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Development of Aerosol Microparticles for the Treatment of Pulmonary Hypertension

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DEVELOPMENT OF AEROSOL MICROPARTICLES FOR
THE TREATMENT OF PULMONARY HYPERTENSION

BY

SARAH BROUSSEAU

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

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ABSTRACT

Pulmonary arterial hypertension (PAH) is an incurable cardiovascular disease characterized by high blood pressure in the arteries leading from the heart to the lungs. Over 2 million people in the United States are diagnosed with PAH annually and the typical survival rate is only 3 years after diagnosis (Archer, Weir et al. 2010). Current treatments are insufficient because of limited bioavailability, toxicity, and cost associated with approved therapeutics. Aerosol delivery of drugs is an attractive approach to treat respiratory diseases because it increases localized drug concentration while reducing systemic side effects. Dry powder inhalers (DPIs) allow for increased physicochemical stability of drugs and increased patient compliance. Dry powder aerosols can be easily designed to meet certain specifications including size, morphology, and crystallinity via spray drying (Meenach, Vogt et al. 2013). Tacrolimus (TAC) is a drug that has recently been found to be useful in treating PAH (Spiekerkoetter, Tian et al. 2013). TAC interacts with bone morphogenic protein receptor type II (BMPR2), which is often mutated or underexpressed in patients with PAH (Humbert, Morrell et al. 2004). The expression of BMPR2 inhibits vascular remodeling, thereby reducing blood pressure. Unfortunately, TAC is poorly water-soluble and toxic when delivered systemically over a long period of time, this driving the need for improved formulations and delivery of the compound. Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) are phospholipids naturally present in the lungs that act as a biodegradable surfactant when used in a 3:1 ratio as an excipient in dry powder microparticles. DPPC and DPPG can improve particle migration in the lungs and increase lung residence

time (Hadinoto, Phanapavudhikul et al. 2007). I hypothesize that targeting the delivery of tacrolimus to the lungs using phospholipid-based dry powder aerosol microparticles will result in increased localized drug concentrations, improving the treatment of pulmonary arterial hypertension and decreasing the severity of side effects experienced by patients.

In this study, phospholipid-based aerosol microparticles were developed via spray drying. These particles were shown to be smooth and spherical in size, ranging from 1-3 μm in diameter. The microparticles exhibited thermal stability and were found to be amorphous after spray drying. Water content in the microparticles was under 10%, which will allow successful aerosol dispersion and long-term storage stability. *In vitro* aerosol dispersion showed that the microparticles could successfully deposit in the deep lung, as they exhibited favorable aerodynamic diameters and high fine particle fractions. *In vitro* dose-response analysis showed that TAC is nontoxic in the low concentrations that would be delivered to the lungs. Overall, this work shows that tacrolimus-loaded phospholipid-based microparticles can be successfully created with optimal physicochemical characteristics.

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DEDICATION

I would like to dedicate my thesis to my mom, Deborah Marie Brousseau, and my memère, Florence Brousseau, both of whom I know would be so incredibly proud of me for all of my accomplishments. Thank you for always loving and supporting me and for being my biggest cheerleaders. Don't worry memère, I'll tell my dad to put it in the Pawtucket Times.

PREFACE

This thesis was written and formatted in accordance with guidelines provided by the University of Rhode Island Graduate School and contains four chapters: Introduction, Materials and Methods, Results and Discussion, and Conclusions. I hope you enjoy reading about the work I have completed over the past year.

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CHAPTER 1

INTRODUCTION

1.1 Background and Treatment of Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is a chronic, incurable cardiovascular disease with a 15% annual mortality rate. PAH involves an increase in pulmonary vascular resistance due to pulmonary arterial obstructions and can eventually lead to right ventricular failure and ultimately death. Annually, 2.4 new cases are diagnosed per million people (Archer, Weir et al. 2010) and there is a mean life expectancy of 3 years after diagnosis with PAH (Saigal, Ng et al. 2013). It is likely that the worldwide impact of pulmonary arterial hypertension is much more widespread than current diagnoses suggest due to the difficulty of accurately diagnosing patients.

Vasoconstriction, remodeling of the pulmonary vessel wall, and thrombosis cause increased pulmonary vascular resistance in PAH patients. The characteristic pathogenesis of PAH is pulmonary arterial obstruction caused by vascular proliferation and remodeling, which occurs in all layers of the vessel wall (Humbert, Morrell et al. 2004). In **Figure 1**, the heart and lungs of a healthy adult are compared to those of an adult with PAH. Pulmonary arterial hypertension is defined by a mean pulmonary artery pressure of 25 mm Hg at rest and 30 mm Hg while exercising (Rosenblum 2010). PAH can be idiopathic, familial, related to connective tissue disease, or caused by drug or toxin exposure. Idiopathic, familial, and anorexigen-associated PAH have similar clinical, functional, and hemodynamic characteristics

(Humbert, Sitbon et al. 2010). Features of PAH include intimal fibrosis, distal localization and proliferation of vascular smooth muscle, and pulmonary arterial occlusion. PAH is also associated with alterations in the rates of cellular proliferation and apoptosis, resulting in thickened arteries. Severe end stages of PAH often result in plexiform lesions due to disorganized endothelial cell proliferation (Humbert, Morrell et al. 2004). Hormones, growth factors, neurotransmitters, and environmental stresses can promote the development of PAH. One of the primary causes of PAH progression is an imbalance of the neurochemical mediators that help maintain the vascular tones of pulmonary arteries (Gupta, Rawat et al. 2010). The prostacyclin, nitric oxide, ion channels, endothelin-1, and serotonin pathways have been connected with PAH.

