeze and can remain suspended in the air for hours (Nicas et al., 2005).

After inhalation of droplet nuclei has been achieved in a susceptible host, bacteria are deposited in the lung, where the *Mtb* will be phagocytosed by alveolar macrophages (Philips & Ernst, 2011). Once inside the macrophage, *Mtb* employs strategies to prevent bacterial death by preventing formation of the phagolysosome, which gives the bacteria an environment to multiply and a means to manifest disease outside of the lungs (Marino et al., 2014). The TB infection can potentially migrate out of the lungs via the lymphatic system and will most often disseminate to the liver, spleen, kidney, bone and brain (Philips & Ernst, 2011), (O'Garra et al., 2013). Within 4 weeks of infection taking residence within macrophages, a cell-mediated immunity is initiated which recruits cytotoxic T-cells to kill the *Mtb*-infected macrophages. This cascade of events triggers a delayed-type hypersensitivity (DTH) response, promoting invasion of phagocytic cells which release fluid and digestive enzymes to the sites of infection. This DTH

response causes inflammation which is the primary source of damage to the lungs and other tissues that occurs in TB infection (Price & Muttil, 2016), (Ehrt & Schnappinger, 2009).

Another fate of *Mtb*-infected macrophages is isolation within granulomas, which is generally a cluster of macrophages, lymphocytes, fibroblasts and giant cells. These granulomatous formations will generally create a hostile, anaerobic environment for the *Mtb*, which restricts the bacteria's ability to multiply, slowly decreasing the bacterial burden and allowing the granuloma to form fibrous caps



(Marino et al., 2014), (Russell, Barry, & Flynn, 2010). Figure 1.1 shows the potential pathway that a bacterium from an inhaled droplet can manifest inside a granuloma which could ultimately result in the release of Mtb to cause active infection.

Primary Tuberculosis Treatment

Although TB may require treatment durations of at least 6 months, it is a treatable infection for many inflicted with this disease. There are many ATDs approved for the treatment of TB and are shown in Table 1.1. However, other agents which are currently not approved are used when preferred treatment fails (Table 1.1). First line therapy for the treatment of susceptible *Mtb* consists of isoniazid (INH), rifamycins such as rifampicin or rifampin (RIF), pyrazinamide (PZA), and ethambutol (EMB). For the first 2 months of treatment, the *Mtb*-infected patient will take all 4 of these ATDs together which is known as the initial treatment phase. For the remaining 4 months of treatment, the patient will receive only RIF and INH in combination in what is known as the continuation phase (CDC, 2014b).

Treatment of pulmonary, susceptible TB may seem to be a relatively straight forward regimen, however aspects of the disease greatly complicate outcomes such as the emergence of variably resistant *Mtb*, access to healthcare, a patient's willingness to complete the lengthy and rigorous treatment, and the marked side effect profile of these medications (Table 1.1). Various treatment modalities, including newer approved ATDs, are available for the treatment of these complex infections; yet one of the goals of current TB therapy is to extend the longevity of first-line ATDs. Understanding the mechanisms of tolerance and resistance is key

to achieving this goal. Recent strategies of *Mtb* treatment have been targeted at these aforementioned mechanisms, and will be discussed further in the coming sections.

Table 1.1. Anti-Tubercular Drug Chart: Routes of Administration, Side Effects, and Special Considerations
Table adapted from Uptodate, IDSA, and Sanford Guidelines
* Dosing based on most frequent dosing strategies. Other dosing strategies may be available for each individual agent

First-Line Anti-Tubercular Agents			
Agent:	Mechanism of Action:	Dosing Strategy*:	Common and/or Serious Adverse Effects and Special Considerations:
Isoniazid	Inhibits mycolic acid synthesis	Daily	Hepatotoxicity, neurologic reactions (pyridoxine can prevent neurologic side effects), monoamine oxidase inhibitor-like activity (special care with food high in tyramine)
Rifampin	Inhibits transcription	Daily	Red-orange discoloration of body fluids, hepatitis, cutaneous vasculitis, red cell aplasia, leukopenia, agranulocytosis, thrombocytopenia, P-gp and CYP3A inducer (many drug interactions)
Rifabutin	Inhibits transcription	Daily	Red-orange discoloration of body fluids, hematologic and hepatotoxic effects similar to rifampin, uveitis, UGT1A1 and CYP3A inducer (many drug interactions)
Rifapentine	Inhibits transcription	Once Weekly	Red-orange discoloration of body fluids, GI toxicity, hepatotoxicity, hyperuricemia, LFT elevations, CYP system inducer, do not use in patients with HIV
Pyrazinamide	Converted to pyrazinoic acid; acidifies *Mtb* environment	Daily	Hepatotoxicity, GI toxicity, cutaneous hypersensitivity, thrombocytopenia, anemia

Ethambutol	Inhibits arabinosyl transferase; impairs mycobacterial cell wall synthesis	Daily	Optic neuropathy, neutropenia, thrombocytopenia, GI toxicity, hepatotoxicity, CNS toxicity, cutaneous hypersensitivity
Second-Line Anti-Tubercular Agents:			
Fluoroquinolones			
Levofloxacin	Inhibits DNA Gyrase and DNA Topoisomerase II; prevents supercoiling	Daily	GI toxicity, CNS toxicity, rash, glycemic changes, tendonitis/tendon rupture, QT prolongation
Moxifloxacin	Inhibits DNA Gyrase and DNA Topoisomerase II; prevents supercoiling	Daily	GI toxicity, CNS toxicity, rash, glycemic changes, tendonitis/tendon rupture, QT prolongation, hepatotoxicity
Injectable Agents			
Amikacin	Binds 30S ribosomal subunit; inhibits protein synthesis	Daily	Ototoxicity, nephrotoxicity, electrolyte abnormalities, IM injection site pain
Kanamycin	Binds 30S ribosomal subunit; inhibits protein synthesis	Daily	Ototoxicity, nephrotoxicity, electrolyte abnormalities
Capreomycin	Inhibition of protein synthesis	Daily	Ototoxicity, nephrotoxicity, electrolyte abnormalities, IM injection site pain
Streptomycin	Binds 30S ribosomal subunit; inhibits protein synthesis	Daily	Ototoxicity, nephrotoxicity, electrolyte abnormalities, IM injection site pain
Additional Core Second-Line Agents			
Ethionamide	Inhibits peptide synthesis	Twice Daily	GI toxicity (premedicate with antiemetics), hepatotoxicity, metallic taste, neurotoxicity (pyridoxine can help prevent neurologic side effects), hypothyroidism
Cycloserine	Inhibits bacterial cell wall synthesis	Twice Daily	CNS toxicity, peripheral neuropathy (pyridoxine can help prevent neurologic side effects), serious cutaneous hypersensitivity
Linezolid	Binds 50S ribosomal subunit; inhibits bacterial protein synthesis	Daily	GI toxicity, neuropathy (pyridoxine can help prevent neurologic side effects), myelosuppression

Clofazimine	Exact mechanism unknown	Daily	Red discoloration of body fluids, GI toxicity, photosensitivity, requires application to the US FDA
Add-On Agents			
High-Dose Isoniazid	See Isoniazid above	Three Times Weekly	See Isoniazid above
Bedaquiline	Inhibits mycobacterial proton transfer chain; interrupts ATP generation	Daily	QT prolongation, hepatitis, GI toxicity
Delamanid	Inhibits mycolic acid synthesis	Twice Daily	QT prolongation, GI toxicity
Para-aminosalicylic acid	Inhibits folate synthesis	Three Times Daily	GI toxicity, hepatotoxicity, hypothyroidism
Imipenem-cilastatin	Inhibits transpeptidation of peptidoglycan; inhibits bacterial cell wall synthesis	Twice Daily	GI toxicity, seizures
Meropenem	Inhibits transpeptidation of peptidoglycan; inhibits bacterial cell wall synthesis	Three Times Daily	GI toxicity, seizures
Amoxicillin-clavulanate	Inhibits transpeptidation of peptidoglycan; inhibits bacterial cell wall synthesis	Three Times Daily	GI toxicity
Thioacetazone	Inhibits mycolic acid synthesis	Daily	GI toxicity, myelosuppression, hepatitis, peripheral neuropathy, do not use in patients with HIV

Resistance Mechanisms and Strategies of Treatment

Resistance in *Mtb* is most often defined as a chromosomal change which emerges only after repeated exposure to ATDs, suboptimal prescription access, or poor patient adherence (Zhang & Yew, 2009). In particular, multi-drug resistant TB (MDR-TB), which is defined as *Mtb* which is resistant to INH and RIF, and extensively drug-resistant TB (XDR-TB), which is defined as *Mtb* which is resistant to INH and RIF plus any fluoroquinolone and at least one injectable agent (Table

1.1), have been of concern regarding medication changes to first-line treatments and negatively impacting therapeutic outcomes (CDC, 2014a). When it has been determined that a patient is infected with resistant TB, the course of treatment as well as the effective agents used in the course of treatment are drastically affected. This new ATD regimen will, however, consist of first and second-line agents based on multiple factors (Table 1.1) (CDC, 2014b).

The mechanisms of resistance for these antibiotics has been well studied, and exact gene mutations have been identified for the drugs which are most often implemented in drug resistance (Table 1.2). Understanding these precise mutations and the underlying physiological effect these mutation have allowed TB research efforts to create medications specifically for MDR and XDR TB, such as bedaquiline and delamanid (Koch, Mizrahi, & Warner, 2014), (Costa-Gouveia, Aínsa, Brodin, & Lucía, 2017). Even with these advances to combat resistant TB, *Mtb* has been able to utilize other mechanisms, such as efflux pump overexpression, to gain resistance against these newer agents (Gupta et al., 2014). This emphasizes the importance of understanding and inhibiting these mechanisms in order to potentiate the use of first line ATDs as well as decrease resistance against the newer agents specifically designed to combat resistant *Mtb.*

Table 1.2. Mechanism of drug resistance in Mtb-active agents. (Adapted from Zhang & Yew, 2009)

Mechanism of Drug Resistance in *Mtb*				
Drug	Gene involved	Gene function	Role of Gene	Mechanism of Action
INH	*katG* *inhA*	Catalase-peroxidase Enoyl ACP reductase	Pro-drug conversion Drug Target	Inhibits mycolic acid synthesis
RIF	*rpoB*	β subunit of RNA polymerase	Drug Target	Inhibits RNA synthesis

PZA	*pncA*	Nicotinamidease/ pyrazinamidease	Pro-drug conversion	Depletes membrane energy
EMB	*embB*	Arabinosyl transferase	Drug Target	Inhibits arabinogalactan synthesis
Streptomycin	*rpsL* *rrs* *gidB*	S12 ribosomal protein 16S rRNA rRNA methyltransferase	Drug Target Drug Target Drug Target	Inhibits protein synthesis
Amikacin/ kanamycin	*rrs*	16S rRNA	Drug Target	Inhibits protein synthesis
Capreomycin	*tlyA*	2'-O-methyltransferase	N/A	N/A
Quinolones	*gyrA* *gyrB*	DNA gyrase subunit A DNA gyrase subunit B	Drug Target	Inhibits DNA gyrase
Ethionamide	*etaA/ ethA* *inhA*	Flavin monooxygenase Enoyl ACP reductase	Pro-drug conversion Drug Target	Inhibition of mycolic acid synthesis
Para-aminosalicylic acid	*thyA*	Thymidylate synthase	Drug Activation (?)	Inhibits folic acid/ iron metabolism (?)

Tolerance Mechanisms: Efflux Pumps

Resistance, however, is preceded by changes within the *Mtb* physiology which does not require mutated gene expression; rather some changes which may result in overexpression. This phenomenon is known as tolerance and fully understanding these mechanisms provides additional methods of combating the selection for resistant *Mtb*. One mechanism of tolerance in *Mtb* is overexpression of efflux pumps which results from various insults to the *Mtb* environment, such as macrophage phagocytosis or ATD exposure (Adams et al., 2011), (Gupta et al., 2013).

Efflux pumps are transmembrane proteins which are found in almost every bacterial species (Sun, Deng, & Yan, 2014). These highly ubiquitous proteins can be categorized into multiple superfamilies based on structural morphology, substrate preference, and the source of energy used to facilitate efflux (Pule et al., 2016). These include the ATP-binding cassette (ABC), the major facilitator superfamily (MFS), the small multidrug resistance (SMR), and the resistance-nodulation-cell division (RND). Figure 1.2 depicts the classes of the aforementioned efflux pumps as well as substrate preference and the source of energy to facilitate efflux.

