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Nebulization of mycophenolate mofetil inhalation suspension in rats: Comparison with oral and pulmonary administration of Cellcept®

Hélène L. Dugas[a,*], Jay I. Peters[b], Robert O. Williams III[a,*]

[a] College of Pharmacy, Pharmaceutics Division, The University of Texas at Austin, TX, USA
[b] Department of Medicine, Division of Pulmonary Diseases/Critical Care Medicine, The University of Texas Health Science Center at San Antonio, TX, USA

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ABSTRACT

In this study, a suspension of mycophenolate mofetil (MMF) suitable for inhalation was developed using the emulsion template process and characterized for particle size and aerosolization performance. To evaluate the benefits of this suspension over a solution, the IV Cellcept® solution was also characterized *in vitro*. Both formulations exhibited excellent aerosolization performance. The aerodynamic diameters for the solution and the suspension were within the respirable range (below 5 μm) and their fine particle doses were nearly equivalent, suggesting the same drug exposure during *in vivo* experiments. Single dose 24-h pharmacokinetic studies following inhalation of the formulations and oral administration of oral Cellcept® were performed in rats. Following oral administration, MMF was completely and rapidly metabolized into its active metabolite, mycophenolic acid (MPA) and partial metabolism was observed following pulmonary administration. Inhaled MMF suspension displayed more favorable pharmacokinetics than inhaled IV Cellcept® solution, but the MPA drug levels in each compartment were much lower than those obtained with oral Cellcept®. The dose normalized MPA levels in the lung, thymus gland and plasma following inhalation of the MMF suspension with the oral control suggested that pulmonary delivery of a MMF suspension could be beneficial in preventing lung allograft rejection.

1. Introduction

Lung transplantation is a life-saving intervention for patients suffering from end-stage pulmonary diseases. Currently, however, the median survival is only 5.5 years (Christie et al., 2011). Beyond the first year after lung transplantation, graft failure, non-cytomegalovirus infections and bronchiolitis obliterans syndrome are the leading cause of death (Benden et al., 2012), suggesting that improvements in the long-term management of acute and chronic rejection are necessary.

Mycophenolate mofetil (MMF) is the ester prodrug of mycophenolic acid (MPA), an anti-metabolite immunosupressant. MPA is a non-competitive inhibitor of the *de novo* purine biosynthesis of guanosine nucleotides necessary for the production of activated T- and B-lymphocytes (Stepkowski, 2000). MPA has proven its efficacy among transplanted patients (Bardsley-Elliot et al., 1999). Upon absorption, MMF is rapidly hydrolyzed into MPA by the carboxylesterases present in the intestine wall and in the liver (Fujiyama et al., 2010). Due to its rapid excretion, a high daily oral dose (up to 3 g/day) is necessary, leading to GI tract toxicity and myelosuppression (Parfitt et al., 2008; Ting et al., 2006). These side effects often force the patients to decrease their daily dose and even sometimes to stop the treatment, increasing the risks of allograft rejection (Kaushik et al., 2006). Therefore, developing new therapeutic strategies for the delivery of MMF is critical to improve patient outcomes.

Pulmonary therapy offers an interesting approach to drug delivery thanks to its large area of absorption and high vascularization, its capability to avoid the hepatic first-pass metabolism, and its ability to achieve high lung deposition and reduced systemic drug concentration. This noninvasive route of administration has the potential to minimize toxicity without compromising efficacy and therefore improves patient compliance. The inhalation route has already been used widely in the treatment of respiratory diseases (Carvalho et al., 2011; Dailey, 2007; Iacono et al., 2006; Tolman and Williams, 2010). Consequently, the pulmonary delivery of MMF could benefit lung transplant patients by decreasing the systemic side effects.

Particle engineering techniques for pulmonary drug delivery have significantly improved in the recent years, enabling more efficient drug deposition to the lungs (Chow et al., 2007; Patravale

* Corresponding authors at: College of Pharmacy, Pharmaceutics Division, The University of Texas at Austin, 2409 West University Avenue, Mail Stop A1920, Austin 78712, TX, USA. Tel.: +1 512 471 4681; fax: +1 512 471 7474.
E-mail addresses: hdugas@utexas.edu (H.L. Dugas), williro@mail.utexas.edu (R.O. Williams III).

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et al., 2004). The pulmonary delivery of microparticles allows the drug to reach the deep lung when their aerodynamic diameter is less than about 5 μm. Besides, microparticles offer the advantage of providing prolonged drug release (El-Sherbiny et al., 2011) and promoting alveolar macrophages uptake (Ahsan et al., 2002; Makino et al., 2004; Sharma et al., 2001), when their size is between 0.5 μm and 3 μm (Kreyling and Scheuch, 2000; Makino et al., 2003). In the case of an immunosuppressive therapy for lung transplanted patients, since the actors of the immune response are present in the transplanted lung as well as in the lymphatic system, it is important to achieve simultaneously high immunosuppressant levels in the lungs and in the lymphatic system. Therefore, the pulmonary administration of microparticles could deliver MMF to the lungs and the lymphatic tissues *via* alveolar macrophages uptake.

A recent study in our laboratory has demonstrated the ability of human lung cells to hydrolyze MMF *in vitro* into it active metabolite, MPA. This work hypothesized that a micron size MMF suspension, with particles within the 1–2 μm range, can be developed and achieve high and sustained drug levels in the lung and the lymphoid tissues while keeping a low systemic concentration following inhalation. To the best of our knowledge, tissue distribution of MPA after oral administration of oral Cellcept®, the currently commercialized product, has not been reported yet, hence this study reports the distribution of MPA in the lung, thymus gland and plasma following administration of oral Cellcept® by mouth. A micron size MMF suspension containing particles within the 1–2 μm range was developed using particle engineering techniques. Its *in vitro* physicochemical and aerodynamic properties were evaluated. Finally, the pharmacokinetic profile and systemic bioavailability of MMF after pulmonary administration of the micron size MMF suspension were investigated in rats and compared to the profiles obtained after nebulization of the IV Cellcept® solution and oral administration of oral Cellcept®.

2. Materials and methods

2.1. Chemicals and reagents

Mycophenolate mofetil and mycophenolic acid were purchased from Trademax Pharmaceuticals & Chemicals Co., Ltd (China). Mycophenolate mofetil reference standard was bought from USP (Rockville, MD) and mycophenolic acid reference standard from Spectrum (Gardena, CA). Acetonitrile (ACN) for HPLC, triethylamine (TEA) for HPLC, ethanol absolute (200 proof) molecular biology grade, methylene chloride stabilized and certified ACS, spectranalyzed methanol and ortho-phosphoric acid for HPLC were obtained from Fisher Scientific (Pittsburgh, PA). Potassium phosphate monobasic, indomethacin (the internal standard, IS) and tyloxapol were purchased from Spectrum (Gardena, CA). 1,2 dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was purchased from Genzyme Pharmaceuticals (Cambridge, MA). All other chemicals and solvents were of the highest grade commercially available.

2.2. Formulation preparation

2.2.1. Cellcept® suspension

The Cellcept® suspension for oral gavage was prepared as follows. Nine 250-mg Cellcept® capsules, containing 250 mg of the active pharmaceutical ingredient with 50 mg of excipients (croscarmellose sodium, magnesium stearate, povidone (K-90) and pregelatinized starch), were opened and poured into 30 ml of 0.1% (w:v) xanthan gum solution. The suspension was probe sonicated for 10 min at 50% duty cycle and 5 output control, to uniformly disperse the powder in solution. To rinse the sonication probe and reach a final MMF concentration of 50 mg/ml, 15 ml of 0.1% (w:v) xanthan gum solution was added. The xanthan gum was used to prevent particle aggregation prior to administration. The final product will be referred hereafter as oral Cellcept® suspension.