Bone morphogenic protein receptor type II (BMP2) is an active serine/threonine kinase receptor, which signals via the formation of heterocomplexes in response to ligands (Humbert, Morrell et al. 2004). BMP2 plays a large role in familial and idiopathic PAH, but is not likely to be the root cause of PAH. Genetic and environmental factors are thought to play a larger role (Hong, Lee et al. 2008). BMP2 gene mutations are found in more than 70% of cases of familial PAH and 10% to 40% of apparently sporadic or anorexigen-associated PAH (Humbert, Sitbon et al. 2010). In cases of PAH without a BMP2 mutation, BMP2 expression is still significantly reduced (Humbert, Morrell et al. 2004). Improving the signaling of BMP2 reduces damaging vascular remodeling and can potentially improve patient symptoms and outcomes (Spiekerkoetter, Tian et al. 2013).

Current therapies for the treatment of pulmonary arterial hypertension include prostanoids, endothelin receptor blockers, phosphodiesterase type 5 (PDE5) inhibitors,

and soluble guanylate cyclase (SGC) stimulators that are typically delivered orally or intravenously. These therapeutics are often paired with anticoagulation and diuretic drugs due to the risk of thrombosis and edema associated with these treatments (Archer, Weir et al. 2010). Prostanoids, such as Flolan[®] and Remodulin[®], are often used to reduce inflammation and vasoconstriction by targeting the prostacyclin pathway. These compounds inhibit cells in the middle of blood vessels from growing into lesions (Feldman). Prostanoids are typically considered the best treatment option, but the dosage forms used limit patient compliance and cause negative systemic side effects (Saigal, Ng et al. 2013). Endothelin receptor blockers, such as Letairis[®], inhibit the endothelin pathway, which normally causes vasoconstriction by promoting the growth of cells in the middle of blood vessels resulting in the formation of lesions.

PDE5 inhibitors and SGC stimulators both target the nitric oxide pathway. PDE5 inhibitors, such as Revatio[®], decrease the effect of PDE5, thereby increasing the concentration of nitric oxide in the blood. SGC stimulators, such as Adempas[®], stimulate SGC in cells, causing an increased production of cyclic GMP, causing the pulmonary arteries to relax (Feldman). These treatments are highly expensive and difficult to deliver due to their limited bioavailability and toxicity of the drugs. Frequent dosing of these drugs is required because they have short half-lives. In addition, current therapies do not inhibit remodeling and patients are often required to receive heart and lung transplantations to increase their chances of survival (Spiekerkoetter, Tian et al. 2013). Unfortunately, the progression of PAH and its treatments are primarily based on assessments of exercise performance, quality of life,

time to clinical deterioration, and physiological performance because lung tissue for diagnosis is difficult to obtain other than upon autopsy (Stacher, Graham et al. 2012).

Figure 1. Comparison of the physiological characteristics of a healthy heart and a heart with pulmonary hypertension from Nationwide Children's Hospital.

<http://www.nationwidechildrens.org/pulmonary-hypertension>

The limited bioavailability of current PAH therapeutics and their associated toxicity results in the need for more sophisticated formulations to aid in patient compliance and health.

1.2 Aerosolized Therapeutics Produced via Spray Drying

Aerosols are a common method used in treating a variety of pulmonary diseases because they allow for a faster onset of action, avoid the first-pass metabolism of the liver, which removes the drug from the body before it can reach the target organ, and result in low systemic drug concentrations. The lungs are an attractive approach for drug delivery because they are highly permeable and have a large surface area (35-140 m²), allowing for higher bioavailability within the lungs (Shoyele and Cawthorne 2006). In addition, they have an elevated blood flow of 5 L/min (Bosquillon, Lombry et al. 2001). The lungs have a small fraction of drug-metabolizing and efflux transporter activity compared to the gut and liver, resulting in drugs delivered through the lungs producing fewer complex metabolites, thereby increasing drug efficacy (Patton, Fishburn et al. 2004).

The bronchial airways in human lungs have pseudostratified, ciliated, mucus-producing cells, covered with approximately 8 µm of mucus (Haghi, Traini et al. 2012). Particles that are delivered to the lungs as aerosols can produce a concentration gradient, causing the particles to undergo dissolution and solute transport across lung mucus and epithelium (Grainger, Greenwell et al. 2009). The rate of absorption in the lungs can improve pharmacokinetic profiles both locally in the lungs and systemically throughout the body (Forbes and Ehrhardt 2005). Targeted delivery to the lungs can be

enhanced by using microparticles as they can effectively deposit in the lungs by physical sedimentation (Li and Mansour 2011). Nebulizers, pressurized metered dose inhalers (pMDIs), soft-mist inhalers (SMIs), and dry powder inhalers (DPIs) are among the most frequently used aerosol delivery devices. Nebulizers are large and need to be used frequently for long periods of time and pMDIs require a propellant, causing these methods to be inconvenient to patients (Suarez and Hickey 2000). DPIs are beneficial because they allow for the delivery of particles designed with specific characteristics (particle size, drug loading, etc.) and the resulting dry powder particles and DPIs provide improved long-term chemical and physical stability (Meenach, Vogt et al. 2013). DPIs are also advantageous because they are propellant-free, portable, easy to operate, and inexpensive (Bosquillon, Lombry et al. 2001). They allow for effective, targeted delivery of drugs to the respiratory tract to treat a variety of pulmonary diseases. Formulations of dry powder particles delivered via DPIs typically consist of an active pharmaceutical ingredient (API) and excipient to improve stability and aerosolization (Guillon, Montharu et al. 2012).