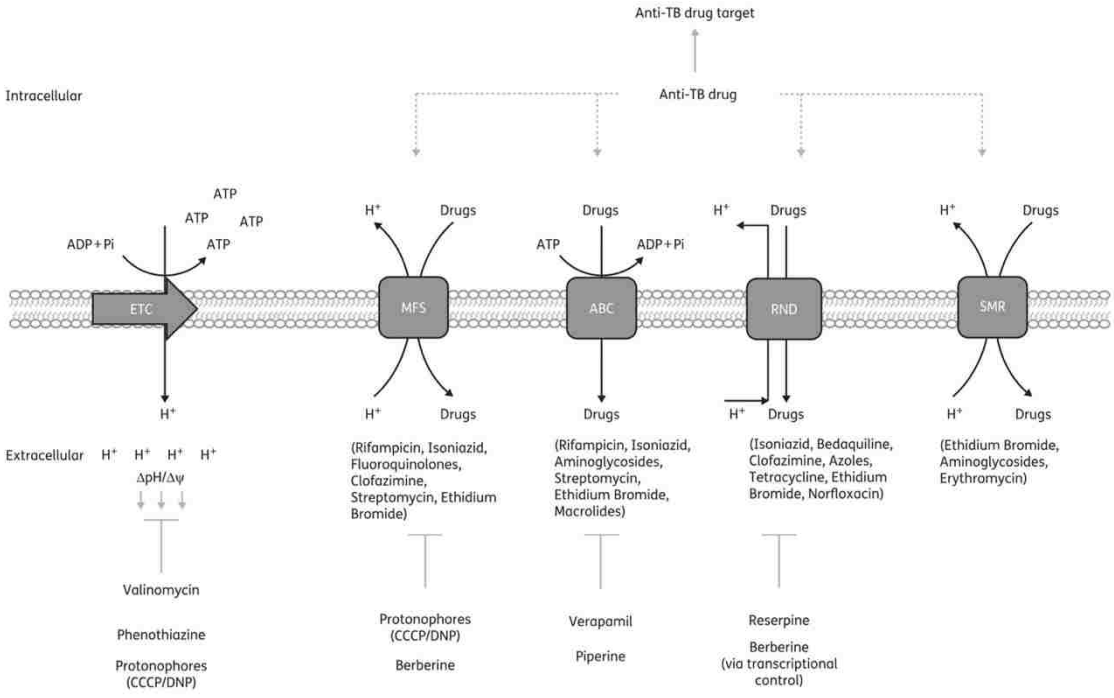


Figure 1.2. Efflux pumps, efflux pump drug targets and efflux pump inhibitors. (From Pule, C. M., Sampson, S. L., Warren, R. M., Black, P. A., van Helden, P. D., Victor, T. C., & Louw, G. E. (2016). Efflux pump inhibitors: Targeting mycobacterial efflux systems to enhance TB therapy. *Journal of Antimicrobial Chemotherapy*, *71*(1), 17–26, by permission of Oxford University Press)

Efflux Pump Inhibitors (EPIs)

Given the limited number of ATDs available, methods of restoring *Mtb* susceptibility to first and second-line agents is crucial given the emergence of MDR and XDR-TB (Pule et al., 2016). Efflux pump inhibition can partially restore susceptibility of a select number of ATDs, extending the longevity of the few agents approved for the use of *Mtb* infections. Some of the efflux pump inhibitors (EPIs) of interest are repurposed drugs which are already approved for separate indications, however have demonstrated great promise in terms of add-on treatment to some *Mtb* treatment regimens. As table 1.3 illustrates, these medications have the drawback of marked side effects as well as dosing strategies that can require a patient to take these medications multiple times a day, however, have much promise in terms of restoring susceptibility in tolerant and resistant *Mtb* infections.

Table 1.3. Repurposed drugs for efflux pump inhibition Repurposed drugs for efflux pump inhibition
Table adapted from Uptodate and Lexicomp
*PO – By mouth; IV – Intravenous; IM; Intramuscular
** Dosing based on most frequent dosing strategies. Other dosing strategies may be available for each individual agent

Efflux Pump Inhibitors (Approved Agents)				
Agent:	Route of Administration*:	Dosing Strategy**:	Therapeutic Indication:	Common and/or Serious Adverse Effects and Special Considerations:
Verapamil	PO, IV	Four times daily	Antianginal, antiarrhythmic, antihypertensive	Headache, gingival hyperplasia, constipation, edema, asystole, bronchospasm, respiratory failure, cannot be used in patients' certain cardiac disorders (i.e. heart failure, certain arrhythmias)

Chlorpro-mazine	PO, IV, IM	Four times daily	First generation antipsychotic	Altered cardiac conduction, extrapyramidal symptoms, anticholinergic toxidrome, agranulocytosis, anemia, leukopenia, cannot use in patients whom use a large amount of CNS depressants, increased risk of death in elderly patients with dementia-related psychosis
Thioridazi-ne	PO	Four times daily	First generation antipsychotic	Dose-related QTc prolongation, anticholinergic toxidrome, blood dyscrasias, CNS depression, extrapyramidal symptoms, increased risk of death in elderly patients with dementia-related psychosis
Reserpine	PO	Once daily	Hypertension, schizophrenia	Cardiovascular toxicity, CNS depression, drug-induced Parkinson's disease, orthostatic hypertension, GI toxicity

Other agents with potential as EPIs are documented in Table 1.4. These agents, however not currently approved, have multiple promising mechanisms of action that are ideal for efflux inhibition. These agents have shown good efficacy at restoring susceptibility to various ATDs, however approval of an ATD

formulation incorporating these agents has the added drawback of approval processes for use in human patients, which has already been overcome in the repurposed drugs used for efflux pump inhibition.

Table 1.4. Experimental efflux pump inhibitor agents and proposed mechanisms

Efflux Pump Inhibitors (Experimental Agents)				
Agent:	Mechanism of action:	Biological Uses:	Mechanism of efflux pump inhibition:	References:
CCCP	Inhibits lactose and amino acid transport by isolated cytoplasmic membrane vesicles	Mitochondrial proton gradient uncoupler	Efficiently disrupts the membrane energy; causes a significant increase in the intracellular accumulation of antibiotic	(Kaback, Reeves, & Short, 1973), (Chevalier, Eyraud, & Pag, 2002)
DNP	Prevents the re-association of coupling enzymes with the electron transport chain	Oxidative phosphorylation uncoupler	Disrupts proton motive force needed by efflux pumps	(Pinchot, 1967), (Zhanel, Hoban, Schurek, & Karlowsky, 2004)
Valino-mycin	Transports potassium ions down their electrochemical gradient	Dodecadesi-peptide antibiotic that conducts ions across membranes	Depletes potassium-generated electrochemic al gradient	(Stillwell, 2016),(Pule et al., 2016)
SILA 421	Eliminates plasmids	Reverses drug-induced resistance	Eliminates plasmids expressing efflux pumps	(Schelz et al., 2007)
Timcodar	Direct efflux inhibition	Increased drug sensitivity cells expressing P-	Direct efflux inhibition; exact mechanism	(Mullin, Mani, & Grossman, 2004),(Gross

		glycoprotein and MRP-1	not currently known	man et al., 2015)
Piperidine	Direct efflux inhibition	Anesthetic, vasopressor action, muscarinic M1 receptor agonist	Ammonio-acetate moiety extends into the antibiotic binding site	(Opperman & Nguyen, 2015),(K.F., B., S.R., & J.C., 1966),(Langmead, 2005)
Piperine	P-glycoprotein, CYP3A4, and glucuronidation inhibitor	Phytochemical that acts as an antioxidant to scavenge for reactive oxygen species	Exact mechanism is unknown; potentially competes for efflux pump binding sites	(Bhardwaj, 2002; Singh, Dubey, & Atal, 1986),(Yaffe, Doucette, Walsh, & Hoskin, 2013)
Berberine	Stabilizes mRNA, inhibits respiration via respiratory complex I in mitochondria	Cholesterol-lowering, improves insulin sensitivity	Limited data	(Li et al., 2004),(Turner et al., 2008)
GEQ Compound Phe-Arg-B-Naphth-amide	RND substrate that occupies the antibiotic-binding site	Novel efflux pump inhibitor	RND substrate that occupies the antibiotic-binding site	(Kourtesi, 2013)

Inhalational TB Treatment and Microparticle Formulation

TB is most often considered a pulmonary disease, as this is the main source of TB infections seen throughout the world. Treatment strategies for TB most commonly use the oral or parenteral route of administration, however these systemic strategies can lead to sub-therapeutic drug levels to the affected organ system which is exaggerated in poorly vascularized regions of the lung (Pham, Fattal, & Tsapis, 2015). Direct pulmonary delivery, however, will allow for high ATD concentration at the primary site of infection (Hickey, Misra, & Fourie, 2013).

Inhalation treatment strategies also have the benefit of effective alveolar macrophage targeting when aerosols deliver particulate drug in the size range of 1 μm to 5 μm (Muttil, Wang, & Hickey, 2009). These inhalational formulations can also decrease marked systemic side effects of the ATDs as well as the EPIs since only a portion of the drug will reach systemic circulation.

Microparticle (MP) formulation strategies are currently of high interest in terms of inhalable delivery of ATDs to the lungs. As previously mentioned, particulate drugs delivered in the volumetric size range of 1 μm to 5 μm are preferable for alveolar macrophage phagocytosis. Particle sizes ranging from 1 μm to 3 μm in aerodynamic diameter have an additional benefit of being deposited in the deep lung; areas rich in alveolar macrophages, thus, the most affected areas of *Mtb* infection (Hickey, Misra, & Fourie, 2016). Formulation strategies have also implemented the use of release-modifying polymers for multiple reasons. First, by controlling the release of ATDs into the lungs this will require the patient to administer these medications less frequently, extending the residence time of these compounds to the affected areas (Misra et al., 2011). Second, by formulating these ATDs and EPIs within biodegradable polymers will help to decrease toxicities of these medications (Vibe et al., 2016).

Verapamil, Rifampicin, and Release-Modifying Polymers: Rationale for Our Formulation

Of particular interest to this project, implementing RIF (ATD) and VER (EPI) into a single, dry powder, MP incorporating a release-modifying polymer offers the ability to restore ATD susceptibility via efflux pump inhibition, as previously

discussed. This dry powder will be formulated using spray drying techniques with the intention for delivery into the lungs with ideal size characteristics for both alveolar macrophage targeting in addition to deep lung deposition. For release-modifying polymers, this project will be implementing Eudragit E PO (EPO). This polymer has many ideal properties for use in this study. It is designed to dissolve below pH 5.0, which is below the physiologic pH of the lungs (pH of 7.38 – 7.43) (Effros & Chinard, 1969), allowing for the polymer to swell and become a permeable polymer matrix (Evonik, 2017). Once the polymer reaches the macrophage microenvironment, we predict that the lower pH inside the macrophage will allow the EPO formulation to act in an immediate release fashion, fully releasing our payload to the site of interest. We hypothesize that because of these properties, this polymer will be an ideal release-modifying polymer for our MP formulation. This polymer also benefits the formulation by being an approved product to be used in humans, although not by the pulmonary route. Other excipients of interest are mannitol and leucine (ML). These excipients will be formulated together to create an immediate-release formulation in contrast to our modified-release formulation. The rationale for our formulation will be to incorporate the EPO polymer with VER, as prolonged inhibition of efflux pumps has been shown to increase intrabacterial levels of ATDs (Adams, Szumowski, & Ramakrishnan, 2014). Since RIF is a concentration-dependent ATD, this agent will be formulated with the immediate-release excipients mannitol and leucine (Gumbo et al., 2007).

Thesis Objectives:

1. Formulate a microparticle formulation of VER and RIF in the size range for deep lung deposition and enhanced alveolar macrophage phagocytosis.
2. Evaluate the properties of this microparticle formulation based on size, aerodynamic diameter, morphology, and drug release profiles.
3. Assess mammalian cell toxicity and mycobactericidal activity of formulation using THP-1 human monocytes as well as *Mycobacterium smegmatis*.

CHAPTER 2: MATERIALS AND METHODS

Materials

Chemicals for dry powder formulation including VER, L-leucine, and D-mannitol were obtained from Sigma Aldrich (St. Louis, MO). RIF was obtained from the Tokyo Chemical Industy (Chuo-ku, Tokyo, Japan), and Eudragit E PO was obtained from Evonik Health Care (Essen, Germany). Chemicals and buffers for HPLC analysis were obtained from the following companies; sodium phosphate monobasic from J.T. Baker Chemical Co (Phillipsburg, NJ), potassium phosphate and OmniSolv acetonitrile for HPLC gradient analysis, spectrophotometry, and gas chromatography from EMD Millipore (St. Charles, MO). The dialysis membrane for the dry powder dissolution analysis, SnakeSkin Dialysis Tubing 10K MWCO 16 mm dry internal diameter, was obtained from Thermo Fisher Scientific (Waltham, MA). Regarding *in vitro* cytotoxicity studies, THP-1 monocyte cell culture was a generous donation from Dr. Eric Neumann's laboratory. RPMI 1640 media (high glucose with L-Glutamine and HEPES) for THP-1 cell culture was obtained from ATCC (Manassas, VA). [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS for the toxicity assay was obtained from Promega (Madison, WI). For *in vitro* efficacy studies, *Mycobacterium smegmatis* was obtained from ATCC (Manassas, VA). Media and agar for *Mycobacterium smegmatis (M. smeg)*, Middlebrook 7H9 with ADC and Middlebrook 7H10 with OADC respectively, were obtained from Becton Dickinson and Company (Sparks, MD).

Microparticle Formulation

Microparticles were prepared using the Buchi-290 mini-spray dryer. For both drugs (VER and RIF), dry powders with individual drugs were formulated. Two types of formulations of VER were made, one with an altered-release formulation utilizing EPO, and another as an immediate release formulation utilizing ML. One formulation of RIF was made which utilized an immediate release formulation of ML. RIF was solubilized in dimethylsulfoxide (DMSO) (10mg/mL), and VER was solubilized using methanol (MeOH) (10mg/mL). EPO was subsequently added to the above formulation by dissolving increasing concentrations into diH_2O at pH < 5.0. pH was adjusted to be < 5.0 for every 10 mg EPO powder added to solution with 1M hydrochloric acid. ML did not require pH adjustments as these excipients are readily water soluble. We used ML in a ratio of 1:1 mannitol:leucine for the immediate release formulations. Specific formulation parameters are as follows; RIF:ML was formulated using 50 mg of RIF powder 25 mg of mannitol, and 25 mg of leucine dissolved in 100 mL of diH_2O. VER:ML was formulated using 25 mg of VER powder with 37.5 mg of mannitol and 37.5 mg of leucine dissolved in 100 mL of diH_2O. VER:EPO was formulated 25 mg of VER and 75 mg of EPO which was dissolved in 100 mL of pH-adjusted diH_2O. For the mixed powder formulations, we wanted to create a 3:1 RIF:VER ratio of the active agents, based on previous data as well as closely representing commonly used oral dosages. This was achieved by combining the spray dried powders in a way that achieved 60% w/w RIF powder with 40% w/w VER powder. These powders were then homogeneously mixed using a 500 μm sieve. RIF:ML, VER:ML powders will from here on be referred to

as RMVM mixed powders. RIF:ML, VER:EPO powders will from here on be referred to as RMVE mixed powders.