2.2.2. IV Cellcept® solution

The IV Cellcept® solution contained the equivalent of 500 mg mycophenolate mofetil as the hydrochloride salt with 25 mg polysorbate 80 and 5 mg citric acid. The solution was prepared by dissolving the IV Cellcept® preparation in a 5% dextrose solution for injection (D5W) as indicated in the package insert. Subsequent adequate dilutions with D5W were performed to obtain a 25 mg/ml solution referred hereafter as IV Cellcept® solution.

2.2.3. Engineered particles of mycophenolate mofetil in suspension

The micron-size MMF suspension with particles ranging from 1 to 2 μm was prepared using the emulsion template process. Briefly, DPPC and MMF were dissolved in 1 ml of ethanol and 6 ml of dichloromethane. This solution was added dropwise to 50 ml 0.2 g/L tyloxapol in D5W solution while sonicating with an analog Branson Sonifier® cell disruptor/homogenizer 450 fitted with a ½″ diameter tapped bio horn at 50% duty cycle and 5 output control and cooled down in an ice bath. After addition of the DPPC/MMF solution, the sonication was continued for 5 min. The solvents were extracted at 27 °C for 10 min by vacuum evaporation using a Buchi® rotary evaporator system consisting of a distillation chiller B-741, a vacuum pump V-700 and a rotavap R-210. The duration of the evaporation was determined by witnessing two boiling events corresponding to the evaporation of dichloromethane and ethanol, respectively. The evaporation was pursued 5 min after the end of the second boiling event to ensure solvent removal; however, residual solvents were not determined quantitatively in the final formulation. Due to the azeotropic nature of the solvent mixture, the volume of the final suspension was readjusted to 50 ml with the 0.2 g/L tyloxapol in D5 W solution and further sonicated for 5 min at 50% duty cycle and 5 output control in an ice bath. The final suspension pH was adjusted to about 7 by the addition of an appropriate volume of 0.5 N sodium hydroxide solution (~40 μl) and left at room temperature to equilibrate before characterization. The final concentration the micron-size MMF suspension was 25 mg/ml and will be referred hereafter as the MMF suspension.

2.3. Formulation characterization

2.3.1. Particle size analysis

The particle size analysis was determined by Dynamic Light Scattering (DLS) using the Malvern Zetasizer Nano S® (Malvern Instrument Ltd, Worcestershire, UK). The measurements were performed at 25 °C with a pre-measurement equilibration time of 1 min. The samples were diluted with 0.2 g/L tyloxapol in D5 W to have the intercept of the correlation function between 0.7 and 1. The refractive indexes for the dispersant (water) and the internal phase (phospholipids) were set at 1.33 and 1.45 respectively. The *z*-average size and the polydispersity index (PdI) were recorded for each formulation and the measurements were done in triplicate.

2.3.2. Morphology

Scanning electron microscopy (SEM) was used to examine the surface morphology of the MMF particles present in the suspension. The suspension was quickly frozen in liquid nitrogen and lyophilized using a Virtis Advantage 2.0 BenchTop freeze dryer (SP Scientific, Warminster, PA) to obtain a dry powder. Samples were loaded onto double-sided carbon tape and sputter coated with a 60/40 Pd/Au target for 4 min. SEM images were captured using a

Leo 1530 SEM or an FEI Quanta 650 SEM under high vacuum mode with an operating voltage of 3–10 kV.

2.4. Aerosol characterization

2.4.1. Aerodynamic droplet size analysis

The formulations were nebulized using the Aeroneb Professional Nebulizer System (Philips Respironics, Murrysville, PA), referred hereafter as the nebulizer. The aerodynamic droplet size analysis was performed using the Next Generation Pharmaceutical Impactor (NGI) (MSP Corp., Shoreview, MN). For each formulation, 6 ml of the formulation were added to the filler port of the nebulizer and attached to the T-piece. The T-piece was then connected to the induction port of the NGI using a molded silicone adapter. The nebulization was started and continued for 15 min at a flow rate of 15 L/min (±5%), as suggested in the General Chapter ⟨1601⟩ of the USP 34-NF 29 on the characterization of nebulizer products (USP 34-NF 29, 2011b). Since the Micro Orifice Collector (MOC) of the NGI fails to operated correctly at that flow rate (Berg et al., 2007), the filter holder placed at the outlet of the NGI was fitted with a type A/E glass fiber filter (nominal pore size: 1 μm) to collect the finest droplets. The drug, collected on each NGI plate and in the induction port, was dissolved in 25 ml of H_2O:ACN:MeOH (2:2:1) (v:v:v) solvent mixture. The glass fiber filter was directly placed in a scintillation vial containing 20 ml of the solvent mixture and vortexed for 30 s. Each solution was diluted with the solvent mixture when necessary, filtered with a 0.22 μm syringe filter and analyzed *via* HPLC analysis for MMF and MPA content.

The aerodynamic parameters were determined as described in the General Chapter ⟨601⟩ of the USP 34-NF 29 on Aerosols, Nasal sprays, Metered-dose inhalers, and Dry powder inhalers (USP 34-NF 29, 2011a). Following HPLC analysis, the total emitted dose (TED), the mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD) were calculated based on the amount of drug collected on the induction port, NGI Plates 1 through 7, the MOC and the back-up filter. The fine particle dose (FPD), which represents the amount of drug included in nebulizer droplets that have MMAD of 5 μm or less and are likely to deposit in the deep lung, was calculated as the sum of the drug amount collected on stage 3 through the back-up filter.

2.4.2. Nebulization performance

To verify if our formulations affect the performance of the nebulizer, we measured the changes in transmission over time with laser diffraction techniques. The Malvern Spraytec® instrument (Malvern Instrument Ltd, Worcestershire, UK), equipped with 300 mm lens, was fitted with the inhalation cell. The outlet of the inhalation cell was fitted to a vacuum tube adapter and connected to a vacuum producing a 1 l/min airflow at the inlet device adaptor portion, therefore mimicking the nebulization conditions of the animal study. The refractive indexes used were identical to the particle size analysis. For each formulation, 6 ml of the formulation was added to the nebulizer reservoir. The nebulizer performance output was measured by looking at the transmission variations of the droplets cloud over a 15-min period. The slopes obtained from each transmission *versus* time plot were compared to each other.

2.5. In vivo studies in Sprague Dawley rats

Adult male Sprague Dawley (SD) rats weighing 300–350 g were used in the present *in vivo* studies. The rats were housed in a 12-h light/dark cycle with food and water available *ad libitum*, and acclimated for 4 days before the start of any procedure. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas at Austin and performed in compliance with all relevant laws, regulations and guidelines governing the humane care and use of research animals.

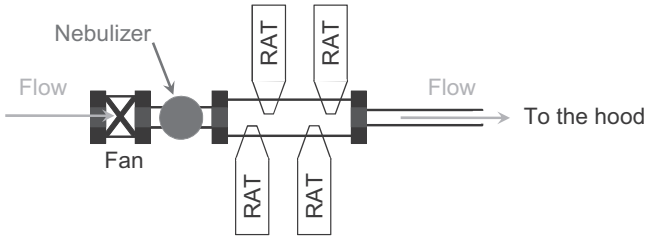

Fig. 1. Nose-only dosing chamber apparatus for inhalation studies in rats.

