European Journal of Pharmaceutics and Biopharmaceutics 77 (2011) 207–215

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Preclinical evaluation of tacrolimus colloidal dispersion for inhalation

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ARTICLE INFO

Article history:
Received 13 August 2010
Accepted in revised form 22 November 2010
Available online 3 December 2010

Keywords:
Tacrolimus
Inhalation
Preclinical
Colloidal
Amorphous
Safety

ABSTRACT

Substantial improvements in transplant therapy have been made in the past four decades resulting in the acceptance of organ transplantation as a viable treatment for late-stage disease and organ failure. More recently, lung transplantation has gained acceptance; however, high incidence of chronic rejection and opportunistic infections has limited success rates in comparison with other transplant procedures. To achieve more targeted therapy, pulmonary administration of nebulized tacrolimus (TAC) colloidal dispersion once daily for 28 consecutive days in Sprague Dawley (SD) rats has been investigated for safety and systemic elimination. A liquid dispersion of colloidal TAC and lactose (1:1 ratio by weight) was aerosolized using a vibrating mesh nebulizer and administered via a nose-only dosing chamber. Blood chemistry and histological comparisons to saline-dosed animals showed no clinically significant differences in liver and kidney function or lung tissue damage. Maximum blood and lung concentrations sampled 1 h after the final dose showed TAC concentrations of 10.1 ± 1.4 ng/mL and 1758.7 ± 80.0 ng/g, respectively. Twenty-four hours after the final dose, systemic TAC concentrations measured 1.0 ± 0.5 ng/mL, which is well below clinically accepted trough concentrations (5–15 ng/mL) for maintenance therapy, and therefore, would not be expected to induce toxic side effects. The propensity for pulmonary retention seen when compared to single dose lung levels may be due to macrophage uptake and the lipophilic nature of TAC. Additionally, three month stability testing of TAC powder for reconstitution showed no changes in amorphous nature or drug potency when stored at ambient conditions. TAC colloidal dispersion proved to be non-toxic when administered by pulmonary inhalation to SD rats over 28 days while providing therapeutic concentrations locally. This delivery strategy may prove safe and effective for the prevention of lung allograft rejection in lung transplant recipients.

1. Introduction

Immunosuppressive therapy has become increasingly important in the success of solid organ and bone marrow transplantation over the past 25 years. With advances in donor matching, continued surgical innovation, and the introduction of calcineurin inhibiting immunosuppressive drugs, the likelihood of allograft rejection has been substantially reduced, increasing a patient's probability of survival and quality of life. Currently, lung transplantation is the least successful of all common solid organ transplant procedures. Despite the improvements in surgical techniques and development of more potent immunosuppressive drugs, the survival rate of lung transplant patients 5 years post-op is only 50% [1]. Many of the complications associated with treatment and maintenance dosing of lung transplant recipients can be attributed to the delicate balance that exists between proper immunosuppression of the lungs and prevention of untoward side effects and opportunistic infection. The immunological function of the lung is poorly understood and unique from any other organ of the body [2], complicating diagnosis and treatment of many immune-related lung diseases. Generally, immunosuppression of the allografted lung is achieved through oral dosing of a three drug regimen consisting of a calcineurin inhibitor, purine synthesis inhibitor, and corticosteroid [3]. Maintenance dosing is continued for the rest of the patient's life and does not routinely involve corticosteroids due to side effects associated with prolonged administration. Tacrolimus (TAC) and mycophenolate mofetil are the most common respective calcineurin inhibitor and purine synthesis inhibitor used for maintenance therapy.

A more logical approach for pulmonary immunosuppression is to administer the therapeutic agent directly to the lung via an inhalable aerosol. Researchers have investigated the delivery of an aerosolized calcineurin inhibitor, cyclosporine A (CSA), in animal models for the determination of its efficacy and safety in lung

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doi:10.1016/j.ejpb.2010.11.013

tissue. In a safety study in rodents and canines, Wang and coworkers determined that inhaled cyclosporine presented no detrimental effects on pathology or lung function, even at 2.7 times the proposed human dose [4]. This strategy has been investigated by various clinicians as well in hopes to improve maintenance therapy and prevent lung transplant rejection [5–8]. Clinical trials involving lung transplant recipients have shown that while no statistical improvements in acute graft rejection were seen when compared to inhaled placebo, prevention of chronic rejection, and ultimately patient survival, was significantly improved with aerosolized CSA [7]. In these trials, nebulization of CSA was facilitated by dissolving it in a viscous, non-toxic solvent, propylene glycol. While recognized as safe for use in pulmonary drug products, high levels of propylene glycol have been shown to cause mild to moderate irritation and cough in a majority of patients, requiring the use of a local analgesic before administration [7]. Additionally, the high viscosity of propylene glycol may prevent proper aerosol production with certain nebulizers, changing aerosol characteristics and possibly limiting deep lung deposition [9]. These factors, as well as a relatively less potent therapeutic effect, may reduce the inclination for clinical acceptance of this therapy.

TAC, first commercialized by Fujisawa, Inc in 1990 as Prograf®, elicits a therapeutic response by inhibiting the functionality of calcineurin and has been shown to be 10–100 times more potent than CSA [10]. Clinical comparisons of oral CSA and TAC in solid organ transplant patients have concluded that TAC improves both short-term graft success [11,12] and overall patient survival [13]. In a pharmacoeconomic comparison, TAC was shown to reduce the associated healthcare and therapy costs by at least 25% in kidney transplant patients when compared to CSA [14]. Other indications where TAC has been investigated for reduction of inflammation include atopic dermatitis (Protopic®), ulcerative colitis, graft-versus-host disease, and asthma [15].

While TAC therapy has noted success clinically and is currently more widely administered for transplantation than CSA [16], a variety of adverse side effects are associated with elevated systemic concentrations over prolonged periods of therapy. Among the most severe and commonly occurring side effects associated with oral TAC are nephrotoxicity, neurotoxicity, hypertension, diabetes mellitus, and increased risk of opportunistic infection; all of which have shown some evidence of dose dependence. Typically, neurotoxicity due to TAC maintenance therapy will result in mild effects such as headache or tremor. Cases of TAC-induced severe neurotoxicity are rare, but are often manifested in seizure, delirium, or coma and most commonly enabled by a reduction in P-glycoprotein efflux function [17]. High sustained systemic concentrations of TAC can also lead to new onset diabetes mellitus (NODM). In a recent study analyzing reports form the past decade of calcineurin inhibitor-induced NODM, evidence showed that TAC was found to be more likely to cause NODM than CSA [18]. A higher concentration of FK506-binding protein (FKBP-12) in the pancreas may explain the greater incidence of NODM in patients receiving TAC. In comparison with maintenance therapy with CSA, cardiovascular events associated with TAC are significantly reduced [19]. Close monitoring of trough blood concentration is critical among patients using oral TAC to avoid severe side effects normally associated with blood levels above 50 ng/ml and to monitor variability of intestinal absorption.