Microparticle Characterization

Dry powders formulated using spray drying were characterized for their volumetric particle size, aerodynamic diameter, particle morphology, and drug loading as follows.

Drug Quantification: HPLC Method

Drug loading and mixing efficiency of the microparticles were quantified using an Agilent 1200 high performance liquid chromatography (HPLC) system using a Phenomenex Gemini-NX 5 micron, C18, 110 Å 150 X 4.60 mm column. We developed an HPLC method to detect both VER and RIF samples, which was used for all samples. The initial isocratic phase consisted of 97% acetonitrile (ACN) and 3% potassium phosphate (KPhos) buffer at pH of 7.7 and remained isocratic until 2.0 minutes. The first gradient phase was set between 2.0 and 2.2 minutes to the following isocratic phase. The second isocratic phase consisted of 55% ACN and 45% KPhos buffer at pH of 7.7 and remained isocratic until 7.0 minutes. The second gradient phase was set between 7.0 and 7.2 minutes to the following isocratic phase. The third isocratic phase consisted of 63% KPhos buffer at pH of 7.7 and 37% KPhos buffer at pH 5.2 and remained isocratic until 10.0 minutes. The final gradient phase was set between 10.0 and 11.0 minutes to the final isocratic phase. The final isocratic phase was the same as the first in order to equilibrate the column for the next sample, which again consisted of 97% ACN and 3% KPhos buffer at pH of 7.7 for a total run time of 15 minutes. All samples were

analyzed at 230 nm wavelength, and 50 microliters of sample were injected per run. All samples were analyzed in duplicate. RIF had an elution time of 5.9-6.1 minutes and VER had an elution time of 8.9-9.1 minutes at a flow rate of 1.5 milliliters per minute. All powder samples were prepared using 50% acetonitrile and 50% diH_2O. Individual standard curves were made for VER and RIF in the concentration range of 0.1-25 ug/mL for every HPLC analysis.

Volumetric particle sizing

Sizing of the particles was performed on a Malvern Mastersizer 3000 (Malvern Instruments Ltd. Malvern, UK) with an Aero S dry powder disperser unit. Measurements were taken in triplicate using a standard operating procedure (SOP) developed in our laboratory.

Aerodynamic particle sizing

Aerosol performance of the powders was characterized using a Next Generation Impactor (NGI). Five milligrams of mixed powder were loaded into hydroxypropylmethylcellulose (HPMC) capsules and pierced using the Aerolizer device. These powders were then aerosolized through the NGI at a flow rate of 60 L/min for 5-7 seconds. Each powder sample was run in triplicate, for a total of 15 milligrams per powder. After the 3 samples were ran through the device, powders at each stage, the Aerolizer inhaler device, capsules, rubber adapter, and all stages were collected using 1.5 milliliters of diH_2O and subsequently diluted 1:1 in acetonitrile for maintenance before HPLC analysis. Samples were then diluted 1:10 in acetonitrile and ran using the VER and RIF combined HPLC protocol.

Particle Morphology: SEM Imaging

The surface morphology of the particles was performed using scanning electron microscopy. In short, samples of each individual powder and mixed powder were desiccated thoroughly overnight. These powders were then lightly brushed onto SEM-specific sampling pegs coated with a sticky tape. Images were captured using a Zeiss Sigma FE-SEM; images were acquired with the SE2 detector using a "classical" SEM detection of secondary electrons with an acceleration voltage of 1.00 kV. Each powder was imaged at 3000X magnification and 7250X magnification.

Drug Release

Release of the drug powders were performing using a modified dialysis membrane protocol. Eight centimeters of SnakeSkin Dialysis Tubing was cut and soaked in 0.05 M sodium phosphate buffer (NaPhos) with an adjusted pH of 7.4 to match that of lung fluid. A 50 mL conical tube was filled with 30 mL of this adjusted-pH solution with a small stir bar, placed on a tube stand on top of a stir plate inside of a 37^{o} Celsius benchtop incubator. Three milligrams of mixed powder were then dissolved into three milliliters of the pH-adjusted NaPhos solution in a 5 mL polypropylene tube and lightly vortexed. One side of the cut dialysis tubing was clamped using a 3 cm dialysis tube clamp. The three milliliters of drug solution were then pipetted carefully into the clamped tubing, and the open end of the dialysis membrane was then clamped after all drug solution was successfully transferred. This clamped dialysis tubing was then submerged into the 30 mL buffer solution within the 50 mL conical tube. Samples of 1 mL from the 30 mL

buffer solution were taken at the time points 0 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours and finally 24 hours. After sampling, the buffer solution was replaced with 1 mL of fresh buffer solution.

Microparticle *In vitro* Cytotoxicity: Toxicity Studies

In vitro cytotoxicity of the powders was determined using an MTS assay in the THP-1 monocyte cell culture. Each of the mixed powders were resuspended and vortexed for 3 minutes in RPMI 1640 medium at different concentrations (1.0-0.0625 mg/mL). The THP-1 cells were differentiated into macrophages by phorbol 12-myristate 13-acetate (PMA) 20 nanomolar in a 96 well cell-culture plate for 24 hours and allowed time to recover after PMA exposure for an additional 48 hours. After exposing the cells to the resuspended dry powders for 24 hours, the drug solution was pipetted off and replaced with 100 microliters of PBS. Subsequently the MTS reagent was added and allowed to incubate for 4 hours at 37^{o} C with 5% CO_2. Absorbance was then read at 490 nm. Loss in cell viability was measured by a reduction in metabolic activity using MTS.

Microparticle Bactericidal Activity: Efficacy Studies

The efficacy of the spray dried powders against *M. smeg* was tested in vitro after 3 hours of incubation at 37°C. We infected PMA-differentiated THP-1 cells (differentiation above) with *M. smeg* for 3 hours. The *M. smeg* CFU was chosen based on our preliminary studies of in vitro infection of macrophages: we showed that the best multiplicity of infection (MOI) was one ($5x10^5$ THP-1 cells/mL cells confluency in a 12 well plate). After infection, we washed the infected cells 3 times in sterile PBS buffer in order to get rid of any extracellular *M. smeg* that did not

manage to be phagocytosed. Different concentrations of the powders were prepared in Middlebrook 7H9 broth medium (40-5 μg/mL of the RIF component) and added to infected PMA-differentiated THP-1 cells. After the aforementioned incubation time point was achieved, we lysed the THP-1 cells using 0.05% SDS buffer in diH2O and plated the bacteria on Middlebrook 7H10 agar plates for CFU counts. These plates were incubated for 48 hours at 37°C and CFU were determined.

CHAPTER 3: RESULTS, DISCUSSION, AND FUTURE DIRECTIONS

RESULTS

Drug loading efficiency

Using the combined drug HPLC protocol, the loading and percentage loading efficiency was quantified. For the RIF:ML formulation an average loading of 517.5 micrograms of RIF per milligram of powder was achieved, with an average loading efficiency of 103.4%±0.8%. For the VER:ML formulation an average loading of 288.1 micrograms of VER per milligram of powder was achieved with an average loading efficiency of 115.2%±4.1%. For the VER:EPO formulation an average loading of 252.2 micrograms of VER per milligram of powder was achieved with an average loading efficiency of 100.9%±3.7%.

Mixing efficiency

Using the combined drug HPLC protocol, the efficiency of dry powder mixing was quantified and assessed for proper drug ratio as well as percent mixing efficiency. We expected to have 300 micrograms of RIF and 100 micrograms of VER per 1 milligram of mixed powder after accounting for amount of excipient in each formulation. For the RMVM powder, we achieved 232.04 micrograms of RIF and 99.56 micrograms of VER per milligram of mixed powder. This gives us a 77.3% mixing efficiency of RIF and a 99.6% mixing efficiency of VER. This also gives us a RIF:VER drug ratio of 2.33:1 (theoretical ratio 3:1). For the RMVE powder, we achieved 227.65 micrograms of RIF and 88.16 micrograms per milligram of mixed powder. This gives us a 75.9% mixing efficiency of RIF and an 88.2% mixing efficiency of VER. This also gives us a RIF:VER drug ratio of 2.58:1 (theoretical ratio 3:1).

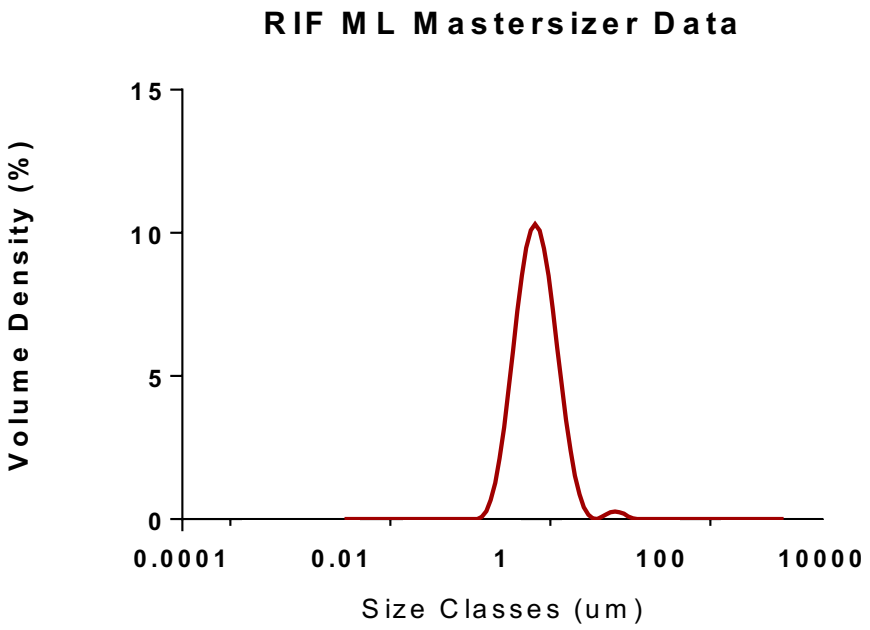
Volumetric size distribution

After formulation of our individual powders, we used the Malvern Mastersizer 3000 to determine their volumetric size distribution (as described above). Approximately 2 milligrams of the spray dried powders were passed through the Aero S dry powder disperser of the Mastersizer in triplicate to get a size distribution (Figure 3.1).

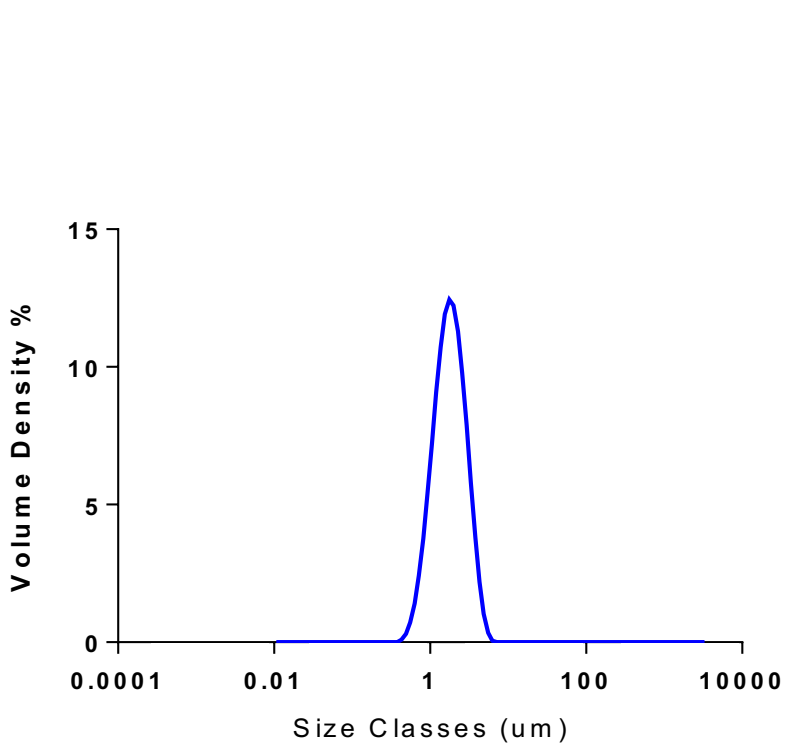
We mixed the different drug containing powders that would give a 1:3 ratio of RIF and VER. This drug ratio is based on the literature and also the oral daily doses of each drug (Parumasivam et al., 2016). After mixing the powders homogenously using a sieving technique, we determined the volumetric size distribution using the above method used for single-drug powders (Figure 3.2). The volumetric size distribution data for all of the formulated powders (individual and mixed) can be found in Table 3.1.

Briefly, we were able to determine that the volumetric size distribution of 50% (D-50) of all spray dried powders fell between 1.77 and 2.63 μm. All powders also demonstrated a span of less than 2 μm except for the RMVE powder, which had a span of 5.74 μm. We attribute this larger span to clumping and potential agglomeration of this particular mixed powder.

a.