2.5.1. Oral gavage of Cellcept®

The oral gavage of the oral Cellcept® suspension was performed using a 16-gauge feeding tube and a 1-ml syringe. To avoid stress during the procedure that could result in an altered metabolism, the rats were trained to take the feeding tube for 3 consecutive days prior to dosing. After the training period, the rats were weighed and administered 50 mg of MMF/kg of the oral Cellcept® suspension. After oral gavage, the rats were placed back in their cage with free access to food and water. Four rats were sacrificed by isofluorane inhalation overdose 0.25, 0.5, 1, 2, 4, 6, 12 and 24 h after dose administration. Blood was collected *via* cardiac puncture with heparinized syringes. Additional blood samples were collected 8 and 10 h after the dose administration from jugular catheters surgically implanted on the 12-h and the 24-h rats, respectively. Upon collection, the blood samples were centrifuged at 3000 × *g* using the centrifuge 5804R with rotor A-4–44 (Eppendorf, Hamburg, Germany) at 4 °C for 10 min. The plasma was transferred into a fresh tube and immediately frozen on dry ice. The lungs and thymus gland were harvested after thoracotomy and frozen on dry ice. The samples were stored at −80 °C until analysis. MMF and MPA were extracted from the samples following the extraction method explained hereafter, and analyzed *via* HPLC with UV detection for drug content.

2.5.2. Pulmonary dosing of the IV Cellcept® solutions and the mycophenolate mofetil suspension

For the inhalation study, the nose-only chamber apparatus capable of dosing 4 rats simultaneously was assembled as shown in Fig. 1. Prior to dosing, the rats were acclimated for 4 consecutive days into the restraint tubes. The residence time was progressively increased to 15 min by 5 min increment each day. This acclimation was performed not only for the well being of the animals, but also to prevent stress and hyperventilation during the actual procedure that could lead to higher variability in the drug levels measured. The day of the experiment, each rat was weighed and placed in the restrainer. The airflow rate was set with an external fan at 1 l/min (±5%) to ensure proper filling of the nose-only chamber during aerosolization. Six milliliters of the formulation was pipetted into the filler port of the nebulizer and aerosolized for 15 min. Once the nebulization time completed, the rats were returned to their cages with free access to food and water. Four rats were sacrificed by isofluorane inhalation overdose 0, 0.5, 1, 2, 4, 6, 12 and 24 h after dose administration. Blood, lung and thymus gland collection and storage were performed as described in the oral gavage of Cellcept® section.

The rat whole body dose was estimated to be 10 mg/kg of MMF for both formulations.

2.6. HPLC analysis

2.6.1. Preparation of the plasma, lung and thymus gland samples

The extraction procedure was similar to the extraction procedure developed by Zivanovic et al. (2008). Briefly, the plasma samples were thawed at room temperature. The volume of 10 μl of the internal standard solution was added to 1.5-ml centrifuge tubes containing 500-μl aliquots of the plasma samples and vortexed for 10 s. An aliquot of 500 μl of KH_2PO_4 (pH 2.4; 0.05 M):ACN (95:5) (v:v) was added to the samples followed by a 30 s vortex mixing. The samples were then centrifuged at 15,000 rpm for 5 min at 4 °C.

The extraction for the lung and thymus gland was as follows. The tissues were weighed and placed into glass test tubes. For every gram of tissue, 4 ml of (2:1) H_2O:ACN solution were added and the samples were homogenized in a bath of ice using an Ultra-Turrax® TP 18/10 Homogenizer with 8 mm rotor blade (IKA-Werke, Staufen, Germany). The volume of 10 μl of the internal standard solution was added to 1.5-ml centrifuge tubes containing 500-μl aliquots of the homogenized tissue and vortexed for 10 s. An aliquot of 500 μl of KH_2PO_4 (pH 2.4; 0.05 M):ACN (2:1) (v:v) was added to the samples followed by a 30 s vortex mixing. The samples were then centrifuged at 10,000 rpm for 5 min at 4 °C and the supernatant transferred into a fresh 2-ml centrifuge tube. A volume of 500 ml of KH_2PO_4 (pH 2.4; 0.05 M):ACN (2:1) (v:v) was added to the pellet, which was then resuspended using an Omni Sonic Ruptor-250® Ultrasonic Homogenizer with 5/32″ (3.9 mm) with a micro-tip probe (Omni International, Kennesaw, GA, USA) for 10 s. After a final 30-s vortex mixing, the samples were centrifuged at 15,000 rpm for 10 min at 4 °C and the supernatant transferred into the 2-ml centrifuge tube containing the first supernatant.

2.6.2. Extraction procedure for plasma, lung and thymus gland samples

The solid phase extraction procedure was performed using 100 mg/1 ml Supelco Discovery® DSC-18 SPE tubes mounted on the Supelco 12-port Visiprep™ SPE vacuum manifold with disposable liners, purchased from Sigma–Aldrich (St Louis, MO). Briefly, the SPE cartridges were conditioned with 2 ml of methanol followed by 2 ml of KH_2PO_4 (pH 2.4; 0.05 M). The prepared plasma and tissue samples were then pipetted into the cartridges and passed through them. The packing was washed with 2 ml of KH_2PO_4 (pH 2.4; 0.05 M). The substances were eluted by the addition of 1.5 ml of KH_2PO_4 (pH 2.4; 0.05 M):ACN (2:8) (v:v). The extracted samples were evaporated under a stream of nitrogen until complete evaporation, reconstituted in with appropriate volumes of H_2O:ACN (3:1) leading to a final internal standard concentration of about 1 μg/ml. The reconstituted samples were analyzed *via* HPLC with UV detection for drug content.

2.6.3. Chromatographic conditions

The HPLC analysis was performed using a Dionex Ultimate 3000 HPLC equipped with HPG-3200SD binary pump with in-line degasser, VWR-3400RS UV-visible detector and WPS-3000SL analytical autosampler. The data were acquired with Chromeleon 6.8 Chromatography Data System (Dionex). Compounds were separated on a reverse-phase Kinetex C18 column (100 mm × 3.0 mm, particle size: 2.6 μm, pore size: 100 Å). Each mobile phase was filtered through 0.45 μm nylon membrane and degassed for 10 min. The HPLC system was operated with a multiple step mode at 0.9 ml/min with the column heater thermostated at 45 °C ± 0.2 °C. The mobile phases consisted of 0.3% triethylamine in water (pH = 5.3):acetonitrile (90:10, v:v) and acetonitrile as mobile phase A and B, respectively. The pH adjustment was done using 85% o-phosphoric acid. The multiple step mode was as follows: from 0 to 0.25 min, 0% B; from 0.25 to 2 min, 9% B; from 2 to 6 min, 33% B followed by 1 min at 90% B to clean the column and 3 min at 0% B to return to the initial conditions. Detection was performed at 215 nm with a 10-min run time. Fig. 2 shows the typical chromatograms of a solution containing MPA, MMF, IS and mycophenolic acid glucuronide (MPAG), the main metabolite of MPA, and a blank rat lung homogenate sample after solid phase extraction. This method was validated in our laboratories following the guidelines of the International Conference on Harmonization and the CDER reviewer guidance on Validation of Chromatographic Methods.

Table 1
Summary of MMF formulation produced by the emulsion template process.

