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# Characterization and pharmacokinetic analysis of tacrolimus dispersion for nebulization in a lung transplanted rodent model

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ABSTRACT

Lung transplantation animal models have been well established and enabled the investigation of a variety of new pharmacotherapeutic strategies for prevention of lung allograft rejection. Direct administration of immunosuppressive agents to the lung is a commonly investigated approach; however, can prove challenging due to the poor solubility of the drug molecule, the tortuous pathways of the lung periphery, and the limited number of excipients approved for inhalation. In this study, we aimed to evaluate a solubility enhancing formulation of tacrolimus for localized therapy in a lung transplanted rat model and determine the extent of drug absorption into systemic circulation. Characterization of the nebulized tacrolimus dispersion for nebulization showed a fine particle fraction (FPF) of 46.1% and a mass median aerodynamic diameter (MMAD) of 4.06 μm. After single dose administration to transplanted and non-transplanted rats, a mean peak transplanted lung concentration of 399.8 ± 29.2 ng/g and mean peak blood concentration of 4.88 ± 1.6 ng/mL were achieved. It is theorized that enhanced lung retention of tacrolimus is due to lipophilic associations with bronchial tissue and phospholipid surfactants in lung fluid. These findings indicate that tacrolimus dispersion for nebulization can achieve highly localized therapy for lung transplant recipients.



## 1. Introduction

Cases of end-stage lung disease such as severe chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), and cystic fibrosis (CF) often show limited response to treatment and pharmacotherapy, leaving very few treatment options for clinicians. Lung transplantation has been accepted as an effective therapeutic option in the treatment of patients with end-stage pulmonary diseases. Annually, approximately 2000 patients undergo lung transplantation when conventional treatments are unsuccessful. While surgical procedures and maintenance therapy can prove quite difficult, recent years have shown the frequency of lung transplant procedures to increase dramatically, nearly doubling in the last ten years (Trulock et al., 2007). Much of this increase has been a result of improved patients outcomes resulting from more effective donor matching, advances in surgical techniques, and development of more effective rejection preventing pharmaceuticals.

The introduction of a more potent immunosuppressive agent, tacrolimus, has been associated with increased success in the prevention of allograft rejection and bronchiolitis obliterans syndrome (BOS) compared to its predecessors (Keenan et al., 1995; O'Grady et al., 2002). Prograf, an oral tacrolimus formulation made by Astellas Pharmaceuticals (Tokyo, Japan), is used in maintenance therapy for approximately 70% of lung transplant patients one year after transplantation (Christie et al., 2008). While its use in lung transplantation is off label (having FDA approval for only kidney, liver, and heart transplant only), oral tacrolimus has been proven more effective as a maintenance (O'Grady et al., 2002) and rescue (Garrity and Mehra, 2004) therapy in lung transplant patients than other oral immunosuppressants, while also showing improved patient pulmonary function in comparison to other treatments (Verleden et al., 2003). Much of these improvements may be a consequence of tacrolimus being up to 100 times more potent than the alternative pharmaceutical, cyclosporine (Kino et al., 1987).

In spite of improvements in surgical technique and therapy, lung transplant rejection rates exceed those of all other forms of solid organ transplant, stemming form the complexity of the pulmonary immune system and the frequency of exposure to foreign antigens. Further complexity is added to dosing regimens of immunosuppressive agents by the prevalence of nephrotoxicity, hypertension, and neurotoxicity (among others) at sustained and/or elevated systemic concentrations. Tacrolimus blood concen-

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tration has also demonstrated high intra-patient variation due to erratic gastrointestinal absorption and low solubility. The delicate balance between therapeutic concentrations and toxic systemic concentrations combined with variable oral bioavailability requires frequent analysis of blood trough levels and often results in untoward complications. Clearly, a reduction in systemic drug levels accompanied with a pharmaceutical product with increased and consistent local bioavailability would alleviate many of the problems encountered in maintenance therapy.

The goal of targeting immunosuppressive therapy to the lungs and reduction of systemic side effects may be realized through the pulmonary delivery of tacrolimus. In transplanted animal models, others have demonstrated that aerosolized tacrolimus is effective in prolonging allograft survival, while maintaining systemic drug concentrations well below the toxic range (Ide et al., 2007; Ingu et al., 2005; Schrepfer et al., 2007). Since nebulization is commonly used in acute care settings for patients with poor breath coordination and lung function, a dispersed formulation for nebulization is investigated. The objective of this study was to characterize a novel tacrolimus formulation for inhalation and determine its pharmacokinetics in a non-rejection lung transplanted rat model. Additionally, pharmacokinetic differences of non-transplanted and transplanted animals were studied. In comparing the drug levels of transplanted and non-transplanted animals, we hypothesized that no significant difference in tissue levels will be observed and that blood levels would remain below the therapeutic range.

## 2. Materials and methods

### 2.1. Materials

Tacrolimus anhydrous (batch #070405) was purchased form Haorui Pharma-Chem, Inc. (Edison, NJ). Sirolimus (rapamycin) was bought from Spectrum Chemical (Gardena, CA). High performance liquid chromatography (HPLC) grade acetonitrile, lactose monohydrate, zinc sulfate heptahydrate, barium hydroxide (0.3N), and phosphoric acid (85%) were all purchased from Fisher Scientific (Fair Lawn, NJ). Heparin sodium (10,000 IU/mL) was purchased from Baxter Healthcare (Deerfield, IL). Cold Perfadex® was purchased for transplant procedures from Vitrolife, Inc. (Englewood, CO).

### 2.2. Formulation preparation

Thin film freezing (TFF) technology was used to produce tacrolimus powder for reconstitution in deionized water. This process is described in detail by Sinswat et al. (2008). Briefly, a 1:1 solution of tacrolimus to lactose monohydrate containing 0.75%, w/v total solids was made by dissolving powders in a cosolvent mixture of acetonitrile and water. The transparent solution was applied to a rotating drum that had been cryogenically cooled to −50 °C, where it was rapidly frozen (Overhoff et al., 2007). The frozen product was removed from the cryogenic surface and subjected to lyophilization using a VirTis Advantage Tray Lyophilizer (VirTis Company Inc., Gardiner, NY) to sublimate aqueous and organic solvents. After 40 h of lyophilization, the product was packaged in a nitrogen rich environment at less than 20% relative humidity. Before aerosolization, tacrolimus powder for reconstitution was dispersed in deionized water by probe sonication.

### 2.3. Formulation characterization

#### 2.3.1. Particle size analysis

Analysis of the particle size of tacrolimus dispersion for nebulization was measured by laser scatter using a BI-ZetaPlus (Brookhaven Instruments, Holtsville, NY) and analyzed by a digital autocorrelator (Brookhaven Instruments, Holtsville, NY). Samples were run immediately after dispersion.

#### 2.3.2. Aerosol characterization

Pharmaceutical aerosols produced by an Aeroneb® Pro (Aerogen, Galway, Ireland) vibrating mesh nebulizer were characterized by cascade impaction and laser diffraction techniques. Aerosol impaction testing conducted with a Next Generation Pharmaceutical Impactor (NGI) (MSP Corporation, Shoreview, MN) was used to determine aerodynamic droplet size, dose emitted, and fine particle fraction. The NGI was operated at a flow rate of 30 L/min. Impacted formulation was collected with mobile phase and injected for HPLC analysis. The method used for HPLC analysis was adapted from Akashi et al. (1996). A Waters 515 liquid chromatograph with a 996 PDA (Waters Corp., Milford, MA) combined with a Lichosphere RP C18 5 μm column (Varian Corp., Lake Forest, CA) was used for analysis. Mobile phase was composed of acetonitrile, water, and phosphoric acid in proportions of 600, 400, and 1, respectively. Data collected was plotted in Sigma Plot (Systat Sofware Inc., San Jose, CA) and fit with a 3-parameter logistic curve for data interpellation. Mass median aerodynamic diameter (MMAD) was calculated based on drug collected on stages 1–7 and micro-orifice collector (MOC) in accordance with USP 31 <601> guidelines. Fine particle fraction (FPF) was calculated as the percentage of the total emitted dose with an aerodynamic size less than 5 μm. A Malvern Spraytec (Malvern Instruments, Worcestershire, UK) was used to determine geometric droplet size distribution of tacrolimus dispersion in deionized water produced by the vibrating mesh nebulizer. An air current of 1 L/min was used to create the plume exiting the device.

### 2.4. Transplant study design

Adult male Lewis rats weighing 250–300 g were used as both recipient and donor in the non-rejection model. All animals received proper care in compliance with an Institutional Animal Care and Use Committee (IACUC) approved protocol. Transplantation procedures were carried out using a cuff technique described by Mizuta et al. (1989). Prophylactic enrofloxacin (Baytril®) was administered after transplantation to prevent possible infection. To confirm successful transplant and survival of allograft lung tissue, pathological review of the transplanted lung in preliminary animals was conducted. This evaluation verified whether or not a successful transplant had been accomplished in the non-rejection model, and that the dosing portion of the study could commence. Histology examples of tissues meeting evaluation criteria are given in Fig. 1. During this study, a total of 24 rats received aerosolized tacrolimus, 12 having undergone unilateral left lung transplantation and 12 non-transplanted control rats. Each animal was given a nebulized dose of 6.4 mg of formulation dispersed in 3 mL deionized water. The dispersion was nebulized by an Aeroneb® Pro and delivered via a four port nose-only dosing chamber (Sinswat et al., 2008). The animals were sacrificed 1, 6, 12, or 24 h after the completion of single dose administration. Whole blood, obtained by cardiac puncture, was collected in heparinized conical vials, while the lungs, liver, spleen, and kidneys were excised and frozen in 20 mL scintillation vials.

#### 2.4.1. Analysis of tacrolimus levels in whole blood

To determine systemic drug concentrations at each time point, a PRO-Trac™ II FK 506 Enzyme-linked Immunosorbent Assay (ELISA) kit (Diasorin Inc., Stillwater, MN) was used. In accordance with the supplied protocol, 50 mL of whole blood, standards, and controls were each combined with 300 μL of digestion reagent in separate conical vials. After incubation, cellular components were centrifuged down and supernatant serum was removed. The sam-

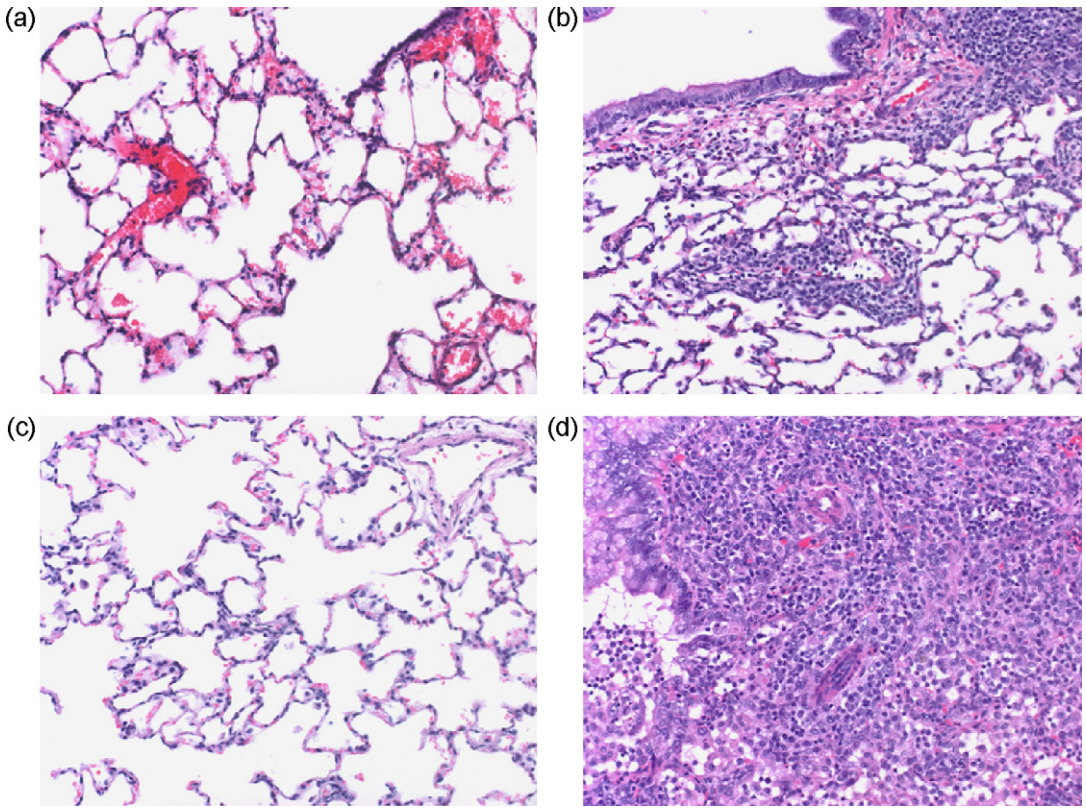


**Fig. 1.** Pathologically reviewed slides of transplanted lung tissue representing: (a) no rejection and (b) acute graft rejection at 3 days post-op and (c) no rejection and (d) acute graft rejection at 6 days post-op.

ples were pipetted into a goat anti-mouse IgG coated 96-well plate along with anti-tacrolimus monoclonal antibody. After further incubation and addition of tacrolimus-horesradish peroxidase conjugate, the wells were rinsed with mild wash solution. Empty wells were filled with chromagen followed by acid stop solution to elucidate a colorimetric response. Spectrophotometric readings at 450 and 630 nm were recorded using a Bio-Tek® Instruments UV/Vis μQuant plate reader (Winooski, VT). A 4-parapmeter logistic fit was applied to the supplied standards and used to determine experimental concentrations.

#### 2.4.2. Analysis of tacrolimus levels in lung tissue

Drug levels in lung tissue were quantified using liquid chromatography/mass spectrometry (LC/MS) after a liquid extraction procedure. Tissue was homogenized in 2 mL normal saline with 1.25 μg of internal standard (sirolimus). Extraction was facilitated by the addition of 0.3N barium hydroxide, 0.4N zinc sulfate heptahydrous, and acetonitrile. Centrifugation was used to separate tissue and precipitated proteins from dissolved tacrolimus. Supernatant was filtered, and lyophilized for 12 h to remove all solvents. The remaining drug was reconstituted with mobile phase, filtered again through a 0.2 μm PTFE filter, and prepared for spectrometric analysis. LC/MS equipment consisted of a Thermo Fisher Surveyor Plus HPLC system with PDA (Waltham, MA) and a Thermo Fisher LTQ FT Ultra Hybrid Mass Spectrometer (Waltham, MA). Other equipment specifications included a C18 3μ Thermal Hypersil Gold 50 × 2.1 column and a gradient flow of acetonitrile and water (5:95 to 80:20 to 5:95).

#### 2.4.3. Statistical analysis

Drug concentration in the lungs and blood of transplanted rats was compared to that of control rats at each time point. Analysis was conducted using Minitab® Release 14 statistical software (Minitab Inc., State College, PA). One-way ANOVA followed by a post hoc Tukey test was used at $p = 0.05$ to determine significant differences between transplanted and non-transplanted groups.

## 3. Results and discussion

### 3.1. Formulation

Tacrolimus, a lipophilic drug molecule ($\log P = 3.3$) with poor water solubility (4 μg/mL), faces formulation hurdles in order to achieve high and reproducible bioavailability. Efforts to improve the oral bioavailability of tacrolimus have been made through the inclusion of dimethyl-β-cyclodextrin to in enhance solubility and reduce p-glycoprotein efflux (Arima et al., 2001) as well as microencapsulation to allow for sustained colonic delivery (Lamprecht et al., 2005). To increase solubility in the commercial product, amorphous drug is stabilized within a hydroxypropylmethylcellulose (HPMC) matrix in the FDA approved oral formulation, Prograf® (Astellas Pharmaceuticals, 2009; Yamashita et al., 2003).

A similar strategy is applied in our novel pulmonary formulation in that the amorphous drug is stabilized; however, use of stabilizing excipients are limited to those that are generally recognized as safe (GRAS) for use in the lungs. In a formulation study involving TFF particle engineering technology, it was found that a nanostructured solid dispersion of amorphous tacrolimus could be created using equal parts of lactose (Sinswat et al., 2008). In vitro testing of this drug product showed a 10-fold improvement in peak saturation of tacrolimus in simulated lung fluid. Pulmonary delivery of the nanostructured powder created by this process is facilitated by dispersion in an aqueous media, and nebulized by a vibrating mesh nebulizer. Particle size analysis of the resulting dispersion measured 5 min after addition to the aqueous phase showed a monodisperse distribution with a mean particle diam-

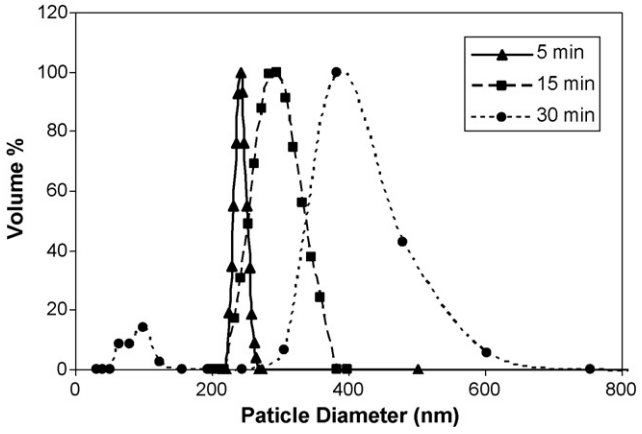


**Fig. 2.** Analysis of resulting particle size of particulate drug formulation within an aqueous media (▲) 5, (■) 15, and (●) 30 min after dispersion via probe sonication. Particle diameter is plotted versus normalized volume distribution.

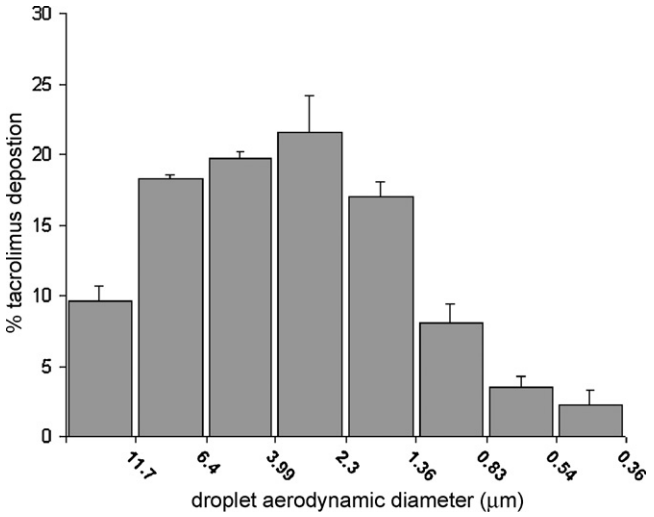


**Fig. 3.** Aerodynamic droplet size of nebulized tacrolimus dispersion for nebulization determined by Next Generation Pharmaceutical Impaction.

eter of 239.2 nm. As seen in Fig. 2, over the course of 30 min, the dispersion particle size and distribution changes, increasing in diameter and becoming more heterogeneous. The mean particle diameter grows from 240 to 352 nm in addition to a bimodal size distribution appearing at 30 min after dispersion. This phenomenon is not completely unexpected since the formulation is in a state of thermodynamic instability and contains no stabilizing polymers or surfactants to provide steric or ionic hindrance of particle interactions. Measured particle growth can be attributed to both particle aggregation and Ostwald ripening. Aggregation results as drug particle size is reduced and overall surface area of that mass is increased, leading to an increase in free energy of the system. Free energy is directly proportional to surface area as made evident by the following equation:

$$\Delta G = \gamma_{s/1} * \Delta A$$

where $\gamma_{s/l}$ represents the interfacial tension of a substance and $\Delta A$ represents the change in surface area. In order to reduce the free energy and become a stable system, particles within a nanodispersion will aggregate to form larger particles with reduced surface area.

An additional reason for measured particle size growth may be due to crystal growth, not aggregation, referred to as Ostwald ripening. While it is clear the vast majority of the formulation remains in the amorphous, particulate state upon dispersion (as seen visually and by light scattering measurements), a small portion of the formulation may go into solution, potentially supersaturating the dispersion media. In a thermodynamically supersaturated state, dissolved drug and excipient will begin to precipitate out of solution, nucleating to form small particles and adsorbing onto existing ones. The rate of Ostwald ripening is dependant on mass transport due to molecular diffusion, interfacial surface tension, and the solute molar volume (Welin-Berger and Bergenståhl, 2000). This is the rationale for bimodal distribution that appears 30 min after powder dispersion. The smaller fraction of particles appearing in the 50–100 nm size range may represent crystalline tacrolimus; having supersaturated, then precipitated out of solution.

Despite the aggregation and growth of particles over time, light scatter measurements confirm that the primary particle size of the formulation immediately after dispersion is approximately 200–400 nm. To prevent particle size growth, nanodispersions for oral use will incorporate excipients such as povidones, pluronics, polysorbates, and cellulose derivatives; however, high levels of these stabilizers are generally not proven safe for pulmonary delivery, and can often result in alveolar epithelial damage (Tátrai et al., 1996). For these reasons, the drug dispersion used in this study was dispersed without stabilizing excipients. To limit changes in particle size distribution, all formulations were dosed within 15 min of preparation, before substantial particle growth and aggregation could occur.

In characterizing the nebulized aerosol produced by the Aeroneb® Pro, both cascade impaction and laser diffraction techniques were used. Since the nebulized drug product is a dispersion, not a solution, it was essential to study impaction results based on drug mass recovery to determine amount of drug actually delivered to the deep lung. The mass median aerodynamic diameter (MMAD) of the formulation according to NGI measurements was 4.06 μm with a geometric standard deviation (GSD) of 2.7 μm. The aerodynamic size distribution of the aerosol is given in Fig. 3. The FPF of the nebulized dispersion was 46.1%. The total emitted dose (drug mass collected in the NGI/drug mass dispersed) was 50.5%, most likely due to dispersion remaining in the formulation vial and nebulizer head. Mass balance of 85% or less are also quite common in NGI testing, and may further contribute to low emitted dose measurements. Interestingly, volume median diameter (VMD), as measured by laser diffraction, for the same formulation was 6.55 μm. Theoretically, given that the dispersion media is water (unit density), all droplets produced should have identical aerodynamic and geometric diameter; however, testing differences may explain why the MMAD and VMD vary slightly.

NGI measurements taken according the USP 31 do not account for the droplet mass impacting in the induction port of the device, which can be assumed to represent droplets with aerodynamic diameters exceeding 11.7 μm (the cut off of the first stage). Laser diffraction analysis collects data on all droplets produced, including droplets larger than 11.7 μm in the calculation of VMD. When adjusted for the drug mass present in the induction port of NGI, the MMAD measured by impaction increases to 5.68 μm, much closer to want was measured by laser diffraction. Other impaction testing conditions may further influence the measured MMAD. Berg et al have studied the effects of temperature and humidity on partial droplet evaporation during impaction testing and concluded that under most ambient conditions (25 °C, 50% RH) evaporation will occur (Berg et al., 2007). In order to prevent skewing toward a lower MMAD, it is suggested that NGI testing be conducted at 5 °C and 100% RH. Since characterization of tacrolimus dispersion for nebulization occurred under ambient conditions, the possibility of underestimation of the MMAD due to evaporation is possible, and may account for the remaining difference between VMD and

**Table 1**
Geometric droplet size distribution of (a) pure deionized water and (b) tacrolimus dispersion for nebulization in deionized water. Laser light transmittance (Trans), volumetric particle size distribution (Dv(10), Dv(50), Dv(90)), surface area moment mean (D[3][2]), volume moment mean (D[4][3]), volumetric concentration (Cv), specific surface area (SSA), and obscuration (Obs) were measured.

| | Average | Standard deviation | Minimum | Maximum |
|---|---|---|---|---|
| (a) | | | | |
| Trans (%) | 50.9 | 5.578 | 36 | 64.1 |
| Dv(10) (μm) | 2.707 | 0.1368 | 1.964 | 2.906 |
| Dv(50) (μm) | 6.243 | 0.1673 | 5.875 | 6.677 |
| Dv(90) (μm) | 12.65 | 0.5501 | 11.54 | 14.86 |
| D[3][2] (μm) | 3.399 | 0.4364 | 2.291 | 4.588 |
| D[4][3] (μm) | 7.059 | 0.2233 | 6.625 | 7.805 |
| Cv (PPM) | 9.765 | 1.533 | 6.437 | 14.63 |
| SSA ($m^2/cm^3$) | 1.765 | 0.24425 | 1.308 | 2.619 |
| Obs (%) | 49.1 | 5.578 | 35.9 | 64 |
| (b) | | | | |
| Trans (%) | 49.1 | 5.595 | 30.9 | 61 |
| Dv(10) (μm) | 2.825 | 0.2274 | 1.741 | 3.063 |
| Dv(50) (μm) | 6.55 | 0.1347 | 5.995 | 6.792 |
| Dv(90) (μm) | 13.33 | 0.4966 | 11.82 | 14.89 |
| D[3][2] (μm) | 3.576 | 0.5369 | 2.042 | 4.728 |
| D[4][3] (μm) | 7.059 | 0.1763 | 6.653 | 7.787 |
| Cv (PPM) | 10.79 | 1.519 | 7.609 | 15.76 |
| SSA ($m^2/cm^3$) | 1.678 | 0.3135 | 1.269 | 2.938 |
| Obs (%) | 50.9 | 5.595 | 39 | 69.1 |

MMAD. Pan coating is another technique that is used to enable more accurate characterization by limiting aerosol "bounce" during NGI testing; however, this effect is thought to have a minimal contribution to liquid aerosol testing (Hardaker, 2008).

Laser diffraction measurements were also used to compare the effect of the nanodispersion on aerosol generation and particle size distribution. As seen in Table 1, a slight increase in aerosol droplet diameter was seen after the inclusion of dispersed tacrolimus formulation. While surface tension has been shown to have minimal effect on the size distribution of aerosols produced by vibratory mesh nebulizers (Ghazanfari et al., 2007), an increase in droplet diameter may be due to the disruption of droplet extrusion through laser-bored holes caused by dispersed particulates in the aqueous phase.

### 3.2. Single dose transplant model

Therapeutic concentrations necessary to provide significant immunosuppression in the lungs have been shown in previous studies. Ide et al. (2007) determined that a tacrolimus concentration of 270 ng/g in an allografted lung is necessary to prevent transplant rejection. Initial lung allograft concentrations of 388.8 ng/g were achieved in this study and were monitored over 24 h to determine subsequent levels. As shown in Fig. 4, peak total lung concentrations shown at 1 h were $294.7 \pm 10.5$ and $301.5 \pm 55.5$ ng/g in transplanted and non-transplanted rats, respectively, showing no difference in overall lung deposition. No statistical difference was noted between lung or blood levels of transplant and non-transplanted rats. Tracheal deposition was low, accounting for approximately 10% of total drug assayed 1 h after dosing (Fig. 5). It is uncertain whether mucociliary action would contribute to an increase or decrease of drug presence in the trachea since it would function to both relocate drug from the trachea into the laryngopharynx as well as add drug to lower regions of the trachea from the right and left bronchus. At the 6, 12, and 24 h time points; however, mucociliary transit should have removed all solubilized and particulate drug in the mucus layer, meaning that only drug absorbed into tracheal tissue remains.

Assuming that lung levels 1 h after dosing are an accurate representation of deposition level, average mass deposition in the right lung, 255.8 ng, was slightly larger than that in the left lung, 176.5 ng. Under normal breathing conditions a greater difference in deposition would be expected since the rodent right lung is on average twice as massive as the left, and is capable of higher tidal volumes. When peak tissue concentrations are compared in control rats, it is evident that deposition in the left lung is higher when adjusted for tissue weight. Mean tacrolimus concentration in the right lung was 272 ng/g, while in the left lung it was 356 ng/g. This discrepancy in lung concentration could be attributed to erratic breathing behavior. Disruptions in normal breathing in this study may have been due to animal excitement/anxiety during dosing, thoracic constrains of the dosing chamber restraint tubes, residual pain from surgical procedures, or a combination of the three. This would result in a shallow-rapid breathing rate as described by Valberg et al. (1982). It has been noted that shallow breathing in rodents can lead to increased deposition in the central airways and heterogeneity in aerosol distribution (Sweeney and Brain, 1991). Since the right lung volume of the rat is much greater than the left, it is conceivable that shallow breathing may have a greater limitation on deposition in that right lung. The combination of shallow breathing with a MMAD that is slightly greater than the normal respirable range may have magnified this deposition difference.

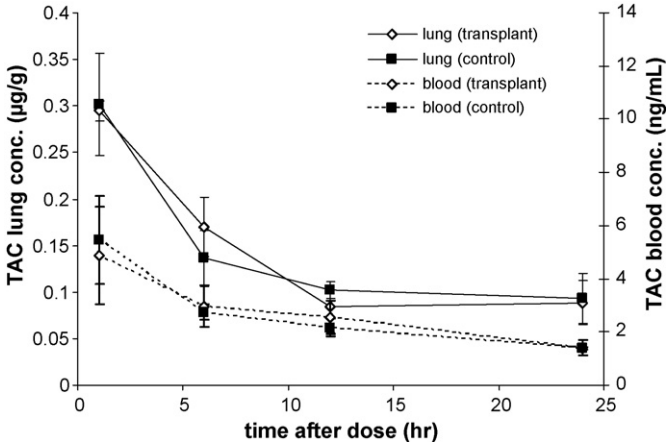


**Fig. 4.** Total lung and whole blood drug concentration in transplant and non-transplanted (control) Lewis rats.

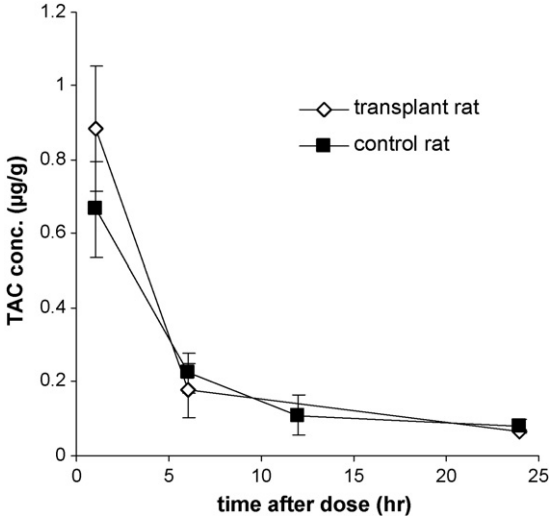


**Fig. 5.** Drug concentration in the trachea of transplanted and non-transplanted (control) Lewis rats after a single dose of tacrolimus dispersion for nebulization.

Blood concentration measured after dosing confirmed findings made my other authors that tacrolimus delivered directly to the lung produces minimal systemic drug levels. Specifically, in a lung transplant study where aerosolized tacrolimus was delivered via pressurized metered dose inhaler (pMDI), the ratio of tacrolimus concentration in the lungs to that in the blood was 55:1 1 h after the final dose (Ide et al., 2007). In a similar study conducted by Schrepfer et al. (2007), delivery of tacrolimus nebulization to tracheally transplanted rats resulted in a lung to blood ratio of 63:1. When delivered orally in the same animal model, lung concentrations of 580 ng/g with a corresponding blood level of 67.1 ng/g were reached after 1 h, giving a lung to blood ratio of only 9:1. As demonstrated in this rodent model, therapeutic lung concentrations are possible through oral delivery, however; it has been demonstrated in multiple clinical evaluations that excessive systemic concentrations will lead to untoward side effects, resulting in the reduction or elimination of the maintenance use of oral tacrolimus (Watts et al., 2009). Preferential accumulation of solubilized tacrolimus in the lungs may be due to its potential to associate with pulmonary tissue and phospholipids rather than partition into the aqueous media of the blood (McAllister et al., 1996). Evidence of this preference has been observed with aerosolized cyclosporine, a drug molecule sharing a similar lipophilicity (Burckart et al., 2003). Specifically, cyclosporine dosed to human lung transplant patients showed biphasic absorption where initial absorption into the blood was followed by slow sustained partitioning into systemic circulation.

In this study, lung to blood ratios of 55:1 and 59:1 were shown in non-transplanted and transplanted rats, respectively. While results proved similar in our study and the two mentioned previously, it has not been demonstrated in vivo if the entire deposited drug mass in the lungs is indeed bioavailable, or whether it is merely present in particulate form on lung epithelia. Characterization of tacrolimus dispersion for nebulization in vitro provides substantial evidence that our drug formulation is in fact bioavailable on the epithelial surface. Enhanced solubility in simulated lung fluid stemming from the nano-amorphous nature of this drug product has been demonstrated in previous studies and may impart superior drug availability to the lung epithelial layer in comparison to crystalline and/or micronized drug products. Future animal studies are needed to confirm that the enhanced solubility in simulated lung fluid translates to improved solubility and drug absorption in vivo.

## 4. Conclusion

It is clear that tacrolimus dispersion for nebulization can be effectively nebulized to the lungs of lung transplanted rats to obtain therapeutic concentrations locally. As hypothesized, resulting systemic concentrations remained low, not exceeding 6 ng/mL after a single dose and avoiding potentially toxic levels. Although it has not been demonstrated in vivo whether or not tacrolimus dispersion for nebulization remains as particulates in the airway or is fully bioavailable as a solution in pulmonary fluid, previous studies have demonstrated in vitro that the formulation possesses the capability of supersaturating simulated lung fluid (Sinswat et al., 2008). This suggests that tacrolimus dispersion for nebulization may be more locally bioavailable than previously studied formulations. This combined with the natural lipophilicity of the molecule may allow for high pulmonary bioavailability with low resulting system drug levels, as shown in this study.

Several studies have shown the benefits of localized therapy in pulmonary delivery for maintenance or rescue immunosuppression in lung transplant recipients. Tacrolimus, a more potent and clinically effective immunosuppressive agent, is the therapy of choice for lung transplantation; although it presents some formulation challenges. Application of solubility enhancing technology adapted with a method of localized therapy may prove to further enhance the therapeutic potential of tacrolimus for lung transplant recipients.

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