RIF ML Mastersizer Data
Volume Density (%)
15
10
5
0
0.0001
0.01
1
100
10000
Size Classes (um)

b.


VER ML Mastersizer Data
Volume Density %
15
10
5
0
0.0001
0.01
1
100
10000
Size Classes (um)

c.

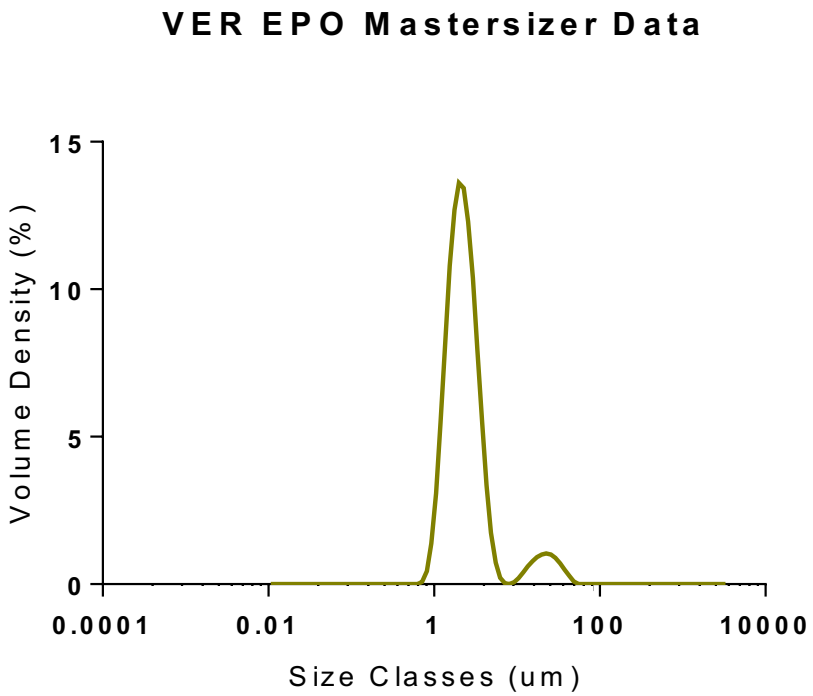
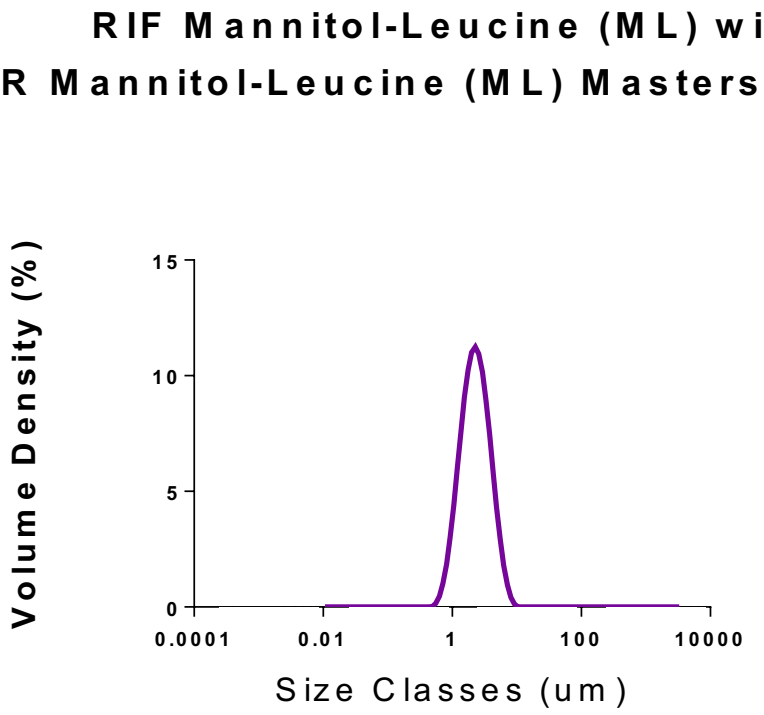


Figure 3.1. Spray dried powder (individual drug) volumetric size distribution. Individual powders which were analyzed were RIF with ML (a), VER with ML (b) and VER with EPO (c).

a.



b.

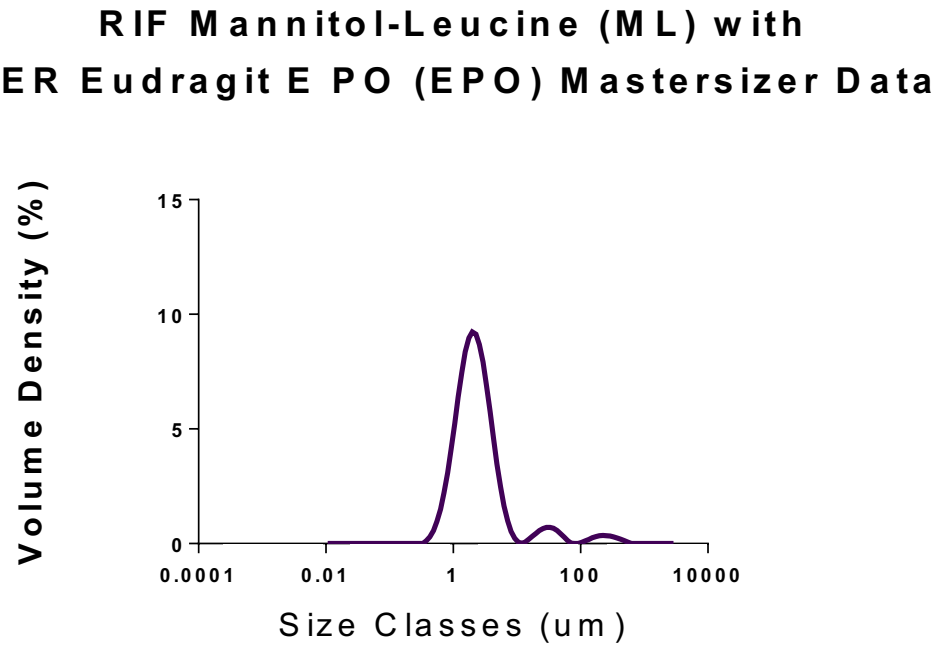


Figure 3.2. Spray dried powder (mixed drugs) volumetric size distribution

Table 3.1. Volumetric size distributions and span of all the formulated powders

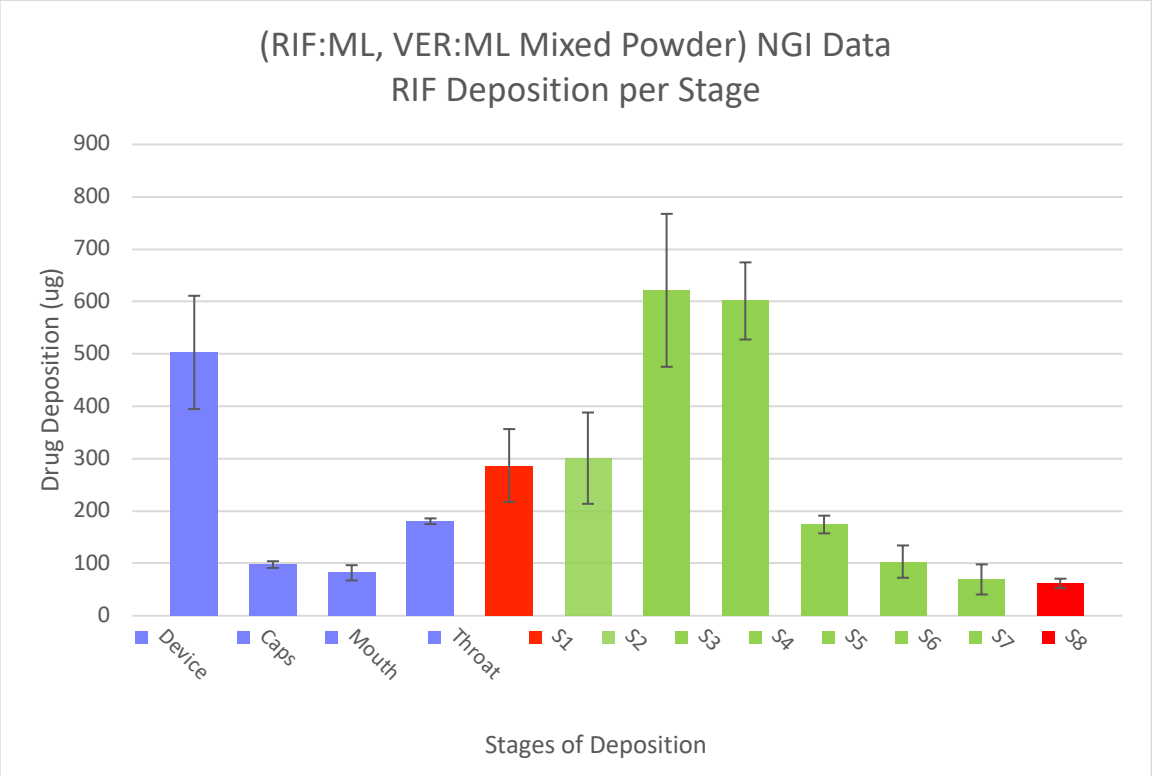
Powder Sample	D-50	Span
RIF ML	2.63±.012 μm	1.65±0.018 μm
VER ML	1.77±0.005 μm	1.27±0.032 μm
VER EPO	2.22±0.022 μm	1.45±0.237 μm
RIF ML:VER ML	2.26±0.029 μm	1.45±0.015 μm
RIF ML:VER EPO	2.48±0.144 μm	5.74±5.29 μm

Aerodynamic diameter

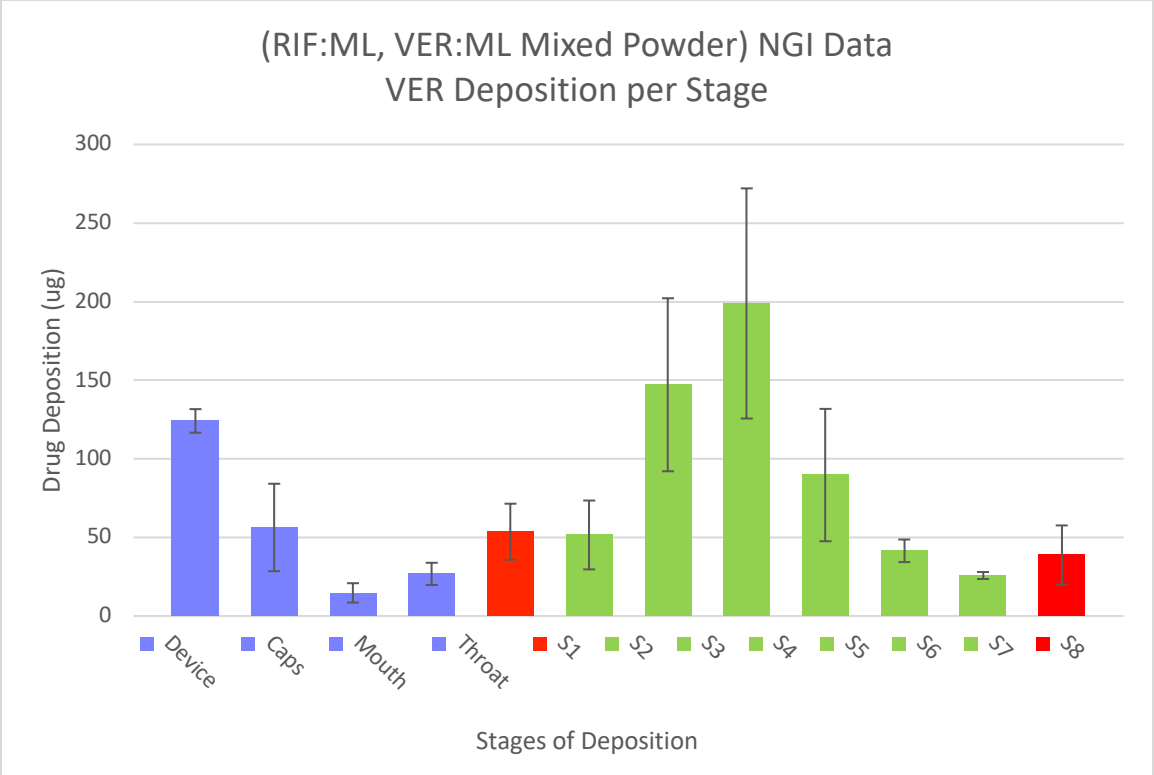
After mixing the individual drug containing powders, we determined that an ideal formulation would consist of a modified-release EPI in combination with an immediate release ATD. Our goal is to achieve elevated anti-TB drug concentration inside the bacteria, with prolonged efflux pump inhibition (Adams et al., 2014); this will be achieved with an immediate release of RIF due to the concentration-dependent killing activity against *Mycobacterium tuberculosis* (Gumbo et al., 2007). We also created an immediate release formulation of both the EPI and ATD for comparability of dissolution, efficacy, and toxicity. The mixed powders of 'RIF:ML, VER:EPO' as well as 'RIF:ML, VER:ML' were chosen for aerodynamic testing. After aerosolizing 15 mg of the mixed powder through the NGI (as previously mentioned), the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were determined using the combined HPLC protocol, which allowed us to analyze RIF and VER together for both mixed powder formulations. For the RMVM mixed powder we determined the MMAD to be 3.05±0.095 μm with a GSD of 1.87±0.155 μm for RIF and determined the MMAD to be 2.45±0.175 μm with a GSD of 1.93±0.025 μm for VER. For the RMVE mixed powder we determined the MMAD to be 3.01±0.08 μm with a GSD of 1.87±0.035 μm for RIF and we determined the MMAD to be

4.95±0.015 μm with a GSD of 2.35±0.02 μm for VER. For the RMVM mixed powder, we determined the fine particle fraction (FPF) of RIF to be 85.6% and the FPF of VER to be 84.3%. For the RMVE mixed powder, we determined the FPF of RIF to be 89.3% and the FPF of VER to be 70.8%. The overall distribution of particle deposition for RIF and VER in each of the mixed powders is illustrated in Figure 3.3.