In order to avoid systemic side effects that might lead to dosing protocol complications, additional medication, and elevated patient discomfort, a more targeted method of drug delivery in lung transplant patients providing a reduced systemic TAC concentration is needed. Additionally, local concentrations exceeding those provided by current oral therapy could potentially increase pulmonary therapeutic levels, resulting in enhanced suppression of the local immune response. This study investigates the safety and elimination of once-daily TAC colloidal dispersion in a rodent model over 28 consecutive days. Blood chemistry, complete blood cell count, and tissue histology were conducted to evaluate the physiological effect of the dosing regimen. Evaluation of the stability of TAC powder for reconstitution is also reported. By providing organ-targeted therapy, multiple doses of TAC should provide heightened lung concentration, with minimal systemic blood levels and no resulting systemic toxicity.

2. Materials and methods

2.1. Materials

Tacrolimus anhydrous (batch #070405) was purchased from Haroui Pharma-Chem (Edison, NJ). High Performance Liquid Chromatography (HPLC) grade acetonitrile, lactose monohydrate, zinc sulfate heptahydrate, barium hydroxide (0.3 N), magnesium chloride, sodium phosphate dibasic anhydrous, sodium sulfate anhydrous, calcium chloride dihydrate, sodium acetate trihydrate, and phosphoric acid (85%) were all purchased from Fisher Scientific (Fair Lawn, NJ). Sodium chloride and normal buffered formalin solution (10%) were purchased from Sigma–Aldrich, Inc. (St. Louis, MO). Potassium chloride was purchased from EM Industries, Inc. (Gibbstown, NJ). Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar-Lipids, Inc. (Alabaster, AL). Sterile 0.9% sodium chloride for injection, USP was purchased from Hospira, Inc. (Lake Forrest, IL). Heparin sodium (10,000 IU/mL) was purchased from Baxter Healthcare (Deerfield, IL).

2.2. Formulation composition and preparation

TAC powder for reconstitution was prepared using the ultra-rapid freezing (URF) process described by Sinswat [20]. Equal amounts of TAC and lactose (1:1 ratio by weight) were dissolved in acetonitrile/water (60/40) so that the total solids content of the formulation was 0.75% w/v. The solution was applied to a stainless steel rotating drum that had been cryogenically cooled to −50 °C. The frozen solution was collected in liquid cryogen and freeze dried using a VirTis Advantage Tray Lyophilizer (VirTis Company Inc., Gardiner, NY) to remove water and organic solvent. A detailed explanation of this particle engineering technology is given by Overhoff et al. [21]. Product was packaged under nitrogen at 25 °C and less than 20% relative humidity.

2.3. In vitro characterization

2.3.1. Powder X-ray diffraction (PXRD)

X-ray diffraction patterns were determined for the drug product stored for up to 3 months using a Philips 1710 X-ray diffractometer with a copper target and nickel filter. Leveled powder was measured from 15 to 55 2θ degrees using a step size of 0.05 2θ degrees and a dwell time of 2 s.

2.3.2. Dissolution testing

Evaluation of the ability of formulated powder to supersaturate simulated lung fluid (SLF) was performed using a modified United States Pharmacopeia (USP) 31 Type 2 dissolution apparatus. Five small volume vessels were filled with 97 mL of SLF with 0.02% DPPC, maintained at 37 °C, and stirred at 100 rpm. Preparation of SLF was carried out as outlined by Davies et al. [22]. Addition of 0.02% DPPC was performed by creating a thin lipid film via rotary evaporation with a Buchi Rotavapor R-210 (Flawil, Switzerland) and dispersing in SLF at 50 °C [23]. Five aliquots of 20 mg TAC powder for reconstitution were weighed, placed in 20 mL scintillation vials, and dispersed in 3 mL de-ionized water. Dispersion was

facilitated by probe sonication with a Branson Sonifier 450 (Danbury, CT) (output 6, 10% interval) for 2 min under cooled conditions. Immediately after dispersion, each vial was added to a respective dissolution vessel. Aliquots of 2 mL were drawn at 5, 15, 30, 60, 120, and 240 min and filtered through 0.2-μm PTFE teflon syringe filters (National Scientific, Rockwood, TN). Subsequently, 1 mL of the filtrate is added to 1 mL HPLC mobile phase, consisting of acetonitrile, water, and phosphoric acid in a ratio of 600:400:1. Quantitation of each sample was performed by HPLC according to a method adapted from Akashi et al. [24]. A Waters 515 liquid chromatograph with a Water 996 Photo Array (Water Corp. Milford, MA) outfitted with a Lichosphere RP C18, 4 mm × 250 mm, 5 μm column (Varian Corp., Lake Forest, CA) was used for analysis. Analysis conditions such as flow rate, column temperature, and injection volume were 1.5 mL/min, 50 °C, and 20 μL, respectively. Tautomers of TAC eluted at 8.3 and 13.2 min, while the main drug peak eluted at 17 min. All three peaks were measured at 215 nm and represent the active moiety; therefore, all three were used in quantitation. Standards, prepared in mobile phase by serial dilution, ranged in concentration from 100 to 3.125 μg TAC per mL. Standard curve linearity (r^2 = 0.999), system precision (RSD = 1.9%), and accuracy were validated for this method.