Formulation	Composition (mg)		Sonication parameters	
	MMF	DPPC	Power output – duty cycle	Time (min)
MMF021 A	1500	500	5–50%	5
MMF022 A	1500	150	5–50%	5
MMF023 A	1500	100	5–50%	5
MMF024 A	1500	50	5–50%	5
MMF023 B	1500	100	5–50%	15
MMF024 B	1500	50	5–50%	15
MMF023 C	1500	100	7–50%	5
MMF024 C	1500	50	7–50%	5

2.7. Pharmacokinetic analysis

The rats were evaluated for their 24 h plasma, lung and thymus gland pharmacokinetics following administration of the dose. The tissue concentrations *versus* time and the plasma *versus* time were calculated using a non-compartmental model in WinNonlin® version 4.1 (Pharsight®, St. Louis, MO). Since the absorption, distribution, metabolism and clearance profiles of MMF and MPA are quite complex, it was chosen to perform a model-independent pharmacokinetics analysis. Each rat gave an individual data point; therefore the pharmacokinetic parameters were calculated based on the average and the 95% confidence intervals at each time point. At least 4 points were used on the lambda-*z* curve generated for the calculation of the parameters and the goodness of fit statistic for the elimination phase had to be more than 0.9 to be acceptable.

2.8. Statistical analysis

The statistical analysis was performed using JMP 9.0® (SAS institute Inc., Cary, NC, USA). One-way ANOVA, with Tukey–Kramer *post hoc* test where necessary, was used to statistically compare the *in vitro* characterization of the formulations and the *in vivo* pharmacokinetic parameters. Results with *p*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Formulation characterization

For the MMF suspensions, the effects of DPPC concentration, sonication times and sonication output on the particle size and polydispersity index (PdI) were investigated. The formulation parameters are summarized in Table 1. From the *z*-average size obtained for each formulation, a decrease in particle size was observed as the ratio DPPC:MMF increased. The *z*-average sizes for MMF021 A, MMF022 A, MMF023 A and MMF024 A were 0.44 ± 0.03 μm, 0.76 ± 0.04 μm, 1.28 ± 0.29 μm and 1.38 ± 0.32 μm, respectively. MMF023 A and MMF024 A were the only formulations for which the *z*-average sizes were not statistically different ($p = 0.594$). The PdIs of each formulation were 0.39 ± 0.03, 0.39 ± 0.09, 0.41 ± 0.09 and 0.43 ± 0.12, respectively, characterizing an unimodal particle size profile. The statistical analysis showed no statistical differences among the formulations

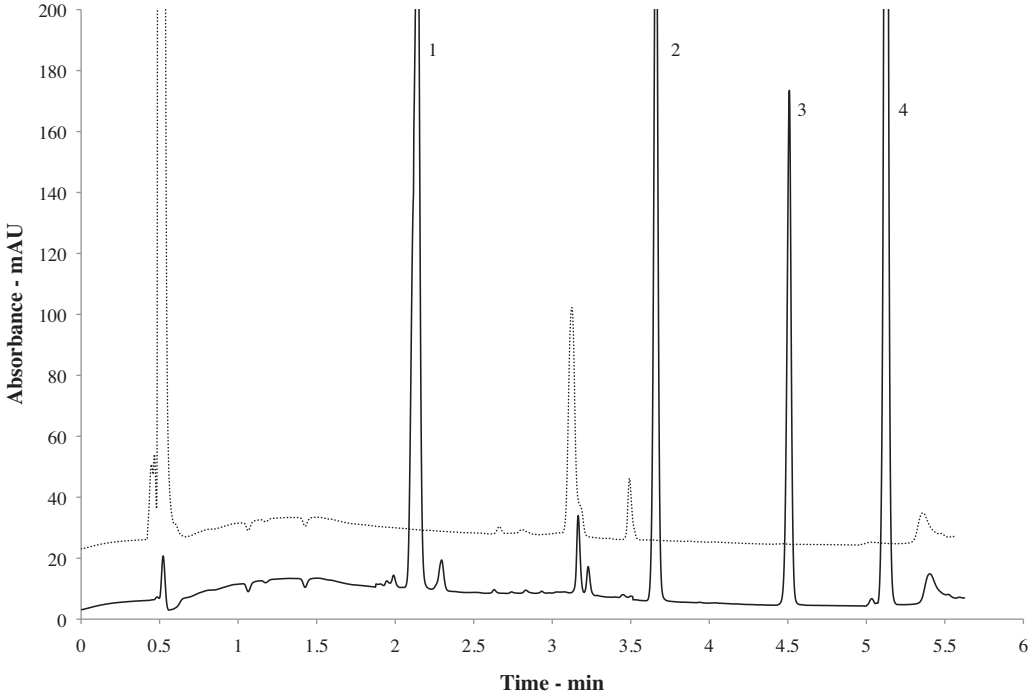

Fig. 2. Chromatograms of a solution containing MPAG, MPA, IS and MMF (—) and a blank rat lung homogenate after solid phase extraction (. . ..). Peaks: 1, MPAG; 2, MPA; 3, Indomethacin (IS); 4, MMF.

($p < 0.05$). Since a MMF suspension with a particle size ranging from 1 μm to 2 μm was desired, the formulations containing 100 mg and 50 mg of DPPC were chosen to investigate the influence of the process parameters on the particle size.

Increasing the sonication time from 5 min to 15 min, did not significantly affect the *z*-average size. The *z*-average sizes of MMF023 were 1.28 ± 0.29 μm with a PdI of 0.41 ± 0.09, and 1.24 ± 0.12 μm with a PdI of 0.52 ± 0.08, after 5 and 15 min of sonication, respectively. The *z*-average sizes of MMF024 were 1.38 ± 0.32 μm with a PdI of 0.43 ± 0.12, and 1.32 ± 0.29 μm with a PdI of 0.44 ± 0.10, after 5 and 15 min of sonication, respectively. No statistical differences in *z*-averages and PdIs were observed for both formulations (*p*-values > 0.05). When the sonication output was increased from 5 to 7, the *z*-average sizes of both formulations increased to 1.69 ± 0.37 μm with a PdI of 0.40 ± 0.11, and 2.09 ± 0.13 μm with a PdI of 0.47 ± 0.10, for MMF023 C and MMF024 C, respectively. The intra-formulation *z*-average sizes obtained with 5 and 7 sonication output were statistically different with *p*-values of 0.0005 and 0.0001 for MMF023 and MMF024, respectively. On the contrary, the PdIs were not statistically different ($p > 0.05$); suggesting an increase in the particle size as the sonication output increased, but not a broadening of the particle size distribution. Finally, because the average particle size increased at higher sonication powder and because shorter processing times are preferred, formulations MMF23 A and MMF24 A were chosen.

Since no significant differences were observed in regards to *z*-average sizes and PdIs between formulations MMF023 A and MMF024 A, a sedimentation behavior was also investigated (data not shown). Those formulations were left to settle for 3 days at room temperature and their ability to redisperse easily was evaluated. After 3 days, both formulations had a similar appearance: white sediment and opalescent supernatant. After inverting 10 times the recipients, suspension MMF023 A was readily resuspended whereas a layer of particles was still observed at the bottom of the container with suspension MMF024 A. Since suspension MMF023 A was the most promising MMF formulation, it was produced in triplicate to ensure reproducibility of the fabrication process (data not shown). No statistical differences between the *z*-average sizes and the PdIs were recorded among the formulations fabricated (minimum *p*-value obtained 0.1553), confirming the process reproducibility. The *z*-average size was 1.42 ± 0.21 μm with a PdI of 0.43 ± 0.09. Suspension MMF023 A was freeze-dried, characterized *via* SEM for particle morphology and used for aerosol characterization.

Fig. 3 shows the SEM images of lyophilized suspension MMF023 A in 0.2 g/L tyloxapol in water and in 0.2 g/L tyloxapol in D5 W. On one hand, the rapid freezing MMF023 A in 0.2 g/L tyloxapol in water in liquid nitrogen followed by lyophilization resulted in large aggregates particles with well defined, non-spherical and flat micron-size primary particles. On the other hand, the rapid freezing in liquid nitrogen of MMF023 A made in 0.2 g/L tyloxapol in D5W followed by lyophilization lead to large aggregate particles in which smaller primary particles seemed to be fused with each other.