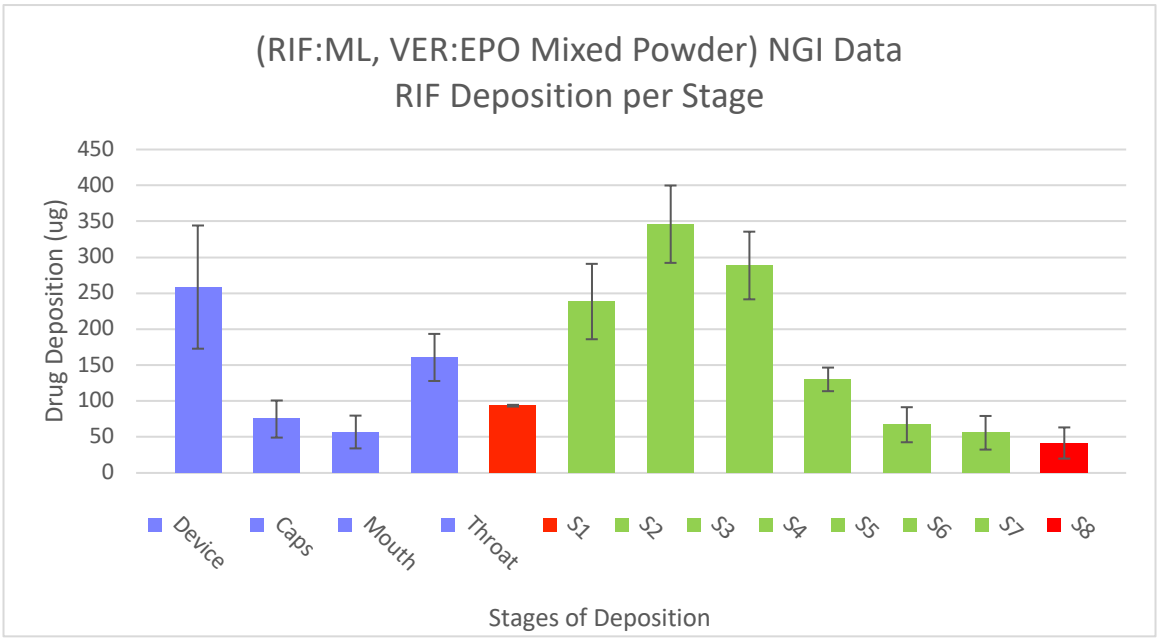
a.


(RIF:ML, VER:ML Mixed Powder) NGI Data
RIF Deposition per Stage
Drug Deposition (ug)
900
800
700
600
500
400
300
200
100
0
Device
Caps
Mouth
Throat
S1
S2
S3
S4
S5
S6
S7
S8
Stages of Deposition

b.


(RIF:ML, VER:ML Mixed Powder) NGI Data
VER Deposition per Stage
Drug Deposition (ug)
300
250
200
150
100
50
0
Device
Caps
Mouth
Throat
S1
S2
S3
S4
S5
S6
S7
S8
Stages of Deposition

c.



d.

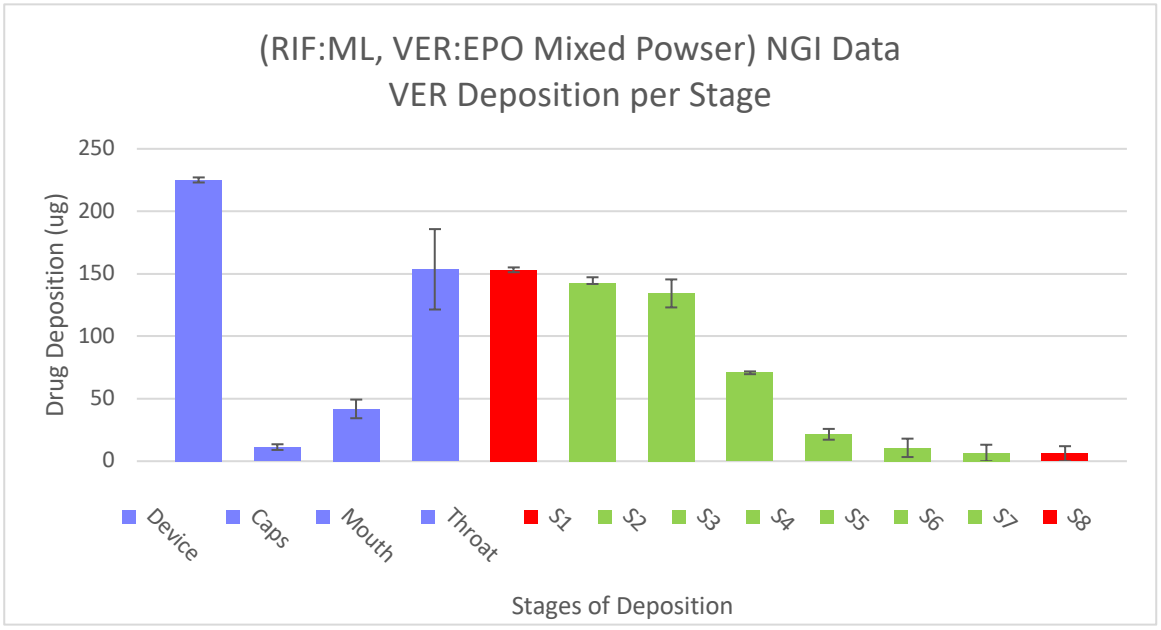


Figure 3.3. Aerodynamic Diameter Data (NGI) for each of the mixed powders. RIF and VER are represented, respectively, in (a) and (b) the aerodynamic performance of the RIF:ML, VER:ML mixed powders and (c) and (d) the aerodynamic performance of the RIF:ML, VER:EPO mixed powders. The Fine particle fraction, that potentially gets depositied in the deep lung, is represented in green bars.

Particle Morphology

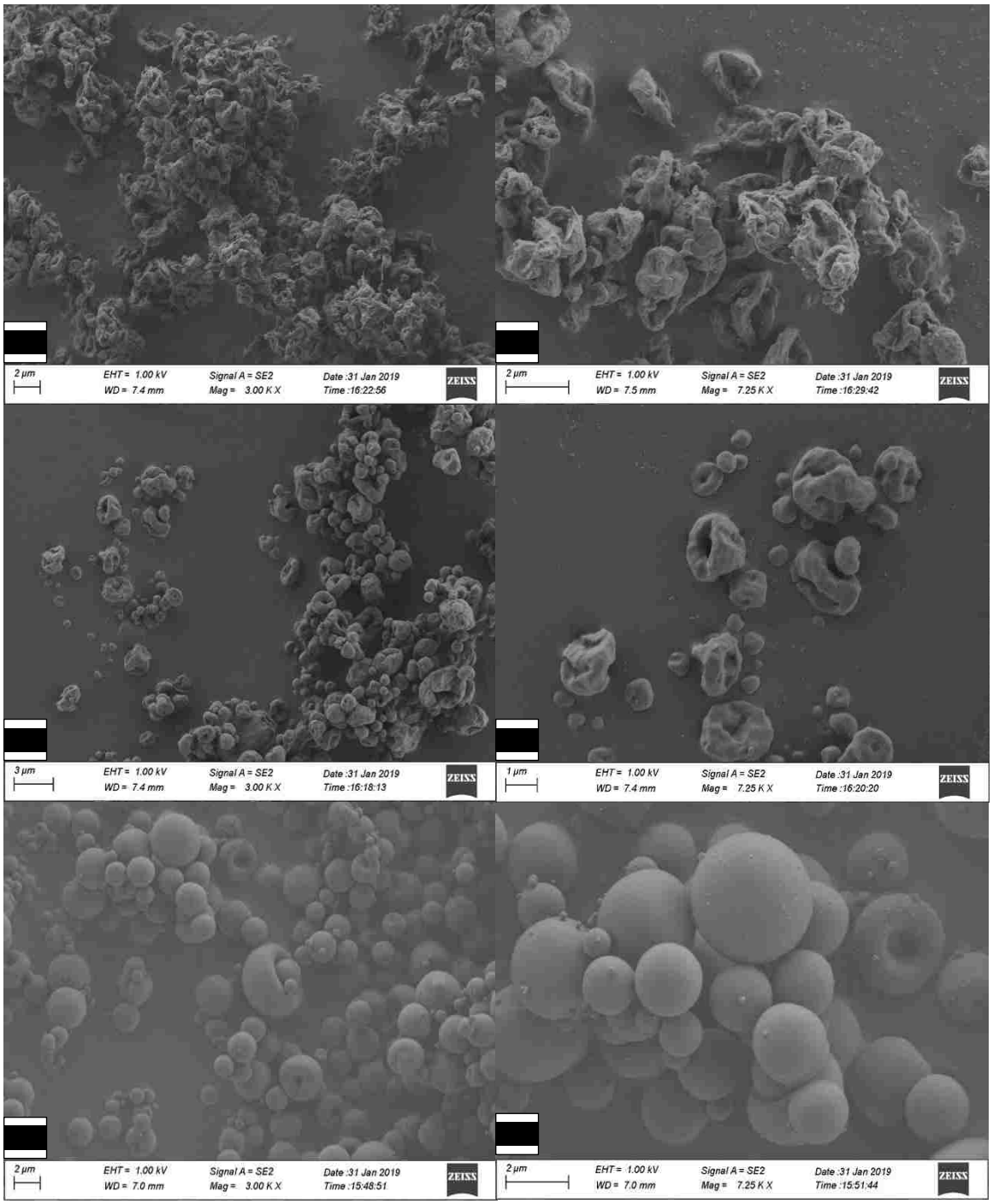


Figure 3.4. Scanning Electron Microscope Images of Individual Powders. SEM images at 3.0K times magnification and 7.25K times magnification, respectively, of (a) and (b) RIF:ML, (c) and (d) VER:ML, and (e) and (f) VER:EPO.