2.3.3. HPLC analysis

Five concentrations, 5, 2.5, 1.25, 0.625, and 0.3125 mg/mL, of bulk TAC in HPLC mobile phase were evaluated for purity using the HPLC method explained in Section 2.3.2. Noise contribution from the solvent front was determined by blank injection and was seen to affect the chromatograph baseline until approximately 3.5 min after injection. All detectable non-tacrolimus peaks occurring after the solvent front were considered impurities and compared to tacrolimus peaks for the determination of percent impurity. The effect of processing, excipients, and storage on TAC purity in the drug product was also evaluated. Product purity was evaluated immediately after production and after 1, 2, and 3 months storage in sealed packaging at room temperature. Samples containing between 4 and 5 mg of drug product were dissolved in 2 mL mobile phase and analyzed by HPLC. Two samples were reconstituted and analyzed for each month storage. For identification of potential degradants, a battery of forced degradation tests were performed on the bulk drug in accordance with the guidelines presented by the International Conference on Harmonization Q3B(R2). Acidic, alkaline, oxidative, and thermal degradation were each induced to a 5 mL solution containing 1 mg TAC in acetonitrile. Acid degradation was accomplished by adding 10 mL 1 N HCl and incubating for 1 h at room temperature. The reaction was quenched by the addition of 10 mL 1 N NaOH. Similarly, base degradation incubated the samples for 1 h with 10 mL 1 N NaOH. The reaction was quenched with 10 mL 1 N HCl. Oxidative degradation involved the addition of 1 mL 30% H_2O_2 to the solution and incubating for 1 h. Thermal degradation was completed by incubation in a 60 °C water bath for 6 h. At the conclusion of quenching and/or incubation, all samples were diluted to 100 mL with water. Two milliliters of the diluted sample was filtered through a 0.2-μm PTFE teflon syringe filter. One milliliter was then added to 1 mL mobile phase and injected for HPLC analysis. Degradation peaks were identified and quantitated by the HPLC method outlined in Section 2.3.2.

HPLC was used to analyze the drug product potency after 1, 2, and 3 months storage. Samples of between 4 and 5 mg of drug product were dissolved in 2 mL mobile phase and analyzed by HPLC. Two samples were reconstituted and analyzed for each month storage. Based on manufacture and batch formula detailed in Section 2.2, theoretical potency is presumed to be 50% of the total drug product weight.

2.3.4. Residual solvent analysis

Gas chromatography (GC) performed with a Hewlett Packard 5890 Series II GC (Hewlett Packard, Palo Alto, CA) was used to determine the drug product residual solvent levels according to a revised USP 467 method for class 2 solvents. A Zebron™ ZB-624, 30 m, 0.32 id, 1.8 μm column (USP phase G43) (Phenomenex, Torrance, CA) was used at a helium flow rate of 2.2 mL/min. Injection temperature was set at 140 °C and oven temperature was set at 40 °C for the first 10 min then increased to 240 °C for another 10 min. Flame ionization detector (FID) temperature was set at 240 °C. Standards for injection contained a concentration of 4, 2, 1, 0.5, and 0.25 μL/mL acetonitrile (ACN) in dimethyl sulfoxide (DMSO). ACN peak elution occurred 6.15 min after injection.

2.4. In vivo tolerability study in a rat model

2.4.1. Pulmonary dosing method and regimen

TAC colloidal dispersion was administered to healthy male and female Sprague Dawley (SD) (Harlan Inc., Indianapolis, IN) rats in clean, humane conditions and in accordance with an Institutional Animal Care and Use Committee (IACUC) approved protocol and meets the standards of EC Directive 86/609/EEC. Aerosol was produced by vibrating mesh nebulization using an Aeroneb® Pro (Aerogen, Galway, Ireland) and delivered to the animals via a four port nose-only dosing chamber [20].

Eight female and eight male rats were given treatments containing TAC (active group) for 28 days, while four male and four female rats were given equivalent volumes of normal saline (control group) for the same period. Six rats in the active group and four of the control group were maintained for 1 week after the last dose in order to evaluate resolution of any pulmonary inflammation that might occur after 28 days of active treatment. All other animals were sacrificed 24 h after the last dose was administered. Eight animals in the active group were designated for the determination of drug elimination. All other animals were designated for histological evaluation.

Before nebulization, 6.4 mg TAC powder for reconstitution was dispersed in 3 mL de-ionized water as described in Section 2.3.2. Dose was selected based on what has been predicted to be efficacious in prevention of allograft rejection in transplanted rats [25]. Rats were exposed to the aerosolized dispersion for approximately 10 min, or until completion. Once-daily aerosol administration was performed at the same time each day for 28 days. Animals were sacrificed by isoflurane (over 10% by volume) inhalation and subsequently confirmed with cardiac puncture. Approximately 3 mL of whole blood was drawn for drug quantitation and hematological testing. Blood for drug quantitation was placed in 1.5-mL conical tubes containing 20 μL of 10,000 IU/mL heparin sodium. Lung tissue was excised from designated rats and frozen at −20 °C until determination of TAC levels by tissue extraction. Liver, spleen, kidney and lung tissue were excised and fixed in 10% formalin solution for histological investigation.

2.4.2. Analysis of tacrolimus levels in whole blood

Heparinized whole blood samples were evaluated for TAC levels using Enzyme-linked Immunosorbent Assay (ELISA). A PRO-Trac™ II FK506 ELISA kit was purchased from Diasorin Inc (Stillwater, MN) and used according to the manufacturer's protocol. Fifty microliters was taken from each of five standards (ranging from 0.3 ng/mL to 30 ng/mL), two controls, and all whole blood samples and added to a 1.5-mL conical tubes along with 300 μL reconstituted digestion reagent. Each tube was mixed, then incubated for 15 min at 75 °C. To separate digested cellular contents, centrifugation was conducted at 1800 g for 10 min, after which 100 μL supernatant was added to duplicate wells of a goat anti-mouse IgG coated 96-well plate. Fifty microliters of anti-tacrolimus monoclonal

antibody was added, followed by plate incubation and shaking at room temperature and 700 rpm form 30 min. After incubation, 50 μL tacrolimus-horseradish peroxidase conjugate was added, and the plate was shaken again for 60 min at 700 rpm. Liquid contents of the wells were then removed by washing, and 200 μL chromogen was added and shaken again for 15 min at 700 rpm. The reaction was stopped by 100 μL of acidic stop solution, and the plate was immediately read at dual wavelengths of 450 and 630 nm on a Bio-Tek® Instruments UV/Vis μQuant plate reader (Winooski, VT). The standard curve was plotted using Sigma Plot Systat Software Inc. (San Jose, CA) and fit with a 4-parameter logistic curve.

