

**Drug- not carrier-dependent haematological and biochemical changes in a repeated dose study of cyclosporine encapsulated polyester nano- and micro-particles: size doesn't matter.**

V. P. Venkatpurwar<sup>1</sup>, S. Rhodes<sup>2</sup>, K. A. Oein<sup>3</sup>, M. A. Elliott<sup>4</sup>, C. D. Tekwe<sup>5</sup>, H. G. Jørgensen<sup>2</sup>, M. N. V. Ravi Kumar<sup>6\*</sup>

<sup>1</sup>Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, G4 0RE, UK

<sup>2</sup> Paul O’Gorman Leukaemia Research Centre, Institute of Cancer Sciences, University of Glasgow, Glasgow, UK, G12 0ZD

<sup>3</sup>Molecular Pathology, Institute of Cancer Sciences, University of Glasgow, Glasgow, UK, G61 1BD

<sup>4</sup>Cancer Research UK Formulation Unit, Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, G4 0RE, UK

<sup>5</sup>Department of Epidemiology and Biostatistics, School of Public Health, 1266 Texas A&M University, College Station, Texas 77843-1266, USA.

<sup>6</sup>Department of Pharmaceutical Sciences, Irma Lerma Rangel College of Pharmacy, Texas A&M Health Science Centre, Texas A&M University, College Station, TX 77843-1114, USA.

**Correspondence**

Professor M.N.V. Ravi Kumar,

Department of Pharmaceutical Sciences, Irma Lerma Rangel College of Pharmacy, 310B Reynolds Medical building, Texas A&M Health Science Centre, Texas A&M University, College Station, TX 77843-1114, USA. \*Phone: +1-979-436-0721 & Email: [mnvrkumar@tamhsc.edu](mailto:mnvrkumar@tamhsc.edu)

## **ABSTRACT**

Biodegradable nanoparticles are being considered more often as drug carriers to address pharmacokinetic/pharmacodynamic issues, yet nano-product safety has not been systematically proven. In this study, haematological, biochemical and histological parameters were examined on 28 day daily dosing of rats with nano- or micro-particle encapsulated cyclosporine (CsA) to confirm if any changes observed were drug or carrier dependent. CsA encapsulated poly(lactide-co-glycolide) [PLGA] nano- (nCsA) and micro-particles (mCsA) were prepared by emulsion techniques. CsA (15, 30, 45 mg/kg) were administered by oral gavage to Sprague Dawley (SD) rats over 28 days. Haematological and biochemical metrics were followed with tissue histology performed on sacrifice. Whether presented as nCsA or mCsA, 45 mg/kg dose caused significant loss of body weight and lowered food consumption compared to untreated control. Across the doses, both nCsA and mCsA produce significant decreases in lymphocyte numbers compared to controls, commensurate with the proprietary product, Neoral<sup>®</sup>15. Dosing with nCsA showed higher serum drug levels than mCsA presumably owing to the smaller particle size facilitating absorption. The treatment had no noticeable effects on inflammatory/oxidative stress markers or antioxidant enzyme levels, except an increase in ceruloplasmin (CP) levels for high dose nCsA/mCsA group. Further, only subtle, sub-lethal changes were observed in histology of nCsA/mCsA treated rat organs. Blank (drug-free) particles did not induce changes in the parameters studied. Therefore, it is extremely important that the encapsulated drug in the nano-products is considered when safety of the overall product is assessed rather than relying on just the particle size. This study has addressed some concerns surrounding particulate drug delivery, demonstrating safe delivery of CsA whilst achieving augmented serum concentrations.

**Key Words:** biodegradable, cyclosporine, microparticles, nanoparticles, nanotoxicology, oral, polyester, toxicity.

## **Introduction**

Cyclosporine (CsA) is a potent immunosuppressive drug widely used for prevention of graft rejection following transplantation (Barbier *et al.*, 2013; Flechner *et al.*, 2013). In addition, CsA alone or in combination with other immunosuppressant drugs is found to have benefit in diverse clinical conditions such as rheumatoid arthritis, psoriasis, traumatic brain injury or glucocorticoid-dependent idiopathic nephrotic syndrome (Gremese *et al.*, 2004; Sullivan *et al.*, 2011; Colombo *et al.*, 2013; Iyengar *et al.*, 2013; Mrowietz 2013). The side effects, however, of CsA such as nephrotoxicity and hypertension often outweigh its benefit.

Beyond immunosuppression, CsA also appears to have activity in metabolic disorders. Recently, preclinical reports suggest that CsA can be beneficial in managing obesity in diet-induced obese mice (Jiang *et al.*, 2013); conversely clinical data in transplant patients suggest CsA can result in hyperlipidemia with marked increases in total and LDL cholesterol as well as triglycerides (reviewed in Wissing and Pipeleers 2013). CsA proved better than other calcineurin inhibitors, e.g. tacrolimus, in avoiding post-transplant diabetes mellitus due to poor allograft outcomes in renal transplants (Choi and Kwon 2013), that could be further improved with better pharmaceutical delivery of CsA (Italia *et al.*, 2006 & 2007; Ankola *et al.*, 2010 & 2011; Park *et al.*, 2013).

Whichever indication, it is widely reported that conventional CsA microemulsion formulations exhibit variable bioavailability with consequently differing biological activity (Ready 2004; Qazi *et al.*, 2006). Moreover, titrating CsA dose whilst avoiding drug interactions within the requisite polypharmacy regimens selected to treat transplant patients with infection can be complicated (Frassetto *et al.*, 2014). Research efforts are therefore aimed at reformulating CsA to maximize benefits by addressing not only the variable

bioavailability but toxicity problems (Azzi *et al.*, 2010; Italia *et al.*, 2007; Ankola *et al.*, 2010 & 2011; Kadam *et al.*, 2012).

There has been much debate in the literature surrounding the question of particle size cut-off of non-conventional (nanoparticle) formulations for drug uptake following oral dosing; recent studies suggest smaller (0.5  $\mu\text{m}$ ) vehicles lead to greater up-take compared to larger (5  $\mu\text{m}$ ) (Reineke *et al.*, 2013). Indeed the vast majority of reports on non-conventional CsA preparations has focussed on drug bioavailability enhancement *in vivo* (Italia *et al.*, 2007; Ankola *et al.*, 2010 & 2011; Park *et al.*, 2013) without consideration of possible influence on organ or system physiology and function (Lucas *et al.*, 2005; Moss and Siccardi 2014). Though the pharmacokinetic/pharmacodynamic properties of CsA and its maintenance within its narrow therapeutic window is important, understanding the pathophysiological effect of nano-carriers themselves has become equally or more important considering the growing concerns of nanotoxicology (Devadasu *et al.*, 2013; Lamprou *et al.*, 2013). Very recently, we demonstrated for the first time the utility of “atomic force microscopy (AFM) for visualizing label-free CsA encapsulated polylactide-co-glycolide (PLGA) nanoparticle distribution within the tissues following intravenous or *peroral* administration (Lamprou *et al.*, 2013).

Since we have obtained the first proof of drug encapsulated nanoparticles being absorbed intact across the intestine (Lamprou *et al.*, 2013), we designed the present study to assess any haematological or biochemical changes consequent to encapsulation of CsA in PLGA nano- (<300nm) and micro-particles (>1  $\mu\text{m}$ ) with a view to establishing the safety profile of long-term dosing with such particulates as well as serum drug concentration as a correlative indicator of bioavailability.

## **Materials and methods**

### ***Materials***

PLGA 50:50 (Resomer<sup>®</sup> RG 503 H; intrinsic viscosity 0.32-0.44 dL/g) was purchased from Boehringer Ingelheim (Ingelheim, Germany, now Evonik Industries). CsA was purchased from Flurochem Ltd., (Hadfield, Derbyshire, UK). Polyvinyl alcohol (PVA) (Mol. Wt. 30,000-70,000) was purchased from Sigma-Aldrich (Irvine, UK). Organic solvents and mobile phases for HPLC such as ethyl acetate and acetone (analytical reagent grade), glacial acetic acid, methanol (MeOH; HPLC grade) and acetonitrile (ACN; HPLC grade) were purchased from Fisher Scientific (Loughborough, UK). Ultrapure Milli-Q<sup>®</sup> water (in house supply) was used for all experiments.

### ***Preparation of CsA encapsulated PLGA nano- and micro-particles***

PLGA (500 mg) and CsA (75 mg) were dissolved in ethyl acetate (25 mL) with stirring at 1000 rpm over a 2 h period. Drug containing polymer solution was then added drop-wise to 50 mL 1% (w/v) PVA solution. The resulting primary emulsion (o/w) was stirred over 1 h at 1000 rpm. For reduction of droplet size, primary emulsions were homogenised (Polytron PT 4000, Kinematica, Switzerland) for 30 min at 15,600 or 8,600 rpm for nanoparticle or microparticle preparation, respectively. The emulsion was transferred to 250 mL of water and stirred overnight to facilitate diffusion of organic solvent and evaporation. The CsA encapsulated particle suspension was pelleted by centrifugation at 14,000g for 30 min. The entrapment efficiency was measured by previously developed HPLC method (Italia *et al.*, 2007; Ankola *et al.*, 2010 & 2011). Blank nano- and micro-particles were prepared as described without addition of CsA.

### ***Freeze drying***

The particle pellets were re-suspended in 30 mL water to which 5% (w/v) sucrose was added and vortexed until dissolution. The particle suspension (4 mL) was placed in 5mL glass vials and frozen at  $-80^{\circ}\text{C}$  overnight. Freeze drying process was carried out using a bench top freeze drier system (MicroModulyo<sup>®</sup>230, Thermo Electron Corporation, Ohio, USA) operating at  $-50^{\circ}\text{C}$  under high vacuum (0.003 mBar) for 48 h to ensure a dried product. The freeze dried nCsA and mCsA were re-suspended in 4 mL of distilled water for further characterization and *in vivo* study.

### ***In vivo study design***

The repeated dose oral toxicity study was carried out at the Biological Procedures Unit (BPU), University of Strathclyde. All procedures on animals were performed according to project licence (PLL 60/3920) under the Animals (Scientific Procedures) Act 1986 (UK).

### ***Animals, housing and feeding conditions***

Eight week old Sprague Dawley (SD) male rats were housed in polypropylene cages under standard conditions of temperature ( $24\pm 1^{\circ}\text{C}$ ) and relative humidity ( $55\pm 10\%$ ), in 12 h light and 12 h dark cycles throughout the experiment. Animals had free access to food and water.

### ***Dosing of formulations to animals***

Forty rats were randomly divided into ten groups as shown in Table 1. The rats received *peroral* dosing of approximately 1mL of each formulation by oral gavage daily for 28 days. The four control groups were dosed similarly with equal volume of vehicle only, blank nano- or micro-particles, or Neoral<sup>®</sup> for 28 days.

### ***General observation, body weight and food intake***

Throughout the study period, observations were made daily for general health of the animals and signs of acute toxicity e.g. locomotor activity, prostration, rapid shallow breathing, pallor, profound dullness. Prior to dosing, the rats' weights were recorded at a fixed time using a calibrated balance. To monitor food consumption, a weighed amount of standard pellet diet was placed in the food tray of the cage. Any leftover pellets were weighed and replaced with fresh pellets each day. Feeding time was fixed throughout the study.

#### ***Blood sampling and measurement of CsA concentration in serum***

At the end of the 28 day study period, the animals were fasted overnight and euthanized the following day by CO<sub>2</sub> asphyxiation. Blood samples were collected by cardiac puncture in heparinized syringes and transferred into EDTA tubes or plasma/serum separation tubes for blood analysis and CsA concentrations respectively. The concentrations of CsA in serum after 28 days study period were determined using commercially available Cyclosporine EIA Kits (Immunotech, Czech Republic) (Ankola *et al.*, 2010 & 2011).

#### ***Estimation of blood urea nitrogen (BUN) and plasma creatinine (PC) levels***

Plasma was separated from whole blood by centrifugation at 3000 rpm for 15 min at 5°C. The BUN and PC were determined calorimetrically using commercially available kits (Cayman Chemicals Ltd.).

#### ***Oxidative stress, inflammation and antioxidant enzyme levels***

The degree of oxidative stress/inflammation caused by the treatment was assessed by measuring fibrinogen (FBG), C-Reactive Protein (CRP), ceruloplasmin (CP) levels and monitoring their effect on the endogenous antioxidant enzyme levels e.g. superoxide

dismutase (SOD) and catalases (CAT). Protein quantification was carried out using commercially available ELISA kits (Cayman Chemicals Ltd.).

### ***Haematological parameters***

A fully automated haematology analyser (Hemavet 950FS, Drew Scientific Inc, USA) was used to measure the following parameters in blood: haemoglobin (Hb), red blood corpuscles count (RBC), white blood corpuscles count (WBC), and differential leukocyte count (DLC). Blood films were analysed after standard haematoxylin and eosin staining.

### ***Flow cytometry for CD90 expression***

Peripheral blood mononuclear cells were stained with phycoerythrin (PE) conjugated mouse anti-rat CD90 (Thy-1) (BD Pharmingen; Clone OX-7) for 15 minutes on ice and unbound antibody washed off in phosphate buffered saline (PBS) by centrifugation at 192g for 5 minutes. The pellet was re-suspended and after red cell lysis with EasyLyse™ (Alere Ltd., Stockport, UK), total mononuclear cells were analysed by flow cytometry on a BD Bioscience FACS Canto II with Diva software with an initial gate on live cells based on the forward (FSC) and side scatter properties (SSC) of viable cells. Doublets were then excluded based on the ratio of the area versus width of the pulse generated by single cells. Within the viable, single cell population, cells expressing CD90 on their cell surface were identified. Finally to determine the percentage of haemopoietic stem/progenitor cells (HSPC) specifically, the cells with high CD90 expression were back gated into FSC/SSC to identify a subpopulation of cells which are likely true HSPC based on their distinct physical properties of size and granularity.

### ***Urine analysis***

Urine samples were collected at the end of the study period and analysed for appearance, pH, protein and blood traces.

### ***Histopathological evaluation***

Heart, kidney, liver, testis, spleen, lung, brain and small intestine were isolated immediately on sacrifice. The tissues were washed with ice-cold saline and weighed before fixing in 10% neutral buffered formalin solution. The tissues were then embedded in paraffin blocks and used for histopathological examination. Five micrometre thick sections were cut, deparaffinised, hydrated and stained with haematoxylin and eosin. The histological sections were examined under microscope (Leitz, Wetzlar, Germany) for any morphological changes. In case of kidney, the diameters of the glomerular capillary tuft (CD) and Bowman's capsule (BD) were measured with the help of an internal micrometer and the CD/BD ratio was calculated as an index of glomerular collapse (Origlia *et al.*, 2006).

### ***Statistical Analysis***

The data are presented as mean  $\pm$  standard deviation. ANOVA models were used to compare the treatment groups. Additionally, the least squares means were obtained for all groups under considerations and multiple comparison tests of the least squares mean differences were performed following the overall test for the treatment effect. The Tukey method was used to adjust the *p* values for the multiple comparisons. A *p* value of less than 0.05 was considered to be statistically significant.

## **Results**

### ***Evaluation of nCsA and mCsA***

The freeze dried nCsA, mCsA or blank (drug void) particles were easily reconstituted in water by simple shaking. The particles had an average size of  $280.2 \pm 25$  nm and  $1.21 \pm 0.22$   $\mu$ m with polydispersity index of 0.231 and 0.301 for nCsA and mCsA, respectively (SI Figure 1a,b). The entrapment efficiency of freshly prepared particulates was found to be about  $55 \pm 6\%$  for both nCsA and mCsA which did not change significantly after freeze drying. To further characterise the microparticle size distribution, mCsA were subjected to differential centrifugation at 2000, 5000 and 6500 g (SI Figure 2a-c). The mCsA preparations were found to contain a large population of nanoparticles.

### ***General observation, body weight of animals and food intake***

Thorough physical examinations were carried out every day during the study period for signs of morbidity. No unusual variations in locomotor activity or signs of intoxication or ataxia were observed during the 28 day study period. Hair loss was noted in high dose nCsA and mCsA (45mg/kg) treated rats. Daily eye examinations did not show any dose related abnormalities in any treated group. During the treatment period, significant loss of body weight (Figure 1a,b) with reduced food consumption (Figure 2a,b) were observed in high dose nCsA and mCsA treated animals during the first two week period; these animals never reached normal weight. The weight loss was not statistically significant between the nCsA and mCsA (45mg/kg) treated groups. The control animals gained weight as did rats treated with 15 or 30 mg/kg CsA.

### ***CsA concentration in serum***

CsA concentrations in serum were measured 24 h after the last administered dose (Figure 3). A dose dependent increase of CsA concentration was observed across all formulations, though nCsA and mCsA did not differ in their levels with the exception of 15 mg/kg group.

### ***BUN and PC analysis***

Significant elevation of BUN was observed across all treatment groups compared to control; a dose response was evident (Figure 4a). However, PC levels were not significantly different across all treatment groups and were similar to controls (Figure 4b).

### ***Oxidative stress, inflammation and antioxidant enzyme levels***

Data was gathered on markers of oxidative stress/inflammation (CRP, FBG and CP) and endogenous antioxidant enzyme levels (CAT and SOD). All the markers, except CP, did not change across all the treatment groups compared to control (Table 2). CP was increased significantly on treatment with high dose nCsA or mCsA (45mg/kg) while the other groups including Neoral<sup>®</sup> remained unchanged compared to control. The increase in CP was found to be independent of inflammatory markers or endogenous antioxidant levels.

### ***Haematological parameters***

Although there was a general, non-statistically significant trend towards lower total WBC in all CsA treatment groups with the exception of low dose nCsA or mCsA, with respect to untreated or blank particle controls (SI Table 1), statistically significant differences in (increased) neutrophil and (decreased) lymphocyte proportions (as percent contribution) were noted when a differential count was performed (Table 3). Erythrocyte numbers and parameters e.g. haemoglobin, were found to be unchanged across all groups when compared to control (SI Table 2). A significant increase in platelet count was only observed in Neoral<sup>®</sup>

treated rats (Table 4) with respect to control; while all other treatments were significantly lower than Neoral<sup>®</sup>. No gross abnormalities or differences in morphology were observed on blood film examination.

#### ***Haematopoietic stem/progenitor (CD90<sup>+</sup>) cells in peripheral blood***

The effect of the particle formulations on more primitive haematopoietic cells were next examined to see whether the treatments had had subtle effects on cells found earlier in the developmental hierarchy i.e. on haematopoietic stem/progenitor (CD90<sup>+</sup>) cells (Figure 5). The Neoral<sup>®</sup> did not significantly restrict homeostatic CD90<sup>+</sup> cell appearance in the peripheral blood (0.07±0.01 versus 0.09±0.05%, Neoral<sup>®</sup> versus untreated control), neither did nCsA/mCsA nor blank (drug void) particles (SI Figure 3).

#### ***Urine analysis***

Urine samples from control and treated rats were collected over time. The urine pH of all the groups was alkaline and traces of protein were found, but not blood.

#### ***Histopathological examination***

Insignificant variations were observed in relative organ weights (SI Table 3), when comparison was made between test and control groups. Histopathological assessment confirmed the integrity of tissue morphology and architecture of various organs after long-term daily dosing over 28 days (Figure 6 and SI Figure 4&5), including target organs for CsA toxicity (e.g. kidney); there were no major areas of confluent tissue necrosis or inflammation. However, subtle sub-lethal cell changes could not be ruled out on examination of these tissue sections.

## Discussion and conclusion

The nano- and micro-particles were prepared in large scale in sufficient quantities for our sub-acute toxicity study. The particles were successfully freeze dried resulting in easily reconstituted cake with comparable attributes of fresh preparations (particle size and entrapment efficiencies). A dose dependent increase in serum CsA levels was noted across the doses and mCsA generally showed much higher levels (almost comparable to nCsA) than anticipated owing to the presence of a significant proportion of nanoparticles in mCsA preparations (Figure 3; SI Figure 2a-c). Although there is no agreement on the cut-off for particle size for optimal *peroral* absorption, it is a relative phenomenon whereby smaller is generally believed to be better (Mittal *et al.*, 2007; Reineke *et al.*, 2013).

The animals on high dose nCsA or mCsA (45 mg/kg/day) showed a significant reduction in the body weight during the first two weeks of the treatment that was proportional to food intake (Figures 1-2). The weight loss correlated with high CsA serum concentration (Figure 3). Notably an equivalent dose of blank (drug void) particles did not cause loss of body weight (Figure 1a,b) and all the other groups showed weight gain or maintained their weight over the duration of the study. This data is in agreement with literature where it is reported that very high doses of CsA (50 mg/kg/day) result in short periods of anorexia and reduced food intake; however when food intake becomes normal, and as we observed in our study, the animals do not regain normal weight (Farthing *et al.*, 1981). A recent report demonstrated that dosing with 10 mg/kg/day CsA for 3 weeks attenuated weight gain of mice fed a high fat diet over the preceding 8 weeks while non-obese mouse weight remained constant (Jiang *et al.*, 2013). The authors went on to claim that CsA could stimulate leptin signal transduction in the hypothalamus of obese mice which was responsible for the weight loss.

The serum concentration measurements made at the end of this current study demonstrated a dose response as the CsA levels in the blood increased proportionally with dose administered in agreement with our previous report (Ankola *et al.*, 2011). Although statistically significant differences were seen between nCsA and mCsA, the numerical differences in absolute values between these two groups were not large especially between groups in the high dose cohort (Figure 3). It has been reported in the literature that particle sizes < 2  $\mu\text{m}$  are absorbed through the lymphatic system in lower numbers than particles up to 500 nm in size (Mathiowitz *et al.*, 1997, Desai *et al.*, 1996; Reineke *et al.*, 2013). The wide size distribution range in our mCsA preparation and comparable serum CsA concentration with either mCsA or nCsA, prompted us to do differential centrifugation to apportion the contribution made of nanoparticles to the mCsA preparation (SI Figure 2a-c). There were significant numbers of nanoparticles within the microparticle formulation that could have led to the comparable though statistically significant concentration profiles of CsA administered as nano- or micro-particles (Figure 3). The serum CsA concentrations reflected the BUN but not PC levels; however, BUN levels across all doses were not too different from Neoral<sup>®</sup> 15 mg dose, while significantly different from untreated (Figure 4). We believe this gives comfort to the possibility of dose escalation offered when administering nano- or micro-particulate CsA, which is in agreement with previous reports (Farthing *et al.*, 1981; Ankola *et al.*, 2011). The terminal urine analysis showed no abnormalities, all the samples were alkaline pH with some traces of protein which was normal (Hall *et al.*, 2008).

Foreign bodies such as nano-delivery vehicles may cause mild or severe hematologic toxicity that can be life-threatening, clinically all the more pertinent in the organ transplant setting when patients are treated with CsA. In our *in vivo* study, leukocyte and erythrocyte counts for blank (drug void) particle treated groups were similar to controls suggesting mitigation of

inherent carrier mediated toxicity because of their size (Table 3 and SI Tables 1-2). A decreasing trend in absolute lymphocyte numbers was observed across the particle treated groups comparable to the lymphocytopenia seen with Neoral<sup>®</sup>, which is a predicted CsA dependent phenomenon. Indeed, the reduction in the proportion of lymphocytes in the total white cell count did gain statistical significance with CsA treatment by differential counts. CsA is known to increase platelet activation/aggregation increasing the risk of thromboembolic complications and hypertension (Reis *et al.*, 2000). Significantly higher platelet counts were observed in Neoral<sup>®</sup> treated rats but not with nCsA or mCsA at any dose; the mean platelet volume (MPV) remained the same across all CsA treatments. No abnormal variations were observed in erythrocyte counts or red cell parameters across all doses which, together with the platelet result, suggested the particulate carrier could deliver higher doses of CsA without increasing off-target toxicity. The antioxidant enzymes and pro-inflammatory cytokines measured did not alter from control with particulate CsA delivery again indicating the safety profile of the formulations. That said, a recent publication (Lv *et al.*, 2013) has linked CP levels to acute Graft versus Host Disease in the allogeneic stem cell transplant setting, a condition associated with immune reactions and inflammatory cytokine storm. The increase in plasma CP seen in our study may be associated with the higher plasma levels of CsA achieved with highest dose particulate drug delivery as Neoral<sup>®</sup> or low dose and blank particles did not precipitate a significant increase in the protein; again reiterating the safety of particles.

Therefore it was interesting to further evaluate the effect of CsA encapsulated particulates on the immune system given not only that the drug is used as an immunosuppressant but importantly, that nano-vehicles innately travel, by virtue of their size, to the organs of the mononuclear phagocyte system such as the spleen or bone marrow. This is not often

considered. Whilst the steady state appearance of rat bone marrow haematopoietic stem/progenitor (CD90<sup>+</sup>) cells in the peripheral blood seemed to be negatively modulated by high dose mCsA, there was no clear relationship to particle size or payload concentration as neither blank (drug void) nano-/micro-particles nor low dose mCsA (15mg/kg) inhibited CD90<sup>+</sup> cell mobilisation. Indeed the latter had significantly higher number of CD90<sup>+</sup> cells detectable in the blood with respect to high dose mCsA (45mg/kg) but notably this increase in number was not significantly different from untreated control. Whether mCsA affected CD90<sup>+</sup> cell turnover and self-renewal could be determined in a colony forming cell assay. We hypothesise however, that our haematological findings reflect the natural range of distribution of CD90<sup>+</sup> cells in the blood and is not linked to pathology or toxicity as the apparent inhibition seen with mCsA was statistically different from the Neoral<sup>®</sup> but not untreated control group which exhibited the widest variation around its mean.

No significant changes observed in percent relative organ weight supported the presumption of normal functioning of target organs, phospholipid metabolism, and enzyme secretion. Indeed post mortem histological examination of kidneys from all the treatment groups revealed normal renal cortex, glomerular tubules and renal papilla comparable to control. Hepatocytes and portal tracts in liver sections seemed normal across all groups suggesting lack of inflammatory infiltration during the study period. All the cardiac tissue sections assessed showed normal myocardium with no pigment deposition. Normal alveolar architecture and cellular infiltrations was observed in lung sections from both control and treated groups. The testis sections of control and all treated groups showed active spermatogenesis and maturation and normal shape and size of seminiferous tubules. Splenic architecture with normal red and white pulp was observed in control groups similar to the treatment groups (Figure 6; SI Figures 4-5).

In summary, our data indicates that daily dosing with CsA encapsulated PLGA nano- or micro-particles over 28 days did not cause any deleterious general health effects attributable to the carrier. Although high dose nCsA/mCsA (45mg/kg) did cause significant loss of body weight, this was in keeping with reported toxicity of Neoral<sup>®</sup>. No significant haematological or biochemical changes were recorded on long-term dosing with either nCsA/mCsA. Despite the presence of nanoparticles in the microparticle formulation, the nCsA product consistently resulted in higher serum drug concentration thus the particulates allow an increase in dose range without accrual of toxicities.

### **Conflict of interest**

None of the authors have any conflicts of interest to disclose relating to this work.

### **Acknowledgement**

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### **Abbreviations**

Cyclosporine (CsA); Poly(lactide-co-glycolide) [PLGA