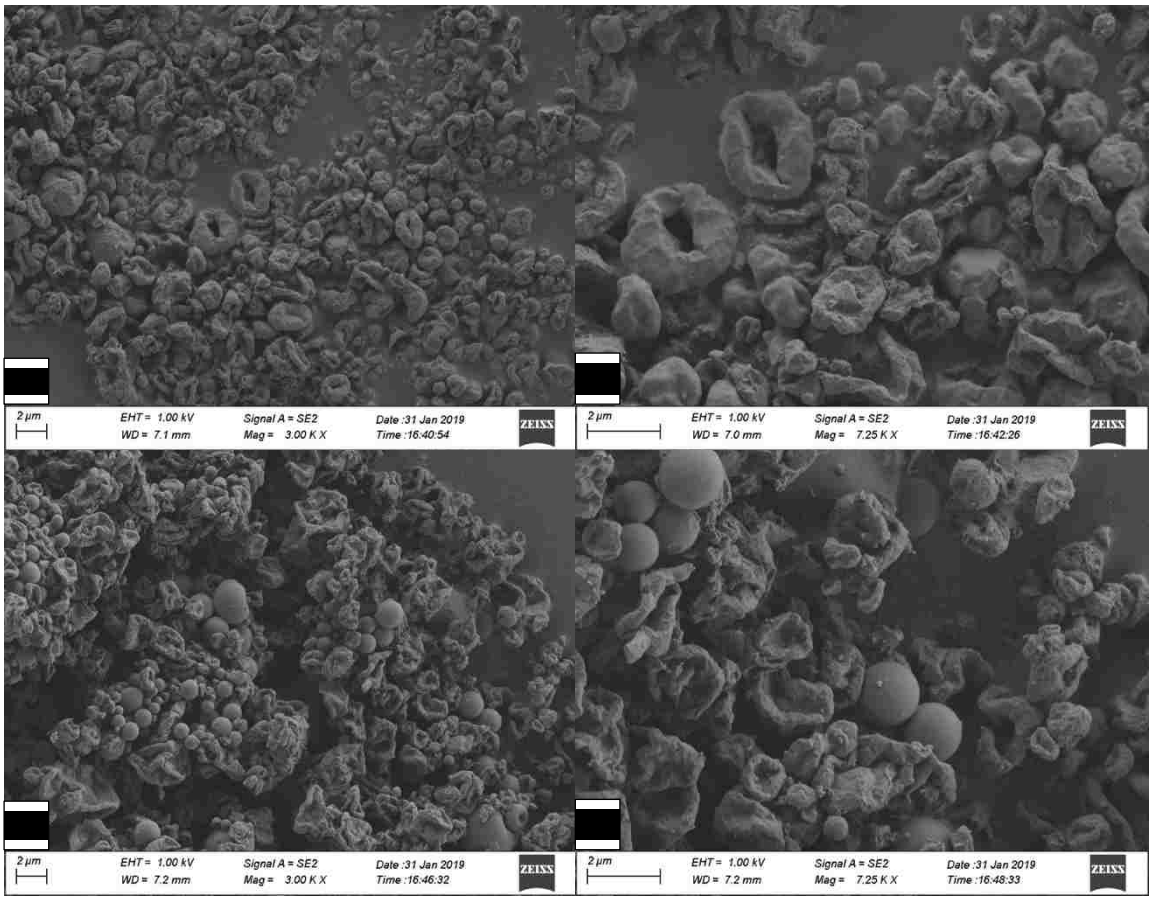


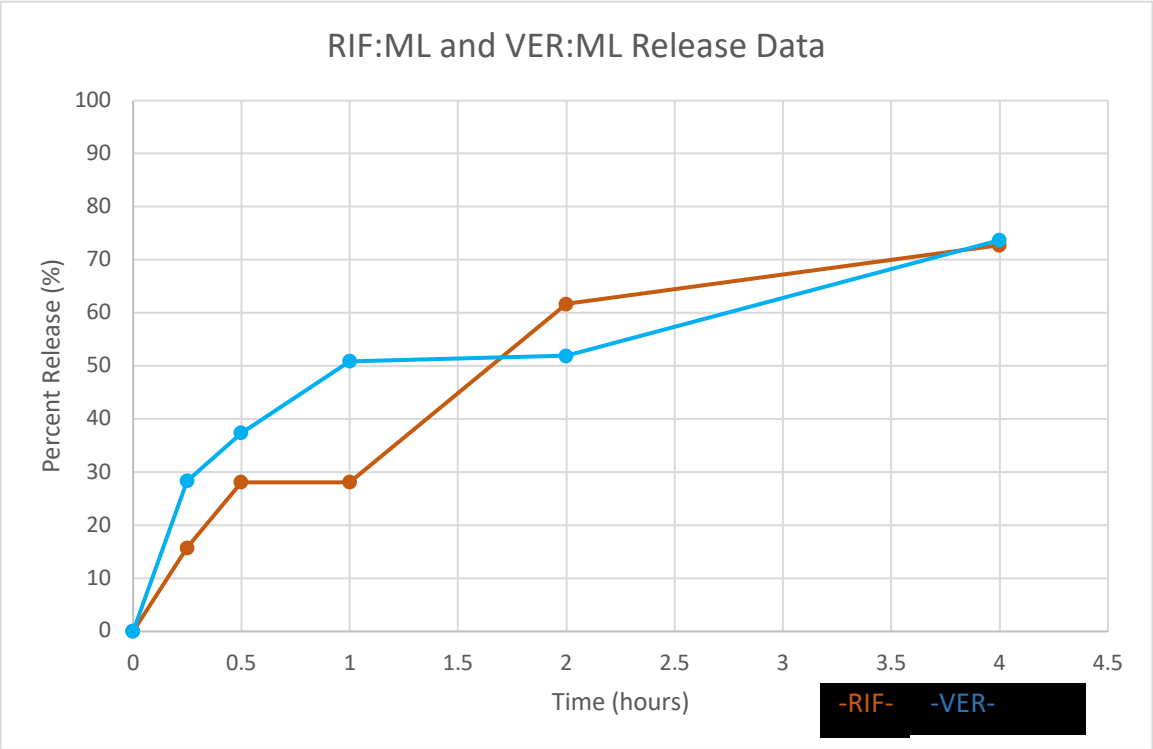
Figure 3.5. Scanning Electron Microscope Images of Mixed Powders. SEM images at 3.0K and 7.25K times magnification, respectively, of (a) and (b) RMVM, and (c) and (d) RMVE

To determine the particle morphology of each formulation, we subjected powders to scanning electron microscopy (SEM) imaging. Figure 3.2 represents the SEM images captured of the individual drug powders. Both RIF and VER formulations using ML showed irregularly shaped particles in the size range that is representative of the volumetric diameter data. The VER formulation using EPO created spherical, regularly shaped particles that are also representative of the volumetric diameter data. Figure 3.3 represents SEM images of the mixed drug powders. Similar to figure 3.2, the mixed powders showed irregularly shaped particles in the RMVM mixed powder, and irregularly shaped particles interspersed with spherical, regularly shaped particles in the RMVE mixed powder.

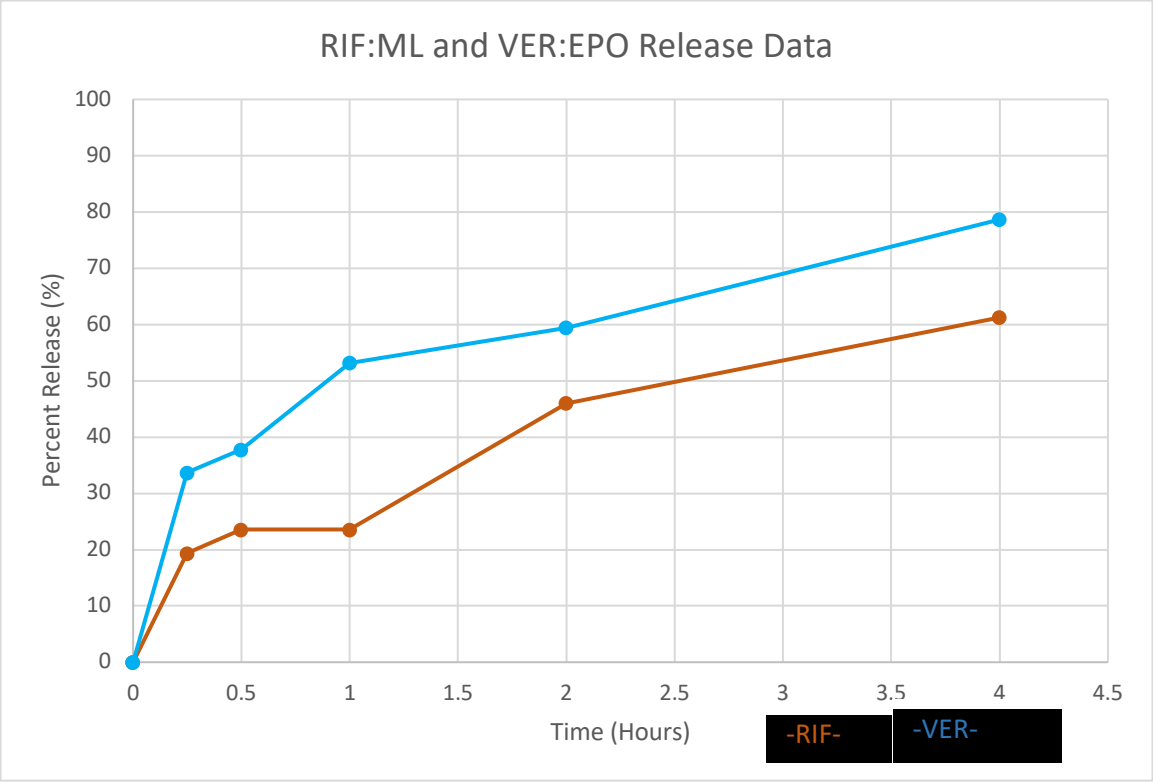
Drug Release

In order to determine the *in vitro* drug release of the mixed powders, we subjected powders to the above-mentioned drug release protocol. In the RMVM mixed powder, we were able to achieve 95.6% drug release of the RIF component and 83.0% drug release of the VER component at 24 hours (figure 3.6c). In the RMVE mixed powder, we were able to achieve 110.6% drug release of the RIF component and 86.9% drug release of the VER component at 24 hours (figure 3.6d). The most noticeable difference of formulation performance, however, was at 4 hours. With the RMVM mixed powder, both drugs showed approximately 70% drug release at 4 hours. With the RMVE mixed powder, however, the RIF component achieved only 60% drug release at 4 hours where the VER component was able to achieve approximately 80% release. This data is depicted in figure 3.6a and 3.6b; with data shown to 4 hours because this is data that demonstrated differences in the release pattern when comparing the two mixed powders.

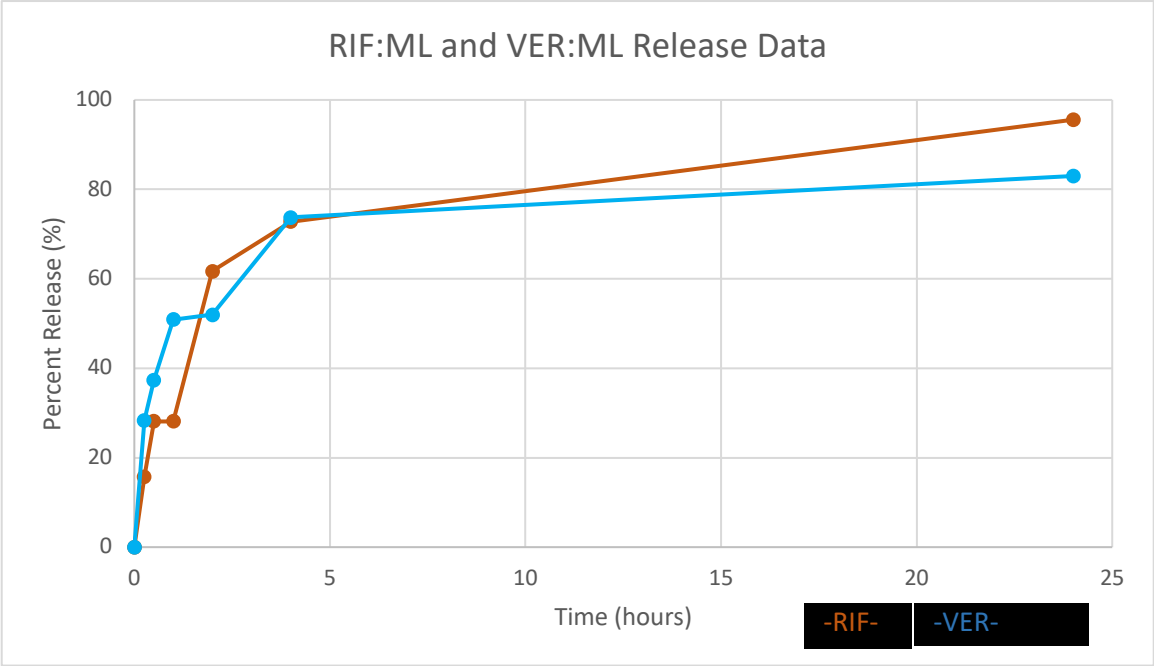
a.


RIF:ML and VER:ML Release Data
Percent Release (%)
100
90
80
70
60
50
40
30
20
10
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
Time (hours)
-RIF-
-VER-

b.


RIF:ML and VER:EPO Release Data
Percent Release (%)
100
90
80
70
60
50
40
30
20
10
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
Time (Hours)
-RIF-
-VER-

c.



d.

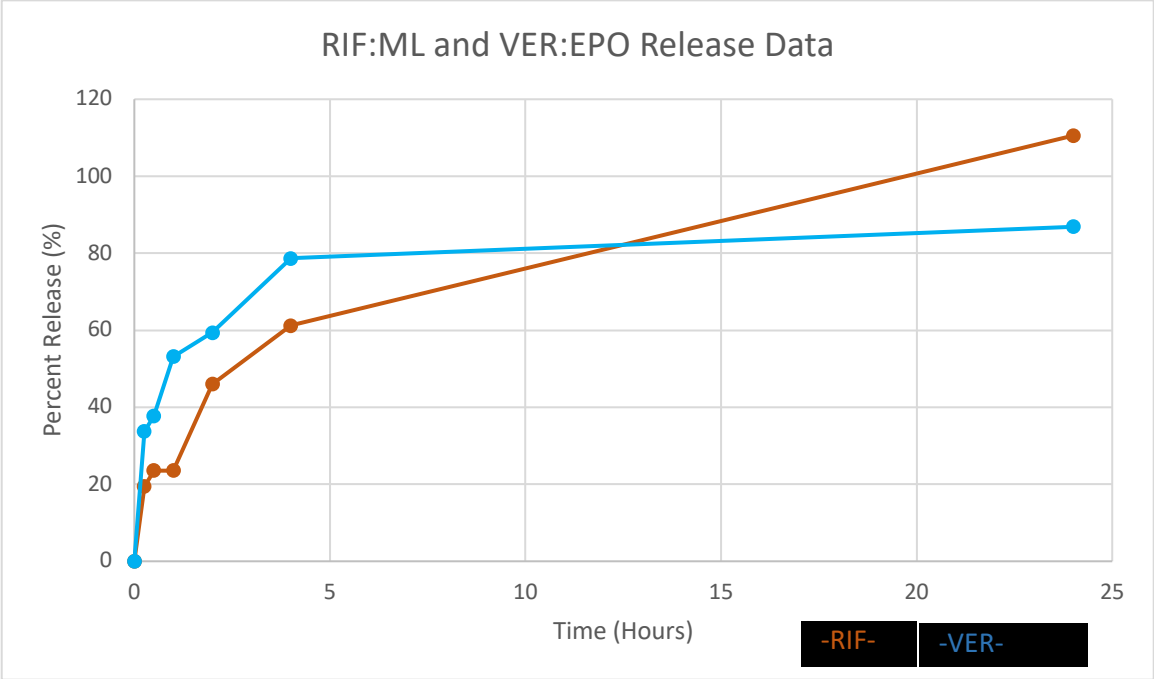
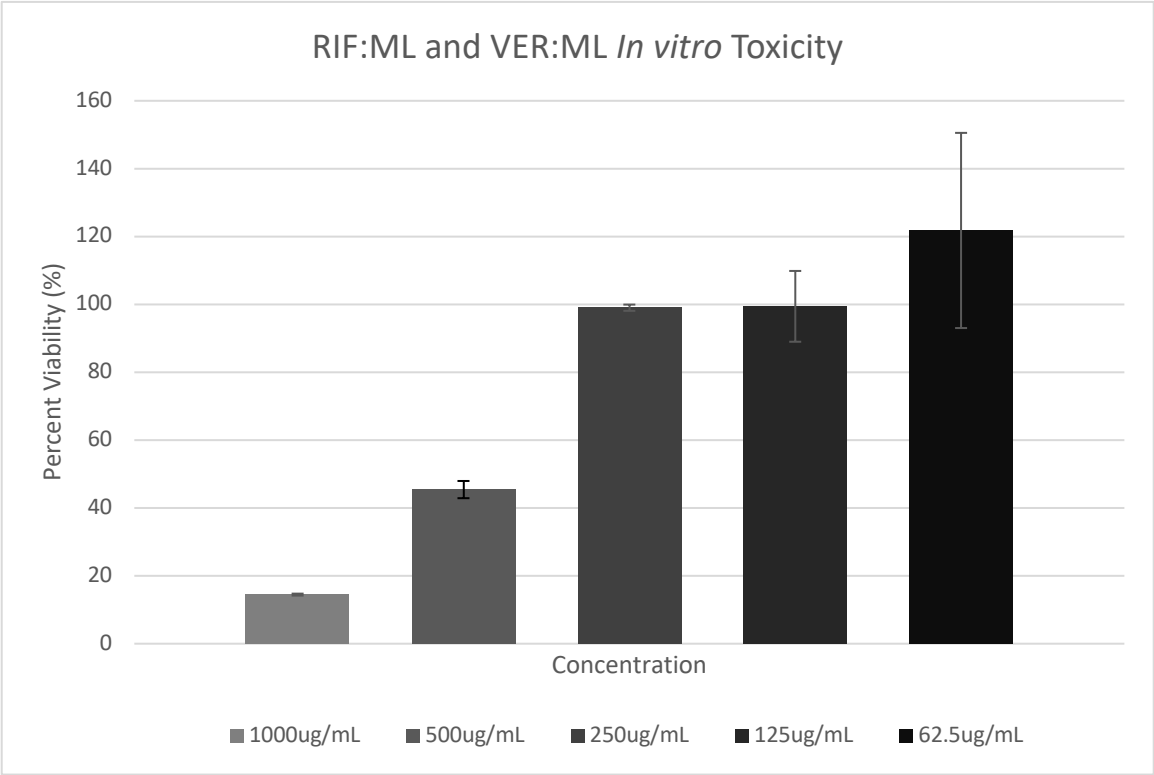