2.4.3. Analysis of tacrolimus levels in lung tissue

TAC was extracted from rodent lung tissue by a liquid extraction procedure and quantitated using liquid chromatography/mass spectrometry (LC/MS). The method was validated by the spiking of blank rodent lung homogenates and subsequent extraction with TAC standards to form a 6-point standard curve ranging from 10.0 μg to 0.05 μg. For experimental sample extraction, 1.25 μg of internal standard (sirolimus) dissolved in 1 mL acetonitrile was added to the tissue before homogenization. Two milliliters of normal saline was also added to the lung tissue before homogenization. Wet lung tissue was homogenized in a scintillation vial for approximately 5 min at cooled temperatures. To extract TAC from bound tissue proteins, 0.1 mL of 0.3 N barium hydroxide, 0.1 mL of 0.4 N zinc sulfate hepahydrous, and 7 mL acetonitrile were added to the homogenate. The homogenate was vortexed for 30 s and placed in 15 mL polypropylene centrifugation tubes (Fisher Scientific, Fair Lawn, NJ). Centrifugation was conducted for 15 min at 3000*g*. Supernatant was then removed and filtered through a Whatman 0.45-μm nylon syringe filter (Fisher Scientific, Fair Lawn, NJ). Water and acetonitrile were removed from the supernatant by 12-hr lyophilization ramping from −40 °C to 25 °C after freezing the sample. The resulting solids were reconstituted in 2 mL acetonitrile and centrifuged again at 3000*g* for 15 min. One milliliter of the supernatant was taken for injection and analysis by LC/MS. A Thermo Fisher Surveyor Plus HPLC system with PDA (Waltham, MA) was used in combination with a Thermo Fisher LTQ FT Ultra Hybrid Mass Spectrometer (Waltham, MA) for sample analysis. The system was outfitted with a C18 3μ Thermal Hypersil Gold 50 X 2.1 column, injected 10 μL of each sample, and run at a flow rate of 10 μL/min. Gradient flow of acetonitrile and water was used where acetonitrile and water concentrations were 5% and 95% initially, changed to 80% and 20% at 17 min, and gradually ramped to back to 5% and 95% at 20 min, respectively. TAC was quantitated by integration of peaks occurring between 825.5 and 827.5 m/z and reported as a concentration in wet lung tissue.

2.4.4. Complete blood count and serum chemistry

After cardiac puncture, approximately 1 mL of blood was used for complete blood count (CBC) and serum chemistry. Samples were drawn and added to vials after the removal of needles to prevent excessive red blood cell lysing. Lavender-topped BD Microtainer® tubes with K_2EDTA (VWR Scientific, West Chester, PA) were filled between 250 μL and 500 μL. Each tube was then inverted 10 times to ensure thorough anti-coagulant mixing. For serum chemistry analysis, whole blood samples between 400 μL and 600 μL were added to yellow-topped BD Microtainer® SST tubes (VWR Scientific, West Chester, PA), inverted 5 times, and allowed to remain at room temperature to coagulate for 30 min. Coagulated blood is then centrifuged at 6000*g* for 90 s. After centrifugation, blood serum was pipetted into a 1.5-mL conical vial. Both CBC and serum chemistry samples were kept at 4 °C until analysis. Analysis was run within 24 h of blood draw by IDEXX preclinical research services (Westbrook, ME).

2.4.5. Tissue histology

At completion of the dosing regimen, lung, kidney, liver, and spleen tissue of both control and experimental groups were reviewed by two different blinded examiners. Eight rats having received 28 days of once-daily TAC colloidal dispersion were evaluated, four of which were sacrificed 24 h after the final dose, the other four sacrificed 168 h after the final dose. Eight control rats followed the same dosing regimen and received aerosolized normal saline instead of TAC colloidal dispersion. Lung tissue was instilled with 5 mL of 10% formalin through cannulation of the trachea, submerged in 40 mL 10% formalin, and embedded in paraffin wax. Lung sections were stained with hematoxylin and eosin (H&E), cluster of differentiation 68 (CD68), and periodic acid Schiff (PAS) and evaluated. Kidney, liver, and spleen were also submerged in 40 mL formalin and fixed in paraffin wax. Non-lung tissue sections were stained only with H&E. Histologic inflammation scores were assigned according to the criteria outlined by Cimolai et al. [26].

2.4.6. Statistical analysis

Statistical analysis of CBC and serum chemistry results was run using Minitab® Release 14 statistical software (Minitab Inc., State College, PA). One-way ANOVA followed by a post hoc Tukey test was used at $p = 0.05$ and $p = 0.01$ to determine significant differences between control and experimental groups.

3. Results and discussion

3.1. In vitro characterization

An amorphous nanoparticulate drug formulation offers advantages over crystalline drug products in that bioavailability may be enhanced by increased solubility, reduced drug particle size, and improved wetting. The marketed oral formulation of TAC, Prograf®, uses this rationale by providing improved solubility through a solid dispersion of TAC and hydroxypropyl methylcellulose (HPMC) [27]. For purposes of pulmonary delivery, lactose is a more attractive choice for stabilization of TAC due to its recognition by the FDA as a safe excipient for pulmonary products. Additionally, lactose is water soluble and has been used as a lyoprotectant [28], making it likely to provide stability to amorphous TAC upon lyophilization and storage. Initially, when dispersed in water for nebulization, the formulation forms a dispersion where only a small quantity of the drug is expected to supersaturate the small volume of water [25]. The dispersion particle size and nebulized aerosol performance have been previously reported [25], where 46.1% of the emitted dose was in the respirable range and the particle size of dispersed TAC within each droplet ranged from 200 to 400 nm. Measurement of osmolarity was attempted in the formulation after filtration; however, sub-200 nm particulates in the filtrate may have caused inaccuracies in osmometer measurements. It is expected that this formulation was hyposmotic since water for nebulization was used. Preparations in future studies should be prepared in normal saline.

When added to a larger volume of aqueous media (approximately 100 mL), the solubilization of lactose and thermodynamic instability inherent in amorphous TAC results in a supersaturated solution during the initial stages of dissolution. After approximately 30 min in dissolution, supersaturated TAC begins to precipitate out of solution to form a more kinetically favored solid state. While it is possible that undissolved TAC particles smaller than 200 nm may pass through the filter during dissolution sampling, it seems more likely that dissolved TAC associates with DPPC liposomes or vesicles in SLF to create a momentarily stabilized supersaturated solution. In order to determine the stability of the

amorphous drug product for reconstitution over 3 months at the conditions previously described, PXRD and supersaturation dissolution testing were conducted. It was determined that the drug formulation maintained its amorphous nature throughout the 3-month period. Figs. 1 and 2 show that amorphous TAC is still present throughout the 3-month period and that the capacity to supersaturate SLF is still present. Based on the stability over 3 months, a ratio of 1:1, lactose to TAC, was adequate to maintain the amorphous nature of the formulation and its ability to supersaturate SLF. The ability to supersaturate SLF containing 0.02% DPPC and the presence of amorphous drug has been shown to increase both the C_{max} and T_{max} in the blood and lungs when compared to crystalline drug formulations dosed to ICR mice [20] and may be instrumental in improving bioavailability in humans.

To determine the effect of varying methods of degradation, a battery of accelerated degradation tests were performed. At absorbance measurements taken between 215 nm and 300 nm, all major degradation peaks showed the highest absorbance at 215 nm, allowing for detection of degradants and TAC at the same wavelength. TAC solution was subjected to acidic, alkaline, oxidative, and heated conditions to induce degradation. TAC was susceptible to both alkaline and acidic hydrolysis, while thermal and oxidative degradation procedures had less effect. Upon storage under ambient conditions for up to 3 months, samples showed less than 0.2% degradants in chromatography. Assuming that the potential human dose of TAC in this drug product is 6 mg once daily, this drug product is below the thresholds for identification (0.5% degradants) and qualification (1.0% degradants) according International Conference of Harmonisation (ICH) guidelines, and only requires that degradants be reported [29]. Measurements of drug purity and potency within the drug product were measured by HPLC and are displayed in Table 1. Percent potency was calculated assuming that the theoretical potency of TAC was 50% of the formulation weight.

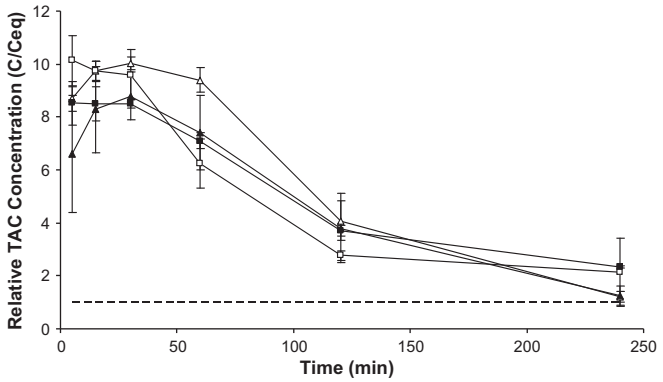

Fig. 1. Supersaturation dissolution profile for TAC colloidal dispersion initially (△) and after 1 month (▲), 2 months (□), and 3 months (■) in simulated lung fluid (SLF) containing 0.02% DPPC at 37 °C and a paddle speed of 100 rpm. Equilibrium solubility (6.8 μg/mL) is represented by the dashed line (- - -).

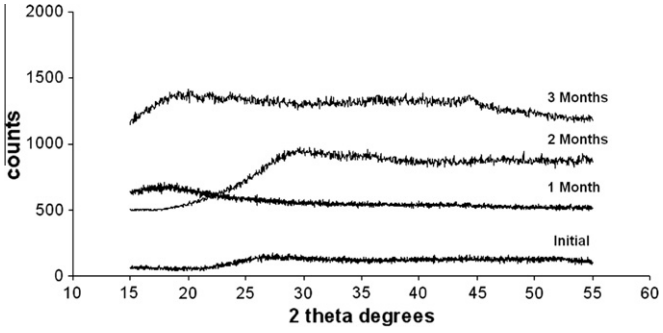

Fig. 2. X-ray diffraction patterns of TAC dry powder for reconstitution over 3 months stability.

Organic solvent remaining after lyophilization is regulated by the FDA in drug products based on a toxicity classification system to ensure patient safety. Residual solvent may also accelerate or contribute to product physical instability due to its solubilizing effect and oxidative stress on the active ingredient. Testing for residual solvent was conducted for quantification of ACN remaining in the drug product after the URF production process. Based on trends in peak height and a chromatograph noise height of 500 counts, the detection limit (based on 3:1 signal to noise ratio) of this system was approximately 0.1 μL/mL for detection of ACN in DMSO. The limit of quantitation (LOQ) for this method based on a 10:1 signal to noise ratio is approximately 0.25 μL/mL for detection of ACN in DMSO. According to ICH guidelines, 410 ppm of ACN is the allowable concentration in a 10 g drug product [30]. While our drug product is approximately 3 g after reconstitution we assumed 410 ppm to be the maximum allowable concentration of ACN residual in TAC powder for reconstitution, not the final TAC colloidal dispersion. As shown in Fig. 3, no detectable amount of ACN was measured when a dose of 50 mg TAC powder for reconstitution was dissolved in 1 mL DMSO. It can therefore be determined that this drug product is below the maximum allowable ACN concentration, even at levels well beyond the anticipated human dose.

3.2. In vivo tolerability

To determine the amount of TAC present in the lungs and corresponding systemic concentrations, blood and lung tissue levels were analyzed by ELISA and LC/MS. After 28 consecutive daily nebulized doses (each delivering approximately 200–300 ng of TAC [25]), analysis of lung tissue and whole blood revealed peak concentrations of 1758.7 ± 80.0 ng/g and 10.1 ± 1.4 ng/mL, respectively. Fig. 4 shows the relationship between lung and blood concentrations at 1, 24, and 168 h after the final dose was administered. In a separate single dose study, rats exposed to the same nebulized dose (6.4 mg TAC colloidal dispersion) showed 1 h tissue and blood levels of 294.8 ng/g and 4.9 ng/mL, respectively [25]. Our results demonstrate that there is substantial drug accumulation in the lungs after multiple pulmonary doses, reaching nearly 6 times single dose levels after 28 consecutive doses. It is most likely that accumulation of TAC is a result of the lipophilic nature of the molecule. TAC, with a Log *P* of 3.8, shows a strong preference for lipid solubilization, while CSA has a Log *P* of 4.3 [31]. With a similar lipophilic nature and molecular weight, it might be expected TAC and CSA would behave similarly in vivo, binding to proteins and lipid membranes. Multiple studies have been conducted to evaluate the efficacy and pharmacokinetics of aerosolized CSA, as mentioned above. Similar to our findings, investigators have determined that cyclosporine exhibits substantial lung retention,

Table 1
TAC powder for redispersion (a) purity and (b) potency after 3 months storage.

a	Purity (%)	Degradants (%)	St. dev.
Initial	99.9208	0.0792	0.0082
1 month	99.8455	0.1545	0.0003
2 month	99.8517	0.1483	0.0314
3 month	99.8268	0.1732	0.0253

b	Potency (%)	St. dev.
Initial	48.92	1.51
1 month	48.16	0.24
2 month	46.86	0.34
3 month	47.99	0.45

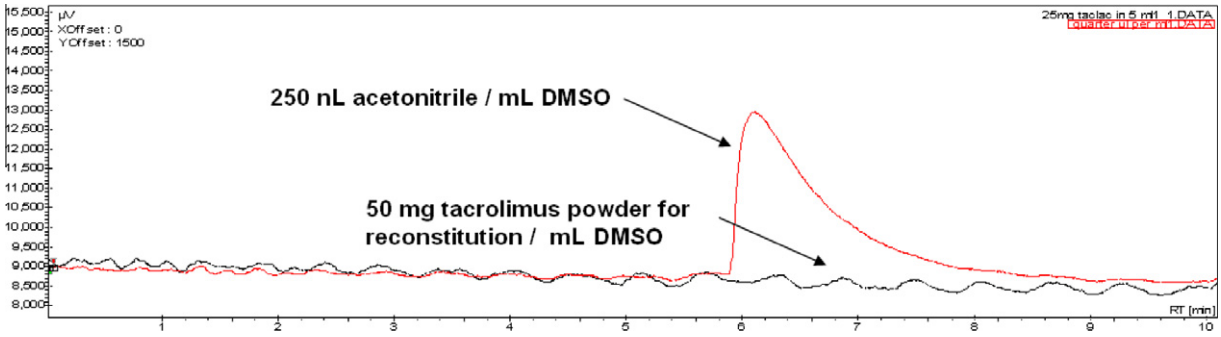

Fig. 3. GC of TAC powder for redispersion dissolved in 1 mL DMSO. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

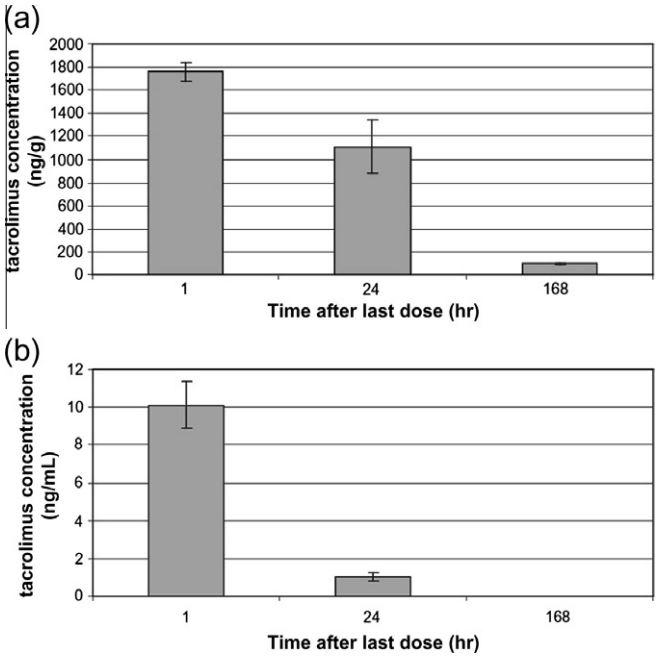

Fig. 4. TAC concentrations remaining in (a) lung tissue and (b) whole blood after 28 daily nebulized doses of 6.4 mg tacrolimus colloidal dispersion.

having a mean terminal half-life of 40.7 h after aerosolization compared to 6.5 h with intravenous administration [32]. Rationale for lung retention can be attributed in part to three potential mechanisms. First, drug interaction with phospholipids [33] and proteins [34] in pulmonary fluid as well as drug association with the pulmonary epithelial has been demonstrated and are believed to be a significant cause in pulmonary drug retention. Additionally, hydrophobic molecules such as CSA [35] and itraconazole [36] in the lung have been shown to experience macrophage uptake, where prolonged release and/or transport to bronchial-associated lymphoid tissue (BALT) may occur. Mechanisms of TAC association with a common alveolar protein, surfactant protein A, have been studied in vitro by Canadas and coworkers. By derivatizing TAC with a fluorescing moiety, the equilibrium association constant and Gibbs binding free energy of TAC to an alveolar lipoprotein were determined. Results showed that, like CSA, TAC preferentially binds to proteins in the alveolar fluid [34]. Finally, pulmonary retention of TAC could also be attributed to P-glycoprotein efflux disruption of absorption. While known to have a substantial influence over variability of intestinal absorption, functional P-glycoprotein has also been recognized in the mucosal barriers of the deep lung and alveolar space [37]. The importance of P-glycoprotein efflux in oral dosing of TAC is well established [38], thus it is reasonable to predict that the same absorption-limiting effects may be seen in pulmonary delivery.

By providing high local concentrations and maintaining systemic levels well below those known to cause adverse side effects, inhaled TAC colloidal dispersion represents an improvement on current therapies for lung transplant recipients. Another potential application of inhaled TAC could be for supplementary dosing to assist with patients showing poor or highly variable gastrointestinal absorption. Administration of large immunosuppressive doses to the lungs may be able to contribute to maintenance of therapeutic blood levels by acting as a biological depot, resulting in slow release from lung tissue, reducing oral dosing requirements, and limiting the impact of poor intestinal absorption. This could be particularly useful in patients showing poor oral bioavailability, such as in cystic fibrosis populations, where the permeability of the intestinal wall is reduced [39]. Investigation of the ability of the lungs to serve as a drug reservoir for sustained release will be considered in future studies.

Comparison of CBC and serum chemistry results of rodents dosed for 28 consecutive days with TAC colloidal dispersion to those administered aerosolized normal saline showed no clinically significant difference for both 1 day and 1 week after the final dose (Table 2). While mean eosinophil count of the group sacrificed one week after the final dose did show significant difference (0.5% vs. 1.7%; $p < 0.01$) from the saline-dosed group, this is still within the normal range [40]. More importantly, the value was not significantly different after 28 days of treatment with inhaled tacrolimus and histological evaluation of the lungs revealed no increase in eosinophils for either treatment group. In fact, no individual animal in this study showed eosinophil levels higher than 196 cells/μL. Decreased platelet count may be due to partial clotting of samples and/or analysis of samples at room temperature rather than 37 °C, which has been reported to cause clumping in EDTA-anticoagulated blood [41]. While TAC has shown to cause cases of diabetes mellitus and hyperglycemia, this study noted hyperglycemia in control rats at statistically similar levels. The increased level of blood glucose can be attributed to the use of high levels of isoflurane during euthanasia, which both decreases glucose clearance and increases glucose production [42].

Normal liver and kidney function were determined by serum chemistry analysis. The hepatic panel, seen in Table 3, while having no difference between groups, showed elevated aspirate amino transferase and alanine amino transferase levels for several potential reasons. Hypoxia after animal euthanasia, red blood cell hemolysis [43], and liver damage [44] caused by excessive isoflurane inhalation during sacrifice all could have contributed to these increased transferase levels. Significant differences were observed in creatinine and albumin levels; however, both showed improved

Table 2
CBC and serum chemistry of SD rats receiving TAC colloidal dispersion for 28 days and sacrificed 24 h (4 weeks) or 168 h (4 weeks + 1) after the final dose.

	Control	4 weeks	4 weeks + 1
Hematologic parameters			
WBC (10^3/uL)	5.1 ± 0.8	4.6 ± 1.1	6.1 ± 2
RBC (10^6/uL)	8.1 ± 0.5	8.5 ± 0.8	8.3 ± 0.4
HGB (g/dL)	15.1 ± 0.7	15.2 ± 1.4	15.5 ± 0.6
HCT (%)	50.6 ± 3.3	51 ± 4.8	51 ± 3.1
MCV (fl_)	62.6 ± 1.4	60.4 ± 1.5*	61.7 ± 2.3
MCH (pg)	18.7 ± 0.6	18 ± 0.6	18 ± 0.6
MCHC (g/dL)	29.9 ± 1	29.7 ± 0.5	30.4 ± 0.7
Platelet (10^3/uL)	743.3 ± 123.5	699.5 ± 186.3	725.5 ± 281.2
Neutrophils (cells/μL)	564.5 ± 232.3	590.1 ± 380.9	705.2 ± 301.8
Neutrophils (%)	11.4 ± 4.5	14.5 ± 13.1	12 ± 5.4
Lymphocytes (cells/μL)	4351.9 ± 730.4	3851.9 ± 1227.3	5153 ± 1820
Lymphocytes (%)	86.1 ± 3.6	81.8 ± 12.4	83.5 ± 6
Monocytes (cells/μL)	78.8 ± 69.4	99.8 ± 58.2	158.3 ± 101.8
Monocytes (%)	1.6 ± 1.5	2.1 ± 1	2.7 ± 1.5
Eosinophils (cells/μL)	27.8 ± 40.2	50.4 ± 37	105.5 ± 55.1**
Eosinophils (%)	0.5 ± 0.8	1 ± 0.5	1.7 ± 0.5**
Basophils (cells/μL)	20.9 ± 29.4	32.9 ± 39	11.3 ± 27.8
Basophils (%)	0.4 ± 0.5	0.6 ± 0.7	0.2 ± 0.4
Blood chemistry			
Cholesterol (mg/dL)	86.6 ± 9.5	73.6 ± 31.6	102.5 ± 21
Glucose (mg/dL)	488.9 ± 70.4	435.5 ± 191.2	488 ± 201.3
Calcium (mg/dL)	11.1 ± 0.4	11.9 ± 1.1	11.3 ± 0.7
Phosphorous (mg/dL)	11.5 ± 3.4	10.9 ± 3.3	11.9 ± 3.2
Chloride (mg/dL)	99.8 ± 1.8	n/a	97.7 ± 3.7
Potassium (mg/dL)	9.2 ± 3.5	n/a	9.5 ± 3.2
Sodium (mg/dL)	145.8 ± 1.4	n/a	146.7 ± 2.3
A/G ratio	1.1 ± 0.1†	1.2 ± 0.1**,††	1 ± 0.1*
B/C ratio	30.1 ± 4.1	63.5 ± 20.7**,††	26.7 ± 5.7

* Significant difference from control; $p < 0.05$.
† Significant difference from 4 weeks + 1; $p < 0.05$.
** Significant difference from control; $p < 0.01$.
†† Significant difference from 4 weeks + 1; $p < 0.01$.

Table 3
Serum chemistry of SD rats receiving TAC colloidal dispersion for 28 days and sacrificed 24 h (4 weeks) or 168 h (4 weeks + 1) after the final dose.

	Control	4 weeks	4 weeks + 1
Hepatic function			
ALP (U/L)	171.8 ± 35.1	190 ± 46.4	167.2 ± 17.4
ALT (U/L)	176.9 ± 303.2	125.8 ± 137.7	111.2 ± 71.4
AST (U/L)	239.4 ± 319.1	173.4 ± 161.4	171.5 ± 151.4
Bilirubin (mg/dL)	0.075 ± 0	0.0625 ± 0.1	0.05 ± 0.1
Renal function			
Albumin (g/dL)	3.2 ± 0.2	3.7 ± 0.4**,†	3.2 ± 0.2
Protein (g/dL)	6.2 ± 0.4	14.7 ± 22.3	6.6 ± 0.5
Globulin (g/dL)	3 ± 0.2	3.2 ± 0.3	3.4 ± 0.3
BUN (mg/dL)	19.8 ± 2.3	20.6 ± 2.1	18.2 ± 2
Creatinine (mg/dL)	0.7 ± 0.1	0.4 ± 0.1**,††	0.7 ± 0.1

* Significant difference from control; $p < 0.05$.
** Significant difference from control; $p < 0.01$.
† Significant difference from 4 weeks + 1; $p < 0.05$.
†† Significant difference from 4 weeks + 1; $p < 0.01$.

kidney function relative to control. Change in serum creatinine was assumed clinically insignificant, since normal maintenance therapy with TAC is often associated with increased serum creatinine due to nephrotoxicity [45]. Interestingly, increased albumin levels have been reported in the treatment of lupus nephritis with low doses of oral TAC [46]. In one study, TAC trough levels of 4–6 ng/mL reversed hypoalbuminaemia in 5 of 6 patients over 2 years of therapy [47].

Evaluation of lung, liver, spleen, and kidney tissues in both experimental and control groups prepared by H&E stain showed no evidence of inflammation, cell lysis, or histologic lesions. Nephrotoxicity, one of the most common occurring side effects of TAC, is defined as interstitial lesions, vasoconstriction, and fibrosis of renal tissue [48]. Over expression of transforming growth factor (TGF)-β, α-smooth muscle actin leads to an overproduction of fibrous tissue, normally associated with wound healing and vasoconstriction [49]. Calcineurin inhibitor-induced nephrotoxicity is defined structurally as any occurrence of lesions within the fixed specimen. Nephrotoxicity can be diagnosed pathologically when tissue biopsy shows evidence of progressive arteriolar hyalinosis, striped cortical fibrosis, or severe tubular microcalcification [50]. In our study, histological examinations of the kidneys showed no evidence of nephrotoxicity and were indistinguishable from controls.

In addition to H&E staining, the lungs were stained with CD68 and PAS for the determination of alveolar macrophage and monocyte presence and airway mucus production, respectively. Fig. 5

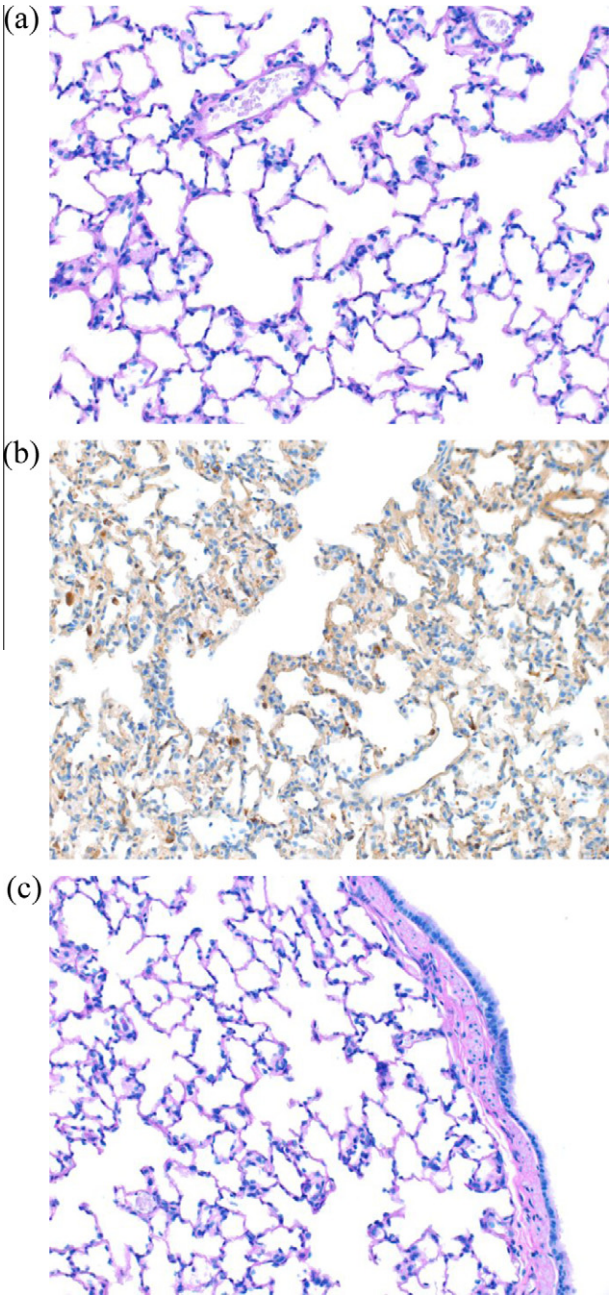

Fig. 5. Histological staining of lungs with (a) H&E, (b) CD 68, and (c) PAS for evaluation of cellular inflammation, monocyte/macrophage presence, and mucus production, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

displays lung sections representative of those evaluated in for each stain. Results in both cases showed no difference to control slides analyzed, meaning inhaled TAC did not cause increased macrophage, monocyte, or mucus production, which may commonly be associated with pulmonary irritation. These results demonstrate that inhaled TAC not only is safe in rodents, but may also cause minimal or no irritation. Considering the only excipient included in the formulation is lactose, an excipient generally recognized as safe for inhalation, one would not expect considerable toxicity in the lung unless it was drug related. As mentioned previously, inhaled CSA, aerosolized by solubilizing CSA in propylene glycol, has been successful in clinical testing in humans [7]; however, it has produced upper airway irritation in a portion of patients, most likely due to the hygroscopicity of propylene glycol. In fact, propylene glycol has traditionally been used to induce cough in patients unable to produce sputum [51]. Administration of aerosolized lidocane and albuterol prior to dosing was needed in order to reduce the irritating effects of propylene glycol on the lungs. By dispersing drug with a GRAS excipient in normal saline, TAC colloidal dispersion may prove to be better tolerated by lung transplant patients and not require the use of a beta-2 agonist or local anesthesia prior to dosing. Furthermore, delivery of amorphous drug particles to fluid lining the lower airways can be expected to reach supersaturated concentrations, increasing the quantity of drug available to permeate the lung's epithelial membrane.

4. Conclusion

It has been successfully demonstrated that TAC colloidal dispersion dosed once daily for 28 days at clinically relevant levels does not induce signs of blood or tissue toxicity. No significant changes were seen in hepatic and renal function panels and lung tissue showed no signs of damage or inflammation. These results lead to the conclusion that multiple doses of 6.4 mg nebulized colloidal dispersion of tacrolimus is non-toxic and safe in SD rats. Furthermore, TAC powder for reconstitution was shown to maintain its physical and chemical properties throughout the 3 month stability testing period. Future studies are needed to confirm formulation safety in larger animal models and/or humans. Most likely as a consequence of its lipophilic nature, TAC was shown to accumulate in lung tissue after pulmonary dosing. Multiple doses showed substantially increased lung levels when compared to a single dose; however, systemic levels measured at trough remained lower than those seen at trough in normal oral dosing (5–15 ng/mL). Localized therapy via pulmonary administration of TAC colloidal dispersion could prove instrumental in reducing incidence of chronic allograft rejection in lung transplant patients and, consequently, improve patient survival rate. With potency up to 100 times that of CSA, TAC shows promise to further improve upon therapeutic benefits seen in clinical trials investigating inhaled CSA. In addition, the novel formulation strategy employed for the development of TAC colloidal dispersion shows evidence for potential improvement in TAC bioavailability through increased aqueous solubility.

Acknowledgement

The author would like to kindly express his appreciation for the financial support provided by the American Foundation for Pharmaceutical Education (AFPE) through the Pre-Doctoral Fellowship in the Pharmaceutical Sciences.

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