Efficient drug delivery to the lungs using a dry powder inhaler depends on the inhaler device, powder formulation, and inhalation maneuver (Kaialy, Alhalaweh et al. 2011). The design specifications necessary for successful implementation in DPIs can easily be met by using advanced spray drying techniques of therapeutics and excipients for form dry powder aerosol particles. Spray drying is a high-throughput pharmaceutical particle engineering technique that efficiently produces respirable dry particles. Spray drying can enable facile control of the size, surface morphology, and shape of spray-dried particles by altering component concentrations, drying

temperature, pump rate, and gas flow of the spray-dryer (Iskandar, Gradon et al. 2003, Hoe, Ivey et al. 2014). There are four steps in a typical spray drying process as seen in **Figure 2**. First, the feed solution is atomized into a spray, followed by spray-air contact (step two). The sprayed droplets are dried at an elevated temperature (step three) and then the dried particles are separated from the air in a collection vial (step four) (Chow, Tong et al. 2007). Particle size and morphology play a large role in drug delivery (Chow, Tong et al. 2007). Optimally sized particles are within 1 to 5 μm for effective whole-lung deposition (Meenach 2014). Particles below 5 μm can be easily deposited into the smaller airways, whereas submicron particles will be exhaled (Chow, Tong et al. 2007). Choosing the correct feed solution when spray-drying poorly water soluble drugs is extremely important (Paudel, Worku et al. 2013). Using an organic feed solution can help achieve smaller sized particles with a lower water content (Li and Mansour 2011). Spray drying also increases the storage stability of particles due to the reduction in water content (Ré 2006). The short half-lives of drugs can be improved by developing spray-dried microparticles encapsulated in excipients such as lipids or polymers (Saigal, Ng et al. 2013).

Figure 2. The main stages involved in the spray-drying process (Ré 2006).

Figure 3. Percent deposition in the different sections of the lungs based on aerodynamic diameter (Laube, Janssens et al. 2011).

1.3 Tacrolimus

Tacrolimus (TAC), first commercialized as Prograf® in 1990, is a poorly water-soluble immunosuppressive agent derived from the fungus *Streptomyces tsukubaensis* (Cho 2014). Normally delivered intravenously or orally, in which it has poor bioavailability, TAC is typically used in the prevention of organ rejection after transplantations as an immunosuppressive agent (Gao, Sun et al. 2012). The aqueous solubility of tacrolimus is low, at 1-2 µg/mL, resulting in challenges in dosage formulations (Park, Ryu et al. 2009). When delivered systemically for a prolonged period of time, TAC causes adverse side effects such as nephrotoxicity, the onset of diabetes mellitus, an increased risk of opportunistic infections, and neurotoxicity. In mild cases of neurotoxicity, the patient experiences headaches and tremors, but patients can experience seizures and delirium in more severe cases (Shin, Cho et al. 2010). TAC has a narrow therapeutic index, making it necessary to closely monitor patients for toxicity. Severe side effects have been shown to occur at blood concentrations greater than 50 ng/mL (Watts, Peters et al. 2011). The target trough range of tacrolimus in the bloodstream is 5-20 µg/L, which requires careful and invasive monitoring (Sallustio, Noll et al. 2011). By targeting the delivery of TAC to the lung, the systemic concentration of the drug will be greatly reduced while increasing local pulmonary drug concentrations and therapeutic levels. Nebulized TAC has already been shown to improve the response of a lung transplant patient while substantially reducing side effects (Hayes, Zwischenberger et al. 2010). Blood levels have been found to remain low after the aerosolization of tacrolimus, reaching

1.6 ng/mL after twelve hours. (Schrepfer, Deuse et al. 2007). Because of this, patients will not have to be monitored as closely for systemic levels of TAC.

TAC has been found to be effective in the treatment of pulmonary arterial hypertension. As mentioned previously, TAC induces the signaling of bone morphogenic protein receptor type II (BMPRII) in endothelial cells, which has been found to be dysfunctional in the pathogenesis of pulmonary arterial hypertension by inhibiting vascular remodeling (Spiekerkoetter, Tian et al. 2013). The chemical structure of tacrolimus can be seen in **Figure 4**.