Figure 3.6. Drug Release Data. The 4-hour data is represented in (a) for RMVM, and for (b) RMVE. The 24-hour data is represented in (c) for RMVM and (d) for RMVE.

Microparticle In vitro Cytotoxicity

Once all powders were formulated and mixed, the toxicity of the formulations against PMA differentiated THP-1 cells (Human monocyte-like cells) was evaluated. We subjected THP-1 cells to various concentrations of each of the mixed formulations for 24 hours, and then washed once with PBS to remove excessive drug powders. Data is shown in figure 3.5, with each bar representing the percent cellular viability. The RMVM immediate release formulation at 1 milligram per milliliter after 24 hours of exposure led to achieve a 14.47% $\pm$ 0.29% THP-1 cell viability. The RMVM formulation at 500 micrograms per milliliter led to a 45.42% $\pm$ 2.54% viability after 24 hours of exposure. The other lower concentrations of the RMVM formulations (250 μg/mL, 125 μg/mL, and 62.5 μg/mL) achieved over 99% cellular viability (Figure 3.7a). The RMVE sustained release formulation at 1 milligram per milliliter led to a 36.20% $\pm$ 3.23% viability after 24 hours of exposure. The RMVE formulation at 500 micrograms per milliliter led to a 29.39% $\pm$ 2.65% viability. The RMVE formulation at 250 micrograms per milliliter allowed the THP-1 cells to achieve a 25.01% $\pm$ 1.84% viability. The RMVE formulation at 125 micrograms per milliliter allowed the THP-1 cells to achieve a 23.69% $\pm$ 1.79% viability. Finally, the RMVE formulation at 62.5 micrograms per milliliter allowed the THP-1 cells to achieve a 26.05% $\pm$ 3.80% viability (Figure 3.7b).

a.



b.

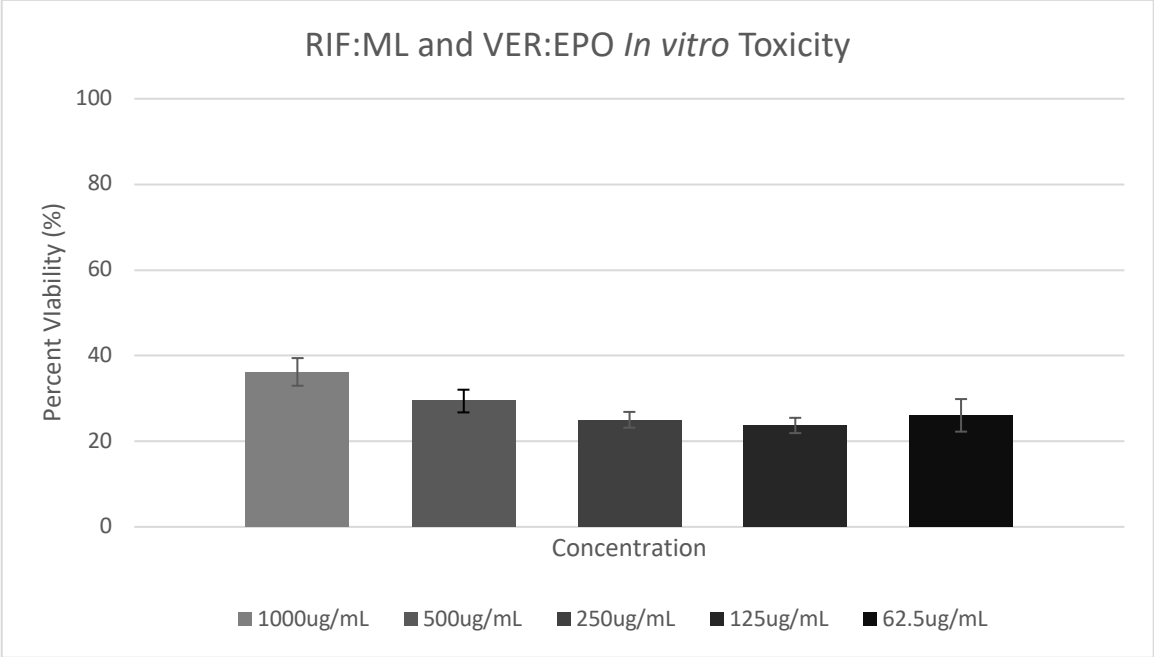


Figure 3.7. In vitro Cytotoxicity Data. Data is shown as percent viability of the THP-1 cells after 24 hours of drug exposure. (a) RIF:ML and VER:ML data at given concentrations (b) RIF:ML and VER:EPO data at given different concentrations

Microparticle Bactericidal Activity in Infected Cells

In order to determine the bactericidal activity of our mixed powder formulation, THP-1 differentiated cells were infected with *Mycobacterium smegmatis*, a *Mtb* surrogate (BSL-1 organism). After the THP-1 cells were infected with *M. smeg* for 2 hours, different concentrations of powder formulations were exposed to the infected cells for 1 hour. The infected cells were washed twice in PBS, lysed in 0.05% SDS buffer, and colony forming units (CFUs) were determined. This data is depicted in Figure 3.6. For controls, one well of THP-1 cells were infected with *M. smeg* and exposed to no drug powders. This control well resulted in 5.4 X 10^5 CFUs of *M. smeg* after infection. For the RMVM immediate release powder exposure, a concentration of 5 micrograms per milliliter resulted in 1.0 x 10^5 CFUs; at a concentration of 10 micrograms per milliliter resulted in 9.0 X 10^4 CFUs; at a concentration of 20 micrograms per milliliter resulted in 3.8 X 10^4 CFUs; and at a concentration of 40 micrograms per milliliter resulted in 4.0 X 10^4 CFUs. For the RMVE sustained release powder, a concentration of 5 micrograms per milliliter resulted in 4.0 X 10^5 CFUs; at a concentration of 20 micrograms per milliliter resulted in 1.1 X 10^5 CFUs; at a concentration of 20 micrograms per milliliter resulted in 1.2 X 10^4 CFUs; and at a concentration of 40 micrograms per milliliter resulted in 1.4 X 10^4 CFUs.

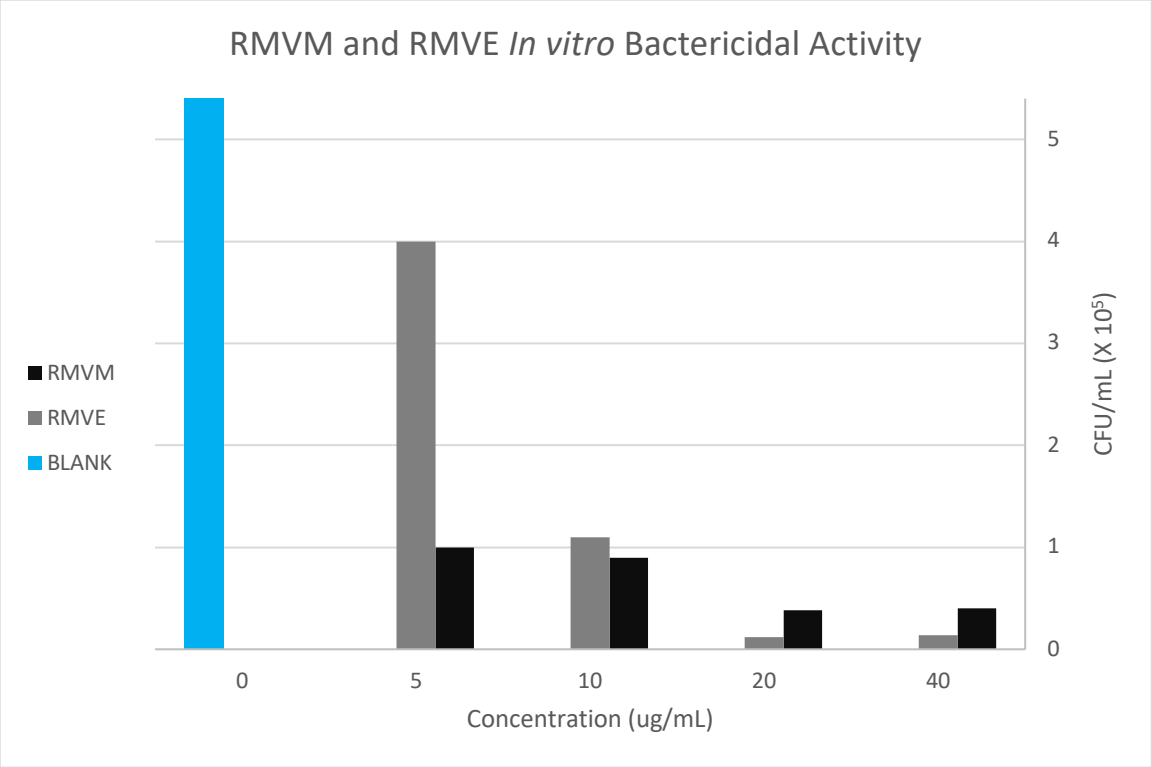


Figure 3.8. *In vitro* bactericidal activity. RIF:ML and VER:ML immediate release and RIF:ML and VER:EPO sustained release formulation data

DISCUSSION

The necessity of combating MDR and XDR-TB cannot be overstated. Understanding novel mechanisms utilized by *Mycobacterium tuberculosis* to evade bactericidal activity of our current antibiotic regimens, such as efflux pump overexpression, has been a crucial step in the right direction. Additionally, the strides taken for pulmonary delivery for TB treatment has been a pivotal goal which will hopefully be implemented in TB endemic regions to achieve global control of this extremely debilitating disease.

We have demonstrated the feasibility to formulate microparticles (dry powders) that are 1-3 microns in aerodynamic diameter, the correct size range to reach the deep lung (Misra et al., 2011). Furthermore, we have shown excellent

drug loading efficiency for both RIF and VER as well as demonstrated homogenous mixing efficiency to create the ideal RIF:VER drug ratio. This formulation will potentially facilitate therapeutic drug concentration to the intended site of action in the lung without the requirement for high ATD oral or parenteral administration.

Interestingly, our formulations displayed vastly different aerodynamic performance regarding the VER component. The “immediate release” formulation of RIF achieved an aerodynamic size of 3.05 micron and VER achieved an aerodynamic size of 1.87 micron. The “sustained-release” formulation of RIF achieved an aerodynamic size of 3.01 micron, similar to the immediate release; however VER achieved an aerodynamic size of 4.95 micron, drastically different from the immediate release formulation. This data contradicts the volumetric diameter data for the same formulation; the aerodynamic diameter of the VER component is higher compared to the volumetric size distribution, possibly due to the differences in the density of MP containing EPO. Another interesting result was the release data. It appears that the Eudragit polymer affected the release of RIF, delaying its release by approximately 10% at 4 hours when compared to the immediate release formulation. The “sustained release” formulation also displayed increased VER release at 4 hours to 80% release. There could be many reasons for this contradicting result and further studies are required to fully understand the release mechanism from our formulations.