3.2. Aerosol characterization

The aerodynamic parameters of the IV Cellcept® solution and the MMF suspension (MMF023 A) were determined in triplicate for intra- and inter-formulations variability. The *in vitro* deposition profiles for each formulation are showed in Fig. 4. Overall, similar intra-formulation profiles were observed. For most NGI stages, the amount of drug deposited showed inter-formulation statistical differences ($p < 0.05$). The main difference observed between the IV solution and the suspension deposition profiles was the higher MMF deposition on stages 4–6, and on stages 2–4, for IV Cellcept® solution and MMF suspension, respectively. This behavior resulted in a lower MMAD in IV Cellcept® solution than in MMF suspension – 2.19 ± 0.33 μm *versus* 4.42 ± 0.19 μm. The intra-formulation aerodynamic parameters, reported in Table 2, were statistically different, but were of the same order of magnitude. Interestingly, although the MMF suspension had a higher aerodynamic

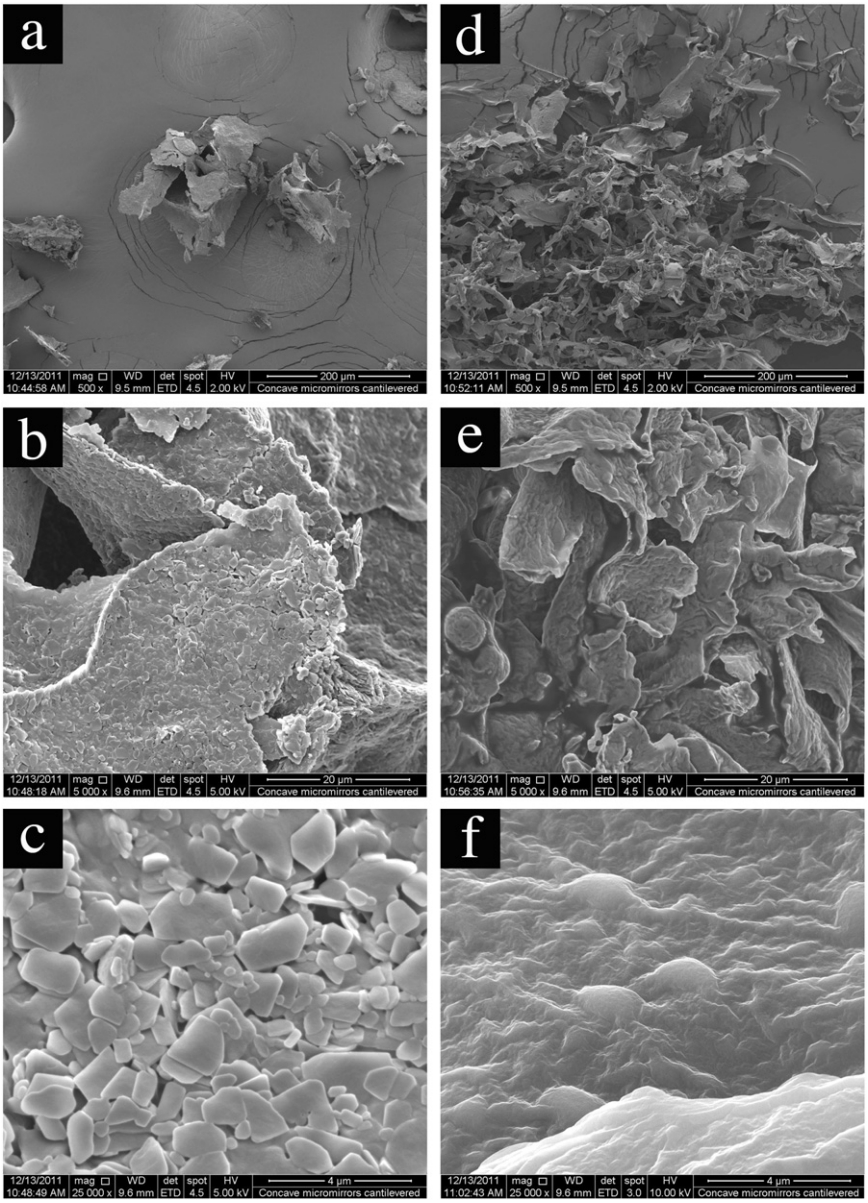

Fig. 3. SEM images of MMF023 A in 0.2 g/L tyloxapol in water (a–c) and 0.2 g/L tyloxapol in D5 W (d–f). (a) and (d) aggregate particle at 500× magnification; (b) and (e) aggregate particle at 5000× magnification; (c) and (f) primary particle structure at 25,000× magnification.

diameter than the IV solution, the FPDs were similar for both formulations and not statistically different: 36.31 ± 2.64 mg of MMF and 38.46 ± 4.83 mg of MMF, for the MMF suspension and the IV Cellcept® solution, respectively.

The transmission plots (data not shown) enabled the comparison of the nebulization performance of each formulation in comparison to saline over a 15-min period. Neither a progressive loss of aerosol performance nor a discontinuous nebulization was observed for each formulation. The slopes of the linear regressions performed on each formulation suggested that the nebulizer's performance was not altered during the test period: they were very similar to the slope obtained for saline, which nebulizes readily.

3.3. *Pharmacokinetics of oral Cellcept® following oral gavage*

The extravascular input non-compartmental model was used to determine 24-h pharmacokinetic parameters in the plasma, lung and thymus gland after oral administration of oral Cellcept®,

Table 2
Summary of the aerodynamic properties of aerosolized IV Cellcept® and MMF suspension at a flow rate of 15 L/min using an Aeroneb Pro® nebulizer.

Formulation (mg)		MMAD (μm)	GSD	FPD (mg)	TED
IV Cellcept® solution	1	1.87 ± 0.09	2.09 ± 0.10	43.22 ± 3.27	47.88 ± 3.78
	2	2.24 ± 0.13	2.28 ± 0.04	36.33 ± 1.25	44.65 ± 0.06
	3	2.55 ± 0.10	2.30 ± 0.04	34.12 ± 1.45	43.98 ± 1.03
MMF023 A	1	4.21 ± 0.11	2.03 ± 0.01	37.75 ± 1.30	64.93 ± 3.46
	2	4.47 ± 0.09	2.08 ± 0.01	37.27 ± 3.31	68.71 ± 4.7
	3	4.58 ± 0.09	2.09 ± 0.02	34.57 ± 1.50	65.23 ± 2.38

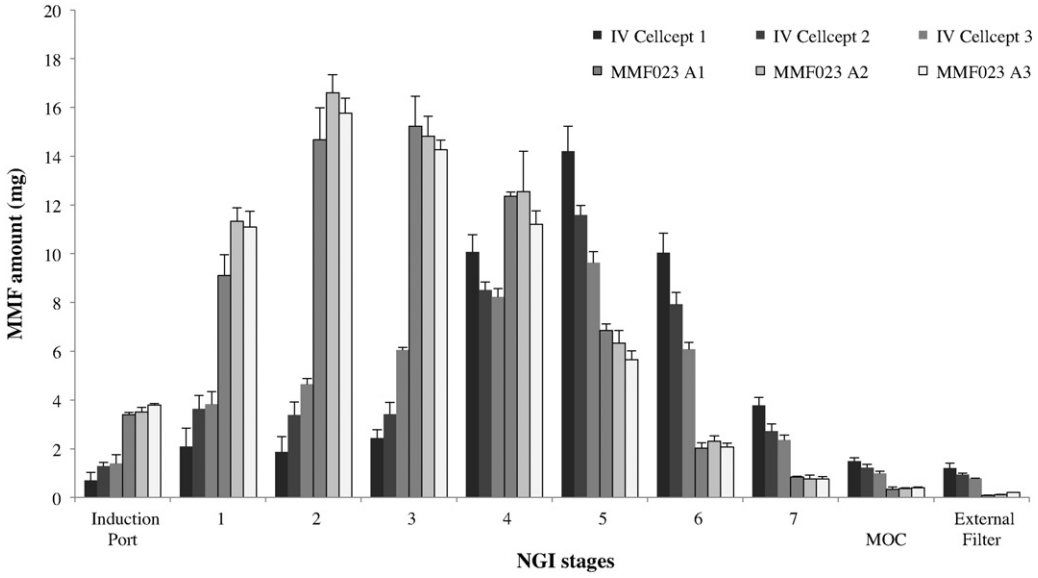

Fig. 4. Inter-formulation *in vitro* NGI deposition profiles of aerosolized IV Cellcept® solution and MMF suspension at a flow rate of 15 L/min using an Aeroneb Pro® nebulizer.

reported in Table 3. The drug concentration *versus* time plots are shown in Fig. 5.

The absorption of MMF through the gut wall is rapid and followed by the rapid hydrolysis of MMF into MPA as the MPA C_{max} occurred within 15 min and no levels of MMF could be detected on the chromatograms.

3.4. Pharmacokinetics of IV Cellcept® and crystalline micron-size MMF suspension following inhalation

For each formulation, the nebulization procedure was exposing the rats to a constant amount of drug during 15 min allowing the use of the constant infusion model available for the non-compartmental analysis. After inhalation of IV Cellcept® and MMF suspension, the pharmacokinetic parameters for MMF and MPA could be calculated and are reported in Table 3. The MPA concentration *versus* time plots are shown in Fig. 5. The MMF concentration *versus* time plots are shown in Fig. 6 for both formulations.

The hydrolysis of MMF was also quick, leading to small levels in the plasma, lungs and thymus gland. No statistical differences between formulations were observed in the MPA and MMF C_{max}, expect for the MMF C_{max} in the thymus gland. The main differences were found in the half-life and AUC values. The MMF $t_{1/2}$ and $AUC_{0-24\,h}$ reported in the three body compartments after MMF suspension nebulization are significantly higher than those after the delivery of IV Cellcept®. The same observation can be made for the MPA $t_{1/2}$ and $AUC_{0-24\,h}$, making the two formulations pharmacokinetically distinct.

4. Discussion

Following activation of the adaptive immune response, T- and B-lymphocytes are activated in the allograft and in its regional lymphoid tissues. When considering the development of an inhaled formulation of MMF for lung transplant patients, it was critical to deliver a high drug amount to the lungs and to the lymphatic system simultaneously, to block the proliferation of T- and B-lymphocytes in both compartments. To target the lymphatic system, the development of the micron-size MMF suspension was based on the evidence of alveolar macrophages uptake of 1–3 μm particles (Geiser, 2002; Makino et al., 2003; Sharma et al., 2001).

The development of pulmonary formulations is typically restricted by the excipients and their amounts (Pilcer and Amighi, 2010; Weers et al., 2010). Two surfactants, DPPC and tyloxapol, were chosen in the present study because of their approved amounts, 108 mg and 8 mg, respectively, (Long et al., 1991) and their physicochemical properties, which should enhance the

Table 3
Pharmacokinetic parameters of oral Cellcept® following oral administration, and IV Cellcept® and MMF suspension following pulmonary administration. Results are expressed as mean ± 95% confidence interval ($n = 4$).

Pharmacokinetic parameters		Oral Cellcept®			IV Cellcept®			MMF suspension		
		Plasma	Lung	Thymus gland	Plasma	Lung	Thymus gland	Plasma	Lung	Thymus gland
MPA	C_{max} (μg/ml)	31 ± 18	9.5 ± 4.5	4.1 ± 2.5	5.8 ± 1.3	1.3 ± 0.2	0.54 ± 0.16	4.3 ± 4.8	0.98 ± 0.3	0.80 ± 0.31
	t_{max} (h)	0.25 ± 0.14	0.25 ± 0.14	0.25 ± 0.14	0.25 ± 0.24	0.25 ± 0.24	0.25 ± 0.24	0.75 ± 0.24	0.75 ± 0.24	0.75 ± 0.24
	$t_{1/2}$ (h)	3.6 ± 0.2	4.2 ± 0.5	4.3 ± 0.5	5.9 ± 0.8	6.1 ± 1.0	9.8 ± 1.2	7.8 ± 0.9	9.6 ± 1.5	10.4 ± 2.3
	AUC_{0-24} (mg h/ml)	116 ± 17	33 ± 5	16 ± 5	25 ± 4	5.4 ± 0.6	4.5 ± 0.4	16.6 ± 1.5	7.7 ± 0.5	9.1 ± 0.4
	AUC_{0-24}/Dose (μg h/ml/mg/kg))	2.3 ± 0.4	0.7 ± 0.1	0.3 ± 0.1	2.5 ± 0.4	0.5 ± 0.06	0.5 ± 0.04	1.7 ± 0.15	0.8 ± 0.05	0.9 ± 0.04
MMF	C_{max} (μg/ml)	–	–	–	0.16 ± 0.01	1.4 ± 0.2	0.74 ± 0.09	0.35 ± 0.03	1.5 ± 0.1	2.7 ± 1.1
	t_{max} (h)	–	–	–	0.75 ± 0.24	0.75 ± 0.24	0.25	1.25 ± 2.7	0.25 ± 0.48	0.25 ± 0.96
	$t_{1/2}$ (h)	–	–	–	4.5 ± 0.8	2.7 ± 1.0	6.6 ± 1.2	27.4 ± 2.5	10.1 ± 3.5	26.2 ± 3.4
	AUC_{0-24} (μg h/ml)	–	–	–	0.8 ± 0.1	11.5 ± 3.4	8.8 ± 2.2	6.8 ± 0.7	24.7 ± 1.0	33.4 ± 1.8

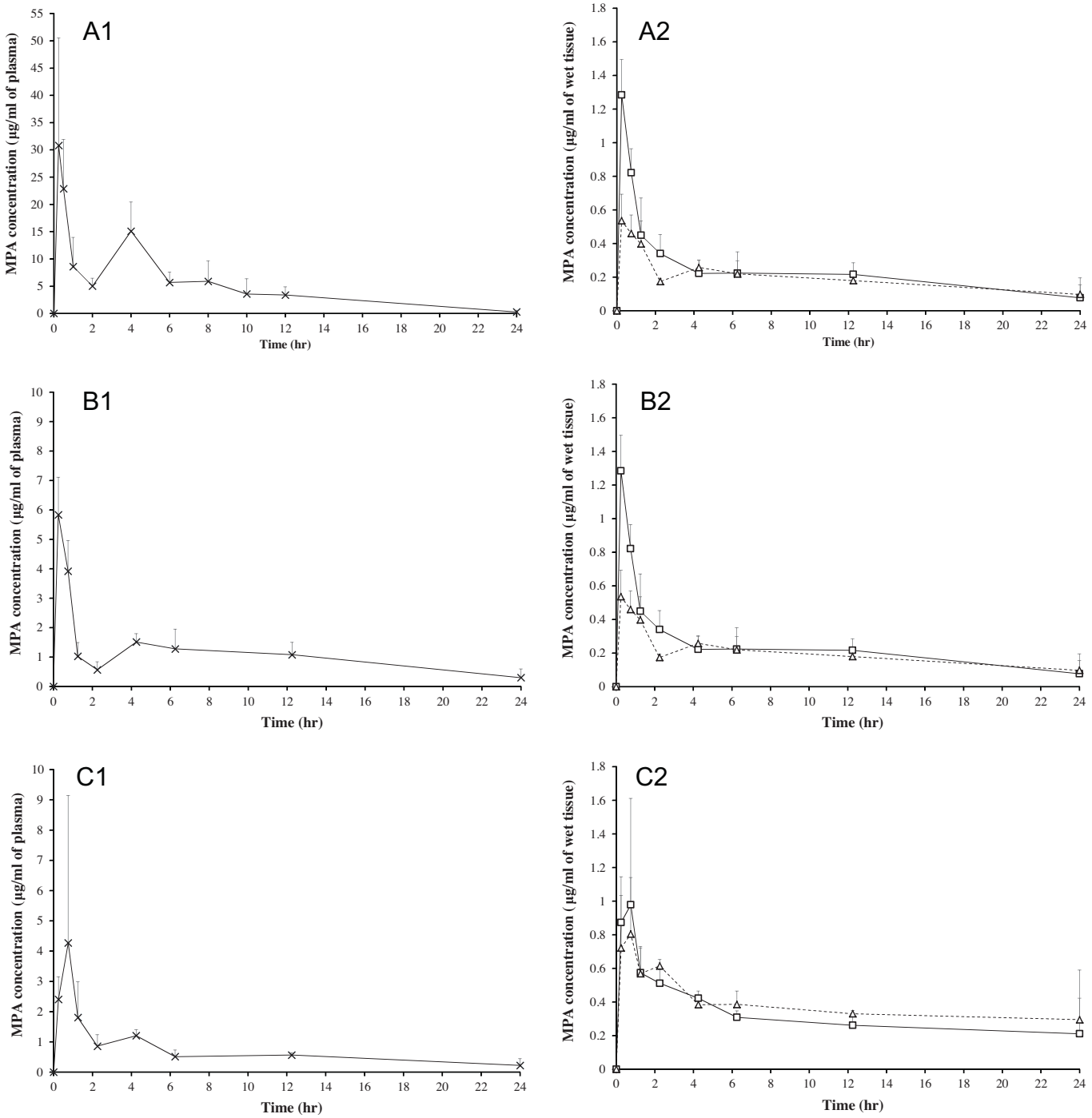

Fig. 5. Plasma, lung and thymus gland MPA concentrations in rats: (A) after oral administration of oral Cellcept® (50 mg/kg); (B) after pulmonary administration of IV Cellcept® solution (10 mg/kg); (C) after pulmonary administration of MMF suspension (10 mg/kg). 1 – plasma (×); 2 – lung (□) and thymus gland (△). Results are expressed as mean ± standard deviation ($n = 4$).

migration of MMF to the lung peripheral region after deposition (Ganguly et al., 2008).

The emulsion template process is based on the formation of a nano- or micro-emulsion followed by solvent evaporation leading to the precipitation of nanoparticles or microparticles in the aqueous phase. The size and the stability of the particles obtained rely on the quantity of energy provided to the system, the amount of surfactants and the nature of the components (Anton et al., 2008). This study showed the influence of increased amount of DPPC on the particle size of the MMF suspension. During the formation of the emulsion, the hydrophobic portion of DPPC strongly adsorbed into the oil phase while the hydrophilic portion extended into the aqueous phase. It decreased the interfacial tension and consequently stabilized the emulsion. Therefore, when the amount of DPPC was increased, stabilization of the emulsion increased and its droplet size decreased leading to smaller particles in suspension. Upon solvent removal, MMF and DPPC precipitate in the aqueous phase due to their low water solubility. Particle stabilization was achieved thanks to the hydrophobic nature of MMF in water leading to the adsorption of the DPPC hydrophobic chain onto the surface of the microparticles. When the concentration of DDPC was sufficient, its hydrophilic portion extended from the surface of the MMF particles.

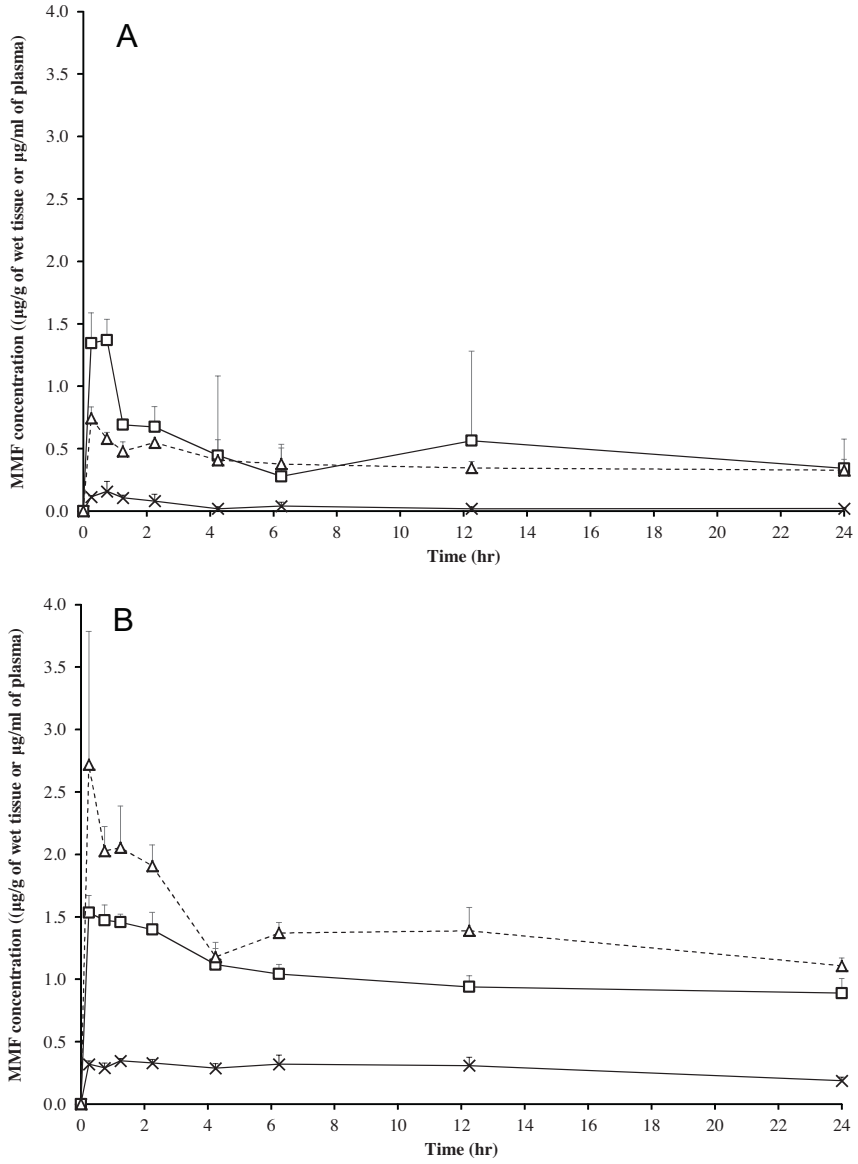

Fig. 6. Plasma, lung and thymus gland MMF concentrations in rats after pulmonary administration of (A) IV Cellcept®; (B) MMF suspension (10 mg/kg). Plasma (×); lung (□) and thymus gland (△). Results are expressed as mean ± standard deviation ($n = 4$).

The distance between the particles increased such that van der Walls attractions became ineffective and steric stabilization was dominant (Burgess, 2005; Somil et al., 2008). The MMF particles were repelling each other thanks to volume restriction and osmotic pressure effect (Tadros, 2009). However, when the concentration of DPPC was too low, bridging flocculation may have occurred as DPPC had multiple points of contact on the surface of the MMF particles causing the hydrophilic portion to lie along the surface rather than extend away from it (Burgess, 2005) and explaining the difficulties encountered to resuspend the sediment in formulation MMF023C. The slight effect observed on the particle size when the sonication time was increased from 5 min to 10 min can be related to the efficiency of the sonication process since for a given composition of the emulsion and sonication power, the emulsion reaches an asymptotic size independent of the fabrication time (Anton et al., 2008).

From the SEM images of the MMF particles obtained from the lyophilization of the suspension in water, the observed clusters were certainly due to the presence of tyloxapol, which is liquid at room temperature. From the SEM images obtained for the suspension in D5W, the product itself had a cotton candy-like texture, similar to the SEM images obtained from freeze-dried sucrose (Imamura et al., 2010; Labuza and Labuza, 2004; Roth et al., 2001). The images were suggesting the fusion of the MMF particles within the sugar matrix and leading to an unclear morphology of the primary particles.

It was important to evaluate the nebulization performance as well as the aerodynamic parameters of each formulation in order to understand their *in vivo* behavior. For the Cellcept® solution, because its nature, its nebulization was expected to have no fluctuation in the produced aerosol cloud and to obtain aerodynamic parameters similar to those reported by Aerogen® (Respironics, 2001). The inter-formulation differences observed in the aerodynamic parameters could be attributed to the difficulty to maintain constant temperature and humidity levels in our laboratories, which, as reported, can significantly influence the results obtained from the NGI analysis (Berg et al., 2007). For the MMF suspension, the use of microparticles was predicted to clog the nebulizer mesh and compromise the aerosol performance. However, the presence of surfactants and sodium salt helped preventing the phenomenon

from occurring. Indeed, as showed by Ghazanfari et al. (2007), formulations with lower surface tension and higher salt concentration resulted in improved aerosol output when using the Aeroneb Pro® nebulizer. Finally, because there were no significant differences in the FPD and aerosol performance between the solution and the suspension, their nebulization should deliver approximately the same whole body dose to the rats in the *in vivo* study. This enabled a proper comparison of the PK profiles.

The oral dose of Cellcept® administered to the rats was similar to a human daily dose. The pharmacokinetic profile showed complete metabolism of MMF into MPA in the intestine and in the liver, as no MMF was measured in the plasma 15 min after oral administration. It correlates with the presence of carboxylesterases 1 and 2 in the liver and intestine, and their involvement in the metabolism of MMF (Fujiyama et al., 2010; Lee et al., 1990). MPA is distributed to the major organs within two hours of the average t_{max}. The second MPA concentration peak observed at the 4-h time point suggests an enterohepatic circulation of MPAG. In human, this phenomenon is due to the presence of the gallbladder. While rats do not have a gallbladder, this pseudo enterohepatic recirculation phenomenon observed here has been reported and studied in rats: MPAG is extensively excreted into the bile and accumulates in the intestines, where it is reconverted into MPA and reabsorbed (Matsuzawa and Nakase, 1984; Saitoh et al., 2006).

The inhalation studies showed the involvement of a pulmonary first-pass effect on MMF. The hydrolysis of MMF into MPA occurred rapidly after pulmonary administration of both formulations. However, carboxylesterases hydrolysis is a saturable process (*in vitro* work, not shown here) and those enzymes are present in the rat lungs at much lower expression levels than in the liver and intestines (Gaustad et al., 1991; Imai et al., 2003; McCracken et al., 1993). As soon as saturation occurred, the unhydrolyzed MMF in solution permeated through the lung epithelium resulting in the small concentration of MMF found in the systemic circulation. In the case of the suspension, an additional dissolution step of the MMF microparticles in the lung fluid was required, leading to the sustained MMF levels observed in all three compartments when compared to the IV Cellcept® solution.

The thymus gland, one of the major lymphatic structure, is vascularized with the systemic circulation and with the lymphatic system (Tilney, 1971). Therefore, measuring the thymus gland drug concentration can give a good estimate of the lymphatic system drug concentration. After inhalation of the IV Cellcept® solution, the levels of MPA and MMF observed in the thymus gland were the result of the systemic distribution, whereas after the inhalation of the MMF suspension, they were the results of both systemic and lymphatic biodistribution, resulting in higher drug levels. The MMF suspension contained particles in the 1–2 μm size range, which is the optimum size for alveolar macrophages uptake (Makino et al., 2003; Sharma et al., 2001). The MMF particles that were not yet dissolved may have been phagocytosed by the alveolar macrophages and translocated into the lymphatic system, while the dissolved drug traveled in the systemic circulation to reach the thymus gland.

Overall, after inhalation of the IV Cellcept® solution or the MMF suspension, the AUCs of MPA in the different body compartments over the 24-h period were significantly lower than the AUCs obtained after oral administration of Cellcept®. However, as the whole body dose administered was 5 times lower for the pulmonary administration than for the oral gavage, it was interesting to compare the dose normalized AUCs (data shown in Fig. 7). No significant differences were observed after oral gavage of Cellcept® and pulmonary delivery of IV Cellcept®. The nebulization of the MMF suspension led to promising dose normalized AUCs when compared to the IV Cellcept® and the oral administration: the sustained release provided by the progressive dissolution of the MMF microparticles in the lung fluid resulted in dose normalized AUCs

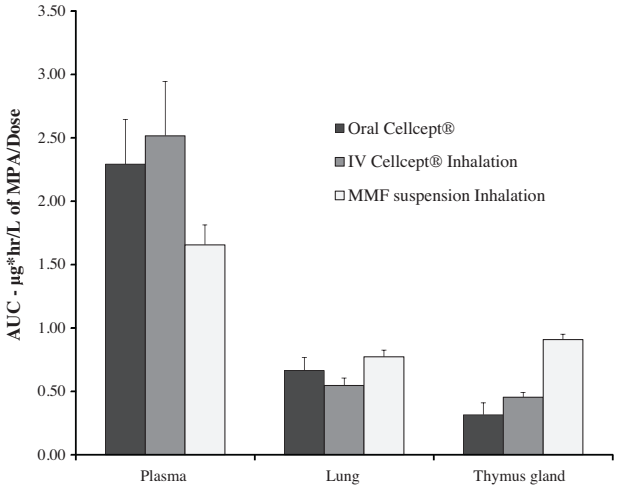

Fig. 7. Plasma, lung and thymus gland dose normalized MPA AUCs after oral administration of Cellcept® (50 mg/kg) and after pulmonary administration of IV Cellcept® and MMF suspension (10 mg/kg).

significantly lower in the central compartment, similar in the lungs and higher in the thymus gland.

5. Conclusion

The objective was to develop a MMF microsuspension capable to achieve high and prolonged drug levels in the lungs and lymphoid system following pulmonary administration. Such formulation was successfully developed. Its *in vitro* characterization showed its suitability for pulmonary delivery *via* aerosol inhalation. The *in vivo* comparison of the inhalation of IV Cellcept® solution and the formulation MMF suspension demonstrated the more favorable pharmacokinetic profile of the MMF suspension. After dose normalization, the pulmonary administration of the MMF suspension exhibited high and sustained levels of MPA in the lung and the lymphatic system, and low levels in the plasma, when compared to the oral administration of oral Cellcept®.

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