1.4 Phospholipid Excipients

In this study, the active pharmaceutical ingredient tacrolimus was spray dried with the excipients dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) to produce dry powder aerosol microparticles. DPPC and DPPG are the primary phospholipid components naturally present in the lungs. Lung surfactant is composed 80-90% of lipids, 70-80% and 5-10% of which is made up of phosphatidylcholines (PC) and phosphatidylglycerols (PG), respectively. Approximately 60% of PC in the lungs is composed of DPPC (Evora, Soriano et al. 1998). Phospholipids such as DPPC and DPPG are necessary to support proper lung surfactant function (Meenach, Vogt et al. 2013) and help protect against infections and inflammation (Mansour, Wang et al. 2001). DPPC and DPPG are often used in lung surfactant replacement therapies and controlled drug release applications (Wu, Hayes et al. 2013). Phospholipids are an efficient excipient because they protect the structural integrity, stability, and fluidity of the drug (Alves and

Santana 2004). Using phospholipid excipients that are biocompatible and biodegradable can aid in the delivery of drugs to the lungs by improving particle and drug migration to the lungs because of the reduction in surface tension from the surfactant (Evora, Soriano et al. 1998). A large portion of inhaled medications fail to reach the deep lung, and it is suspected that phospholipid deposition on the mucosal surface of non-targeted lung areas will cause particles to be transported through the airways via surfactant spreading (Ganguly, Moolchandani et al. 2008). Phospholipid excipients may also cause the spray-dried particles delivered to the lungs to evade recognition and uptake by the immune system, increasing lung residence time (Meenach, Anderson et al. 2013). In addition, it has been found that the co-spray-drying of drugs with DPPC can help reduce particle size and improve the aerodynamic performance. In addition, the presence of phospholipids in particles leads to an improvement in the respirable fine particle fraction (Hadinoto, Phanapavudhikul et al. 2007). The chemical structures of DPPC and DPPG can be seen in **Figures 5 and 6**.

Figure 4. Schematic of tacrolimus (TAC).

Figure 5. Schematic of dipalmitoylphosphatidylcholine (DPPC).

Figure 6. Schematic of dipalmitoylphosphatidylglycerol (DPPG).

1.5 Objectives and Hypothesis

The overall objective of this research was to develop and characterize phospholipid-based aerosol microparticles loaded with tacrolimus for applications in the treatment of pulmonary arterial hypertension. The specific objectives of this research include:

1. Synthesis and optimization of phospholipid-based aerosol microparticles containing tacrolimus using advanced spray drying methods.
2. Evaluation of the physicochemical properties of the spray-dried microparticles including their size, morphology, drug loading, crystallinity, thermal properties, water content, and aerosol dispersion.
3. *In vitro* evaluation of the safety and efficacy of the fully characterized TAC-loaded microparticles.

The development of an improved delivery method of the drug tacrolimus will lead to a more effective treatment method for PAH with fewer side effects. A smaller dose of TAC delivered directly to the lungs can be used than in the oral or intravenous delivery methods, which should minimize toxicity while optimizing the amount of drug that reaches the lungs. Targeting the delivery of tacrolimus to the lungs using phospholipid-based dry powder aerosol microparticles will result in increased localized drug concentrations, improving the treatment of pulmonary arterial hypertension and decreasing the severity of side effects experienced by patients. If delivering dry powder formulated TAC via a DPIs is found to be effective in treating

PAH, this delivery method could be applied to a multitude of other poorly water-soluble drugs with toxic side effects that are used to treat pulmonary diseases.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Synthetic dipalmitoylphosphatidylcholine (DPPC, Molecular Weight: 734.05 g/mol, >99% purity) and dipalmitoylphosphatidylglycerol (DPPG, Molecular Weight: 744.96 g/mol, >99% purity) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Tacrolimus (TAC, Molecular Weight: 804.02) was obtained from LC Laboratories (Woburn, MA, USA). 2-Propanol (HPLC grade, 99.9% purity), Hydranal[®]-Coulomat, methanol (anhydrous, 99.8% purity), and acetonitrile (HPLC grade, ≥ 99.9 % purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ultra-high purity (UHP) nitrogen gas was obtained from Airgas (Radnor Township, PA, USA). Extra pure phosphoric acid (85% pure in water) was obtained from Thermo Fisher Scientific (Waltham, MA). A549 cells were obtained from ATCC (Manassas, VA, USA). Sodium pyruvate was obtained from Fisher Scientific. Dulbecco's Modified Eagle Medium (DMEM), Pen-Strep, and Fungizone[®] were obtained from Life Technologies (Norwalk, CT, USA). Sodium acetate (1 M, pH 5), Triton X-100, and dimethyl sulfoxide (DMSO) were obtained from Wilkem Scientific (Pawtucket, RI, USA). Fetal bovine serum (FBS) was obtained from Atlanta Biologics (Flowery Branch, GA, USA). P-nitrophenyl (pNpp) was obtained from MP Biomedicals (Santa Ana, CA, USA).

2.2 Preparation of Spray-Dried Microparticles

Advanced spray drying of tacrolimus and DPPC/DPPG particles was performed using a B-290 Büchi Mini Spray Dryer (from Büchi Labortechnik AG, Switzerland) in closed mode. UHP dry nitrogen was used as the drying gas and the spray-dried (SD) particles were collected in a sample collector. A nozzle with a diameter of 0.7 mm was used. The feed solutions were prepared by dissolving DPPC and DPPG in a 3:1 molar ratio with varying amounts of tacrolimus, including 0, 25, 50, 75 and 100 mol% TAC to the total amount of DPPC/DPPG in isopropanol to form a dilute solution of 0.5% w/v. The composition of each feed solution can be seen in **Table 1**. The spray drying conditions used during particle formation were a pump rate of 100%, aspiration rate of 100%, and inlet temperature of 100°C. All spray-dried powders were stored in small glass vials sealed with parafilm in desiccators with DrieriteTM desiccant at -20°C under ambient pressure.

2.3 Scanning Electron Microscopy (SEM)

The shape and surface morphology of the spray-dried particles was evaluated via scanning electron microscopy (SEM) using a Zeiss SIGMA VP Field Emission Scanning Electron Microscope (Germany). Dry powder samples were placed on aluminum stubs (Ted Pella Inc., Redding, CA, USA) with double-sided adhesive carbon tabs and then coated with a thin film of a gold/palladium alloy using a BIO-RAD sputter coating system at 20 $\mu\text{\AA}$ for 60 seconds under argon gas. Micrographs were collected at several different magnifications. The size of the spray-dried particles

was analyzed using Image J Size Analysis software with images obtained from the SEM at 10x magnification. Fifty particles per sample were analyzed.

2.4 Differential Scanning Calorimetry (DSC)

Thermal analysis and phase transition measurements of the raw materials and spray-dried particles were carried out via differential scanning calorimetry (DSC) using a DSC Q10 (TA Instruments, New Castle, DE, USA). 1-2 mg of the sample was weighed into aluminum Tzero pans with Tzero hermetic lids, both from TA Instruments. The pans were sealed with Tzero Press from TA Instruments. UHP dry nitrogen gas was the purging gas used. The samples were exposed to heating range of -25 to 300 °C at a heating scan rate of 5 °C/min.

2.5 Powder X-Ray Diffraction (PXRD)

Powder x-ray diffraction (PXRD) patterns of raw material and particle powder samples were measured using a Rigaku Multiflex X-ray diffractometer (the Woodlands, TX, USA) with a slit-detection Cu K α radiation source (40 kV, 44 mA, and $\lambda = 1.5406 \text{ \AA}$). The scan range was 5-45° in 2θ with a scanning rate of 2 °/min at ambient temperature. The sample was placed on a horizontal quartz glass sample holder plate.

2.6 Karl Fischer (KF) Coulometric Titrations

The water content of the raw materials and spray-dried particles was determined by Karl Fischer (KF) coulometric titration. The measurements were

performed with an 851 Titrando KF Coulometer coupled with an 803 Ti Stand (Metrohm Ltd., Antwerp, Belgium). Approximately 10 mg of powder was dissolved in a known volume of anhydrous methanol. The sample solution was then injected into the reaction cell containing Hydranal[®] and the water content was calculated from the resulting reading.

2.7 *In Vitro* Aerosol Dispersion Performance by the Next Generation Impactor[™] (NGI[™])

The *in vitro* aerosol dispersion of the spray-dried microparticles was determined using a model 170 Next Generation Impactor[™] from MSP Corporation (Shoreview, MN, USA) with a USP stainless steel induction port. The NGI[™] was coupled with a Copley HCP5 vacuum pump and a Copley TPK 2000 critical flow controller (Copley Scientific, United Kingdom). The airflow rate was set to 60 L/min in order to model the airflow rate in the lung of a healthy adult (Hickey and Mansour 2009). Glass fiber filters (55 mm, Type A/E, Pall Life Sciences, PA, USA) were placed into the gravimetric insert cups and weighed before and after each experiment to determine particle deposition on each stage. Approximately 10 mg of microparticles were loaded into a hydroxypropyl methylcellulose capsule (HPMC, size 3, Quali-V[®], Qualicaps[®] Inc, Whitsett, NC, USA) and then placed into a dry powder inhaler (HandiHaler, Boehringer Ingelheim Pharmaceuticals, CT, USA). The dry powder inhaler was attached to a rubber mouthpiece attached to the induction port on the NGI[™]. The NGI[™] was operated with a 10 s delay and 10 s run time. The effective cutoff diameters for each stage of the NGI[™] were given by the manufacturer as: stage

1 (8.06 µm); stage 2 (4.46 µm); stage 3 (2.82 µm); stage 4 (1.66 µm); stage 5 (0.94 µm); stage 6 (0.55 µm); and stage 7 (0.34 µm). The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were calculated using a Mathematica® program written by Dr. Warren Finlay (Finlay 2001). The fine particle dose (FPD), fine particle fraction (FPF), respirable fraction (RF), and emitted dose (ED) were calculated using the following equations:

$$FPD = \text{mass of particles on Stages 2 through 7}$$

$$FPF = \frac{FPD}{\text{Initial Particle Mass Loaded into Capsules}} \times 100\%$$

$$RF = \frac{FPD}{\text{Total Particle Mass on all Stages}} \times 100\%$$

$$ED = \frac{\text{Initial Mass in Capsules} - \text{Final Mass Remaining in Capsules}}{\text{Initial Mass in Capsules}} \times 100\%$$

2.8 *In Vitro* Dose-Response Analysis

A549 lung adenocarcinoma cells were cultured in 75 cm² Biolite cell culture flasks (Fisher Scientific) with media consisting of DMEM supplemented with 10% (v/v) FBS, Pen-Strep (100 U/mL penicillin, 100 µg/mL streptomycin), Fungizone® (0.5 µg amphotericin, B, 0.41 µg/mL sodium deoxycholate), and 1 mM sodium pyruvate. The cells were cultured at 37°C and 5% CO₂ in a humidified incubator. The A549 cells were seeded in two 96 well plates (Celltreat Scientific, Shirley, MA, USA)

at 100 µL/well. On day two, the cells were dosed with dilutions of TAC ranging from 0.00001 to 10 µM of a 20,000 µM stock solution of raw TAC in DMSO. 0.1% (v/v) of DMSO was used in each dilution to aid in TAC solubility in media. The control for the calculations was 0 µM TAC in 0.1 % (v/v) DMSO. 100 µL of the dilutions were added to each well (n = 8). At 48 and 72 hours after drug exposure, viability of the cells was assessed using the acid phosphatase assay. A pNpp solution (sodium acetate, 1% Triton X-100, and pNpp) was added to each well and the plate was then incubated at 37°C and 5% CO₂ for two hours. The absorbance was measured at 405 nm with a Cytation 3 plate reader (BioTek, Winooski, VT, USA). Relative viability was calculated using the following equation:

$$Relative Viability (\%) = \frac{Sample Fluorescence Intensity}{Control Fluorescence Intensity} \times 100\%$$

CHAPTER 3

RESULTS AND DISCUSSION

The described study involved the physicochemical and *in vitro* characterization of tacrolimus-loaded DPPC/DPPG particles produced via spray drying. Optimization and design resulted in the formulation of several practical particle systems, which included four systems loaded to tacrolimus (25, 50, 50, and 100 mole% TAC) and one without (pure phospholipids). This study strived to determine the effects on the presence of differing TAC amounts on the particle formulations. The size, morphology, thermal stability and crystallinity, water content, aerodynamic dispersion performance, and *in vitro* effects on pulmonary cells were evaluated.

3.1 Size and Morphology of Spray-Dried Microparticles

The formulated particles and their morphologies were analyzed using SEM micrographs, as seen in **Figure 7**. The geometric diameter of the particles is displayed in **Table 1** and was determined using Image J software. 0TAC particles were agglomerated and as a result their diameter was immeasurable. 25TAC and 75TAC particles were rounder and smoother than 0TAC with some agglomeration and sintering between particles. 50TAC particles were spherical, smooth, and uniformly sized. 100TAC particles were round and smooth, but not uniformly sized. The size of all measurable particles was between 1.20 to 2.83 μm in size as seen in **Table 1**. 50TAC particles were the largest and 100TAC particles were the smallest. All

particles were within the range for optimal lung deposition in children and adults (Meenach 2014). The particles exhibited an ideal size range (approximately 1 μm) for ensuring targeted delivery to specific regions of the lung. In particular, the geometric diameters indicate that the described microparticles are capable of delivering a therapeutic payload to the deep lung (Sung, Pulliam et al. 2007), which is necessary for the treatment of pulmonary arterial hypertension.

Figure 7. Representative SEM micrographs of spray-dried phospholipid-based microparticles: (a) 0TAC; (b) 25TAC; (c) 50TAC; (d) 75TAC; (e) 100TAC, where the number indicates the mole% of TAC in each system. Magnification = 10,000x. Scale bar = 5 μm .

Table 1. Systems of spray-dried phospholipid-based particles and their corresponding mole % of tacrolimus (TAC), dipalmitoylphosphatidylcholine (DPPC), and dipalmitoylphosphatidylglycerol (DPPG), outlet temperature from the spray dryer, geometric diameter, and water content.

System	0TAC	25TAC	50TAC	75TAC	100TAC
TAC (mole %)	0	25	50	75	100
DPPC (mole %)	75	56.25	37.5	18.75	0
DPPG (mole %)	25	18.75	12.5	6.25	0
Outlet T (°C)	45	43	44	42	42
Diameter (μm)	N/A	1.45 ± 0.569	2.34 ± 0.607	2.28 ± 1.06	1.91 ± 0.762
Water Content (%)	10.01 ± 3.29	6.77 ± 1.33	6.04 ± 2.45	6.69 ± 2.88	4.15 ± 1.50

3.2 Thermal Analysis of Spray-Dried Microparticles

DSC studies were performed on the raw materials prior to spray drying (TAC, DPPC, DPPG) and spray-dried formulated microparticles (0TAC, 25TAC, 50TAC, 75TAC, and 100TAC) to determine their thermal behavior and melting points, as seen in **Figure 8**. An endothermic peak is evident at 129°C for raw tacrolimus (TAC), signifying its melting point. The endothermic peak signifying the TAC melting point is not present in the thermograms of the spray-dried particles due to the transition of TAC from crystalline to amorphous forms during the spray drying process. Raw DPPC and DPPG exhibited a characteristic bilayer main phase transition phase at 71°C and 79°C. A bilayer main phase transition was also seen in the spray-dried microparticles at approximately 76°C, becoming less significant with increasing drug concentration due to the DPPC content.

The formulated microparticles were spray dried at an inlet temperature of 100°C at a pump rate of 100% (the highest possible for the described system). As a result, the outlet temperatures of the spray drying process for the microparticles ranged from 42 to 45°C, as seen in **Table 1**. These outlet temperatures are below the phase transition temperatures as indicated in the DSC thermograms, therefore the spray drying temperatures do not negatively impact the formation of the microparticles. The stability microparticles at different storage conditions such as room temperature (22°C), refrigeration (4°C), and freezing (-20°C) is likely since there are no measurable DSC transition peaks present in the data. This is also true at the treatment condition (e.g. body temperature at 37°C).

Figure 8. DSC thermograms of spray-dried microparticles of varying compositions and their corresponding raw materials (DPPG, DPPC, and TAC).

3.3 Crystallinity Analysis of Spray-Dried Tacrolimus Microparticles

X-ray powder diffractograms were collected for the raw pre-spray-dried components (TAC, DPPC, DPPG) and spray-dried microparticles (0TAC, 25TAC, 50TAC, 75TAC, and 100TAC) as shown in **Figure 9**. Strong peaks are seen in raw TAC between 20 and $25^{\circ} 2\theta$ indicating the crystallinity of the material prior to the spray drying process. Raw DPPC exhibited a strong broad peak at $21^{\circ} 2\theta$, which corresponds to the presence of the phospholipid bilayer structure (Alves and Santana 2004). Raw DPPG showed a cluster of peaks between 18 and $25^{\circ} 2\theta$. The spray-dried particles show decreasing peak intensities at $22^{\circ} 2\theta$ with increasing mole percentage of TAC. The peaks corresponding to raw DPPG were not noticeable in the spray-dried formulations, likely due to their limited presence in comparison to raw DPPC, which has a strong peak signal. 0TAC and 25TAC exhibited the most evident peaks of the spray-dried particles at $22^{\circ} 2\theta$, indicative of the presence of phospholipid bilayer structures within the particles. The lack of significant peaks in the 50TAC, 75TAC, and 100TAC particles is likely due to the lower mole percentage of the phospholipids and reveals that the TAC present in the spray-dried particles is amorphous and has limited bilayer formation. Amorphous solids are more soluble and have higher dissolution rates than crystalline solids (Alves and Santana 2004).

PXRD and DSC analysis indicate that the spray-dried phospholipid TAC-loaded microparticles display the characteristics of particles with a lipid bilayer structure (likely multilamellar) owing to the signature peaks in XRD analysis and bilayer phase transition values in the DSC thermograms (Mansour, Wang et al. 2001, Mansour and Zografi 2007).

3.4 Water Content

The residual water content in the spray-dried microparticles can be seen in **Table 1**. The water content in the microparticles ranged from 4 to 10%. It was the highest in 0TAC particles, at $10.01 \pm 3.29\%$ and lowest in the 100TAC particles, at $4.15 \pm 1.50\%$. The remaining formulations had 6-7% residual water, similar to previously reported results (Meenach, Vogt et al. 2013) and within the range of other microparticles in our group (results not published). Low water content in inhalable dry powders is essential for aerosol dispersion properties because it reduces agglomeration (Meenach, Anderson et al. 2013). High water content in aerosol powders and drug formulations can also reduce stability due to the propensity of amorphous powders to recrystallize in aqueous conditions (Wu, Hayes et al. 2013).

Figure 9. X-ray powder diffractograms of spray-dried microparticles with varying compositions and their corresponding raw materials.

3.5 *In Vitro* Aerosol Dispersion Analysis

The most important factor influencing the deposition of particulates to the lungs are the aerodynamic properties of the therapeutic system (Hickey and Mansour 2009). The *in vitro* aerosol dispersion performance properties of the microparticles were evaluated using an NGITM coupled with a DPI device and can be seen in **Figure 10** and **Table 2**. **Figure 10** demonstrates the actual aerosol dispersion performance of the formulated dry powder microparticles by showing the mass fraction of particles on each NGITM stage. The mass mean aerodynamic diameters (MMAD) of the particles were $4.0 \pm 0.6 \mu\text{m}$ for 0TAC, $9.1 \pm 2.2 \mu\text{m}$ for 25TAC, $8.7 \pm 5.2 \mu\text{m}$ for 50TAC, $4.1 \pm 0.7 \mu\text{m}$ for 75TAC, and $6.0 \pm 1.8 \mu\text{m}$ for 100TAC. The geometric standard deviation (GSD) for the spray-dried microparticles ranged from $2.1 \pm 0.2 \mu\text{m}$ to $2.9 \pm 0.9 \mu\text{m}$ and these values were similar to previously reported results (Meenach, Vogt et al. 2013).

The fine particle fractions (FPF) of the particles ranged from 40.6 to 74.2 %, the respirable fraction (RF) ranged from 35.8 to 55.7% and the emitted dose (ED) ranged from 88 to 100%. In addition, as indicated in **Figure 10**, 21-27% of microparticles deposited on stages 2-4 and 1-6% deposited on stages 5-7. There were no clear trends corresponding to the effect of amount of drug in relation to excipient on the aerosol dispersion properties of the microparticles. The particles with the most optimal aerosol performance were the 75TAC particles. Overall, aerosol dispersion was measurable on all of the NGITM stages and as a result these microparticles are predicted to deposit in the deep lung region of the human lung as well as throughout the upper portions of the lung as indicated by the MMAD values.

Figure 10. *In vitro* aerosol dispersion performance as mass fraction of microparticles deposited on each stage of the Next Generation ImpactorTM (NGITM) for spray-dried particles containing DPPC and DPPG with varying PTX content. For $Q = 60$ L/min, the effective cutoff diameters (D_{50}) for each NGITM impaction stage are as follows: stage 1 ($8.06 \mu\text{m}$), stage 2 ($4.46 \mu\text{m}$), stage 3 ($2.82 \mu\text{m}$), stage 4 ($1.66 \mu\text{m}$), stage 5 ($0.94 \mu\text{m}$), stage 6 ($0.55 \mu\text{m}$), and stage 7 ($0.34 \mu\text{m}$). ($n = 3$, Average $\pm$ SD).

Table 2. *In vitro* aerosol dispersion performance using the Next Generation ImpactorTM for spray-dried phospholipid-based systems containing tacrolimus.

Parameters include mass median aerodynamic diameter (MMAD), geometric standard deviation (GSD), fine particle fraction (FPF), respirable fraction (RF), and emitted dose (ED) (n = 3, Average $\pm$ SD).

System	0TAC	25TAC	50TAC	75TAC	100TAC
MMAD (μm)	4.0 $\pm$ 0.6	9.1 $\pm$ 2.2	8.7 $\pm$ 5.2	4.1 $\pm$ 0.7	6.0 $\pm$ 1.8
GSD (μm)	2.1 $\pm$ 0.2	2.5 $\pm$ 0.4	2.5 $\pm$ 0.5	2.1 $\pm$ 0.2	2.9 $\pm$ 0.9
Fine Particle Dose (mg)	8.7 $\pm$ 1.3	6.4 $\pm$ 1.1	5.2 $\pm$ 2.5	9.5 $\pm$ 0.8	8.8 $\pm$ 1.3
Fine Particle Fraction (%)	58.8 $\pm$ 2.9	44.3 $\pm$ 7.2	40.6 $\pm$ 10.9	74.2 $\pm$ 13.4	53.1 $\pm$ 4.7
Respirable Fraction (%)	49.2 $\pm$ 8.4	51.1 $\pm$ 1.2	41.7 $\pm$ 16.1	50.4 $\pm$ 9.2	55.7 $\pm$ 6.8
Emitted Dose (%)	100 $\pm$ 0.3	92.5 $\pm$ 4.9	99.8 $\pm$ 4.6	88.0 $\pm$ 7.3	97.6 $\pm$ 1.0

3.6 *In Vitro* Dose-Response Analysis

The effect of raw tacrolimus on the viability of A549 cells was evaluated at 48 and 72 hours after drug exposure and can be seen in **Figures 11** and **12**, respectively. The relative viability for the A549 cells remained steady for increasing drug concentrations for both the 48 and 72-hour studies, showing that TAC is not toxic to lung cells at concentrations ranging from 0.00001 μM to 10 μM . In greater concentrations, the drug does become toxic (data not shown), but drug concentration in the lungs would not exceed this range, particularly in the spray-dried microparticles (Hayes, Zwischenberger et al. 2010). Delivering TAC to the lung is not expected to cause cell death, minimizing potential negative side effects.

Figure 11. 48-hour dose-response curve for A549 cells exposed to tacrolimus.

Figure 12. 72-hour dose-response curve for A549 cells exposed to tacrolimus.

CHAPTER 4

CONCLUSIONS AND FUTURE WORK

4.1 Conclusions

Dry powder aerosol microparticles containing of tacrolimus (TAC), dipalmitoylphosphatidylcholine (DPPC), and dipalmitoylphosphatidylglycerol (DPPG) were successfully formulated via spray drying and exhibited optimal characteristics for dry powder aerosols used for the treatment of pulmonary arterial hypertension. Five different formulations were made with a 3 to 1 molar ratio of DPPC to DPPG and varying drug concentrations (0%, 25%, 50%, 75%, and 100 mole % TAC). The physicochemical characteristics of the microparticles were evaluated prior to *in vitro* cellular analysis. 0TAC did not form distinguishable microparticles, but the remaining formulations ranged from 1.5 - 2.5 μm in size. 50TAC and 75TAC particles were spherical and smooth with a narrow size distribution. The microparticles were found to be stable in the working temperature range with no measurable thermal degradation. The spray drying process resulted in amorphous microparticles, which can improve drug solubility. Water content in the microparticles was low, which is essential for dry powder aerosolization and formulation stability. The *in vitro* aerosol dispersion showed that these microparticles are optimal for whole, and in particular, deep lung deposition for drug delivery. Dose-response analysis showed that the concentrations of tacrolimus that will be present in the lungs would be nontoxic.

This study successfully showed that tacrolimus-loaded microparticles can be produced that may reduce toxicity. These systems demonstrated the successful use of biocompatible phospholipids as excipients in improving aerosol dispersion and particle size for the targeted delivery of dry powder particles. This method can improve the treatment of pulmonary arterial hypertension by effectively delivering tacrolimus in a way that minimizes side effects, improves stability, and increases patient convenience.

4.2 Future Work

Future work includes the analysis of the drug loading and encapsulation efficiency of tacrolimus in the spray-dried microparticles, the evaluation of impact of the microparticles on the transepithelial electrical resistance of Calu-3 cells, and the dose response of the microparticles on A549 cells. Overall, the use of DPPC and DPPG as excipients in spray-dried microparticles for dry powder delivery could easily be applied to a multitude of other poorly water-soluble drugs with toxic side effects that are used to treat pulmonary diseases.

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