Regarding the *in vitro* performance, the “sustained release” formulation was more toxic to THP-1 cells than the “immediate release” formulation at the same

concentrations. As shown in figure 3.7, at any concentration the RMVE formulation was able to achieve only approximately 20 – 40% cellular viability, whereas the RMVM formulation was able to achieve over 99% viability at concentrations of 250 micrograms per milliliter or lower. As previously mentioned, the only difference between these formulations is the Eudragit E PO polymer, which could be attributed to the increase in toxicity. Interestingly, the "sustained release" formulation displayed greater viability at higher concentrations. This could, however, be due to the residual drug left after the washing step. As the assay is read at 490 nm, residual rifampicin remained in the wells of the "sustained release" formulation, and could have contributed to this higher absorbance. Therefore, this study needs to be repeated with a modified washing protocol. Bactericidal activity was also superior in the immediate release RMVM formulation at lower concentrations, however the bactericidal activity of the sustained release RMVE formulation surpassed that of RMVM at 20 and 40 micrograms per milliliter. Whether the *in vitro* performance of these powders is statistically significant would require repeating the *in vitro* studies.

FUTURE DIRECTIONS

This project would benefit from further tests to better determine the characteristics of our inhalable dry powder formulation. First, by assessing the drug release of our formulation at pH's below the threshold of Eudragit E PO's dissolution, we could determine if a "burst" release profile was present in the EPO formulations, which was hypothesized to occur once the microparticle reached the macrophage microenvironment. Next, *in vitro* testing could be repeated to

determine the effectiveness and toxicity of our formulations, in which various concentrations and sampling times could be tested to further strengthen the conclusion of this project. Finally, the future goal of this dry powder formulation containing an ATD and an EPI is to display *in vivo* activity against *Mtb* in a preclinical TB infection model.

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APPENDICES

Appendix 1: Inhalation Aerosols: Physical and Biological Basis for Therapy, Third Edition Hickey, A.J., and Mansour H.M., (2019)

Chapter 23: Inhaled therapeutics against TB: the promise of pulmonary treatment and prevention strategies in the clinic

Dominique N. Price, Nitesh K. Kunda, Elliott K. Miller, and Pavan Muttil

Contributions to this Publication:

Challenges of inhaled therapy to the clinic

"One of the challenges with the pulmonary route relates to the cost of the combined device-formulation product and the ability of patients to use inhalers consistently and properly to achieve treatment compliance. Since TB primarily affects low- and middle-income countries, it is important to develop treatment and preventive strategies that are cost-effective. Current inhalers in the market (for diseases such as asthma) are expensive and complicated to use without proper medical supervision; this makes it challenging to use a similar strategy against TB, especially if it requires long-term inhaler use. Adherence to treatment also entails patient's satisfaction with the inhaler device, which is influenced by convenience of daily use, patient age, adverse effects observed during treatment, and overall treatment cost. For inhaled therapy to be successful against TB, affordable inhalers should be made available on a large scale in TB-affected nations. In the section below, we discuss the inhaler devices for drugs

and vaccines separately since it requires different development and use strategies."

Devices for pulmonary drug delivery

"Lung deposition of ATDs to the affected areas is determined by the aerodynamic particle size distribution, particle mass, and patients' inspiratory flow. Aerodynamic particle size distribution is optimized when the aerodynamic diameter is between 1 and 3 um, a limitation that is easily overcome in the formulation aspect of inhalable products. Similarly, particle mass is affected by the added excipients, which can be optimized for deep lung delivery, for uptake by alveolar macrophages, and to minimize mucociliary clearance. One major limitation to the pulmonary route of administration, however, is the individual variation observed among patients, such as inspiratory flow rate, tidal volume, ability to hold breath after performing the inhalation maneuver, etc. Such differences in patient-specific factors can be affected by many variables, such as age and disease states (i.e., COPD), and these variations can potentially lead to differences in clinical outcomes between patients despite using the same inhaler device–ATDs combination. Historically, the aerosol devices used for pulmonary delivery have been nebulizers, MDIs, and DPIs (see the section called "Devices available"). One major limitation to using nebulizers is that these systems require lengthy treatment time and waste much of the drug. Here, drug loss to the environment is observed as the patient passively breathes, or the drug is retained within the device; only an average of 10% of the drug is deposited into the lung. In comparison, MDIs promise to provide more drug deposition;

however, the effectiveness of this system is highly variable among patients. Drug deposition is largely affected by the inspiratory flow rate, in which faster inhalations may cause a significant amount of the drug to be deposited via inertial impaction in conducting airways and oropharyngeal regions. Issues regarding a patient's ability to coordinate actuation of the device and inspiration remains a frequent complication while using MDIs. Introduction of spacer and valve holding chambers have helped minimize complications regarding hand-eye coordination; this is achieved by slowing the aerosol velocity, which reduces drug deposited at the oropharynx. However, the use of auxiliary attachments to MDIs has previously led to compliance concerns during chronic use, especially in pediatric patients. In contrast, DPIs are designed to decrease the coordination difficulties associated with MDIs. This system relies on the patient's inspiratory flow rate, however, and can be negatively affected by humidity and changes in temperature. Lung deposition from DPIs is further affected by the ability of the drug to deaggregate from the carrier particles (i.e., lactose), or by the patient's inability to hold her or his breath after the inhalation maneuver, which may be compromised in TB patients. Aerosol ATD formulations are further limited by the excipients that are currently approved for pulmonary delivery. Pulmonary TB therapeutics using novel but nonapproved excipients, such as biodegradable polymers that are used to formulate nano- and microparticles, require additional safety and toxicological data. In addition, many excipients that are generally recognized as safe (GRAS) by other routes of administration are not approved for pulmonary delivery because of the potential safety, toxicity, and pathological

inflammation concerns. Interest in DPIs has arisen more recently due to the delivery platform's ability to generate high local drug concentration in the lungs, as well as the improved stability of the powders compared to drug solutions. Dry powder formulations encompass a few promising strategies for optimal inhalational TB therapy, with liposomal, microparticle, and nanoparticle dry powders at the forefront of this research. Liposomes encapsulating anti-TB drugs can be utilized for pulmonary delivery as they are not immunogenic. Micro-and nanoparticles formulated with polymeric excipients such as poly(l-lactic) acid and poly(lactic-co-glycolic) acid may potentially have a positive impact on the pharmacokinetic and pharmacodynamic parameters of ATDs. Such polymer-based formulations would allow for a less frequent dosing regimen by prolonging the drug release profiles and leading to better patient compliance. Poly(l-lactic) acid microparticles, for example, have displayed a slow in vitro release, where only 70% the encapsulated ATDs was released in 10 days. Conversely, particles delivered in the nanosized range are not suitable for inhalation purposes and must be formulated into a larger, micron-size particles."

Appendix 2: Oral immunization with bacteriophage MS2-L2 VLPs protects against oral and genital infection with multiple HPV types associated with head & neck cancers and cervical cancer. *Antiviral Research.*

Lukai Zhai, Rashi Yadav, Nitesh K. Kunda, Dana Anderson, Elizabeth Bruckner, Elliott K. Miller, Rupsa Basu, Pavan Muttil, Ebenezer Tumban

Contribution to this Publication:

Spray-freeze drying of VLPs into dry powder formulation

"Equal concentrations of MS2-31L2/16L2 VLPs and MS2-consL2(69-86) VLPs were mixed together to obtain mixed MS2-L2 VLPs. Half of the mixed MS2-L2 VLPs (100 μg) was further mixed with 2 μg each of the mucosal adjuvants, cholera toxin (CT) and monophosphoryl lipid A (MPLA); control MS2 VLPs were mixed with the same concentration of adjuvants. The mixed MS2-L2 VLPs and MS2 VLPs, with or without mucosal adjuvants, were added to a 3% w/v MTDL excipient solution (containing Mannitol (M, 75% w/w), Trehalose (T, 7.5% w/w), Dextran (D, 2.5% w/w), and L-Leucine (L, 15% w/w). The VLPs were added at a concentration of 8% w/w to the MTDL excipients and the VLPs-excipients were then spray-freeze dried (SFD) in two steps. In the first step, the VLPs-excipients suspensions were sprayed into a stainless-steel container filled partially with liquid nitrogen. This was achieved by using a two-fluid nozzle (0.7 mm) which is part of a Büchi B-290 mini spray-dryer; the spraying operating conditions were as following: nitrogen flow (Q) between 10 and 15mm, and a VLPs excipients feed

rate of ~ 4 mL/min. Subsequently, the liquid nitrogen in the steel container was allowed to evaporate and the frozen droplets were transferred into a lyophilizer (FreeZone® Triad™ Freeze Dry system, Model 74000 Series) [27] for freezedrying (the second step). Freeze-drying was conducted under the following conditions: pre-freeze for 3 h at -80 °C, primary drying at -10 °C for 24 h with a ramp of 0.25 °C/min, followed by secondary drying at 15 °C for 4 8 h with a ramp of 0.25 °C/min, and vacuum pressure of 1.51 mBar. All SFD products were collected in glass scintillation vials and were stored under refrigeration until further use."

Appendix 3: 2017 AFPE Gateway Award Fellowship Proposal. *Efflux Pump Inhibitors in PLGA Nanoparticles as an Adjunct to Isoniazid.*

Student: Elliott Miller, Pharm.D. Candidate, Class of 2019

Mentor: Pavan Muttil, Ph.D. (Pharmaceutical Sciences), Nitesh Kunda, Ph.D. (Post-Doctorate)

The World Health Organization's 2015 report has identified tuberculosis infections among the top ten causes of death worldwide (1). This disease can remain dormant within the human body for years, and once active it requires complex and extensive multidrug therapies administered over a course which generally requires 6 to 9 months of intense drug regimens (2). This demanding treatment has led to the emergence of drug-resistant *Mycobacterium tuberculosis*, and now therapies require a different approach to combat the further selection and advancement of these dangerous multidrug-resistant (MDR) and extreme drug-resistant (XDR) (1). Identifying the mechanisms of resistance these organisms are acquiring is absolutely crucial to further combat drug resistance and will continue to allow first-line therapies to remain viable, extending the longevity of the few antibiotics known to provide favorable outcomes in tuberculosis infections. It has been observed by many researchers that *M. tuberculosis* is utilizing efflux pumps to promote bacterial survival and is potentially mediating drug tolerance. (3, 4). Researchers have successfully repurposed drugs such as verapamil (VER) and thioridazine (TRZ) and have shown to these pharmaceuticals to be effective efflux

pump inhibitors (EPIs), combating novel drug tolerance mechanisms of tuberculosis as well as potentiate the effects of first-line antibiotics such as rifampin (RIF) and isoniazid (INH) (3,5). Various ways of incorporating these drugs into formulation remains a struggle and determining the best route of administration, release profile, optimized EPIs for each antibiotic, along with a plethora of other characteristics remains an issue. One approach available for formulation is encapsulating the administered drugs (antibiotic plus EPI) into nanoparticles (NPs) made of biodegradable polymer such as poly(lactic-co-glycolic) acid (PLGA). This formulation has the benefit of a sustained-release profile, targeting the macrophages which *M. tuberculosis* reside, and greatly reducing TRZ toxicities *in vivo* (5). Utilizing INH, being one of the drugs implemented in MDR and XDR, we aim to develop an effective formulation incorporating either VER, TRZ or both EPIs into this NP formulation. Each of these EPIs contributes a different mechanism of efflux pump inhibition, as well as providing other novel mechanistic advantages to combating drug resistance. (6,7). Further targeting can also be achieved by creating an inhalational formulation, delivering the therapy directly to the affected organ system. **We hypothesize that a PLGA NP formulation incorporating EPIs and INH into an inhalable delivery system will potentiate INH's antibacterial activity, allowing higher intra-bacterial concentrations of INH, improving killing activity of INH as well as decreasing dosages by direct lung administration, further optimizing tuberculosis treatment and combating drug resistance.**

Aim 1: To optimize a NP incorporating EPI(s) and INH with outstanding characteristics for inhalation, drug loading, as well as macrophage targeting.

Aim 2: To determine the effectiveness and proper dosing of the NP formulation *in vitro* utilizing macrophage cell cultures.

Aim 3: To determine the utility of a novel anti-tuberculosis inhalational formulation *in vivo.*

Aim 1: To optimize a NP incorporating EPI(s) and INH with outstanding characteristics for inhalation, drug loading, as well as macrophage targeting.

Rationale: Nanoparticle formulations enhance targeting of macrophages, which is the place of residence for many *M. tuberculosis* in an active tuberculosis infection. Incorporating EPIs such as VER into this formulation will allow for greater antibiotic concentrations to accumulate within the bacteria, enhancing killing and combating one of the mechanisms tuberculosis utilizes for drug tolerance.

Methods: Formulate, spray dry, characterize

Aim 2: To determine the effectiveness and proper dosing of the NP formulation *in vitro* utilizing macrophage cell cultures.

Rationale: Compare killing of our formulation versus free-drug (without NP) in vitro. Determine dose, effectiveness, etc.

Method: Infect macrophages, treat with various formulations, lyse macrophages and:

1.) quantify CFUs

2.) measure amount of drug intra-bacterial

Aim 3: To determine the utility of a novel anti-tuberculosis inhalational formulation *in vivo.*

Rationale: How well does this formulation perform in a live animal model? Are there notable toxicities? Is there noticeable improvement in the outcomes of these animals?

Method: Infect mice with TB. Treatment:

1.) NP alone

2.) NP-EPI

3.) NP-EPI-INH

4.) Free INH

5.) Free INH+ Free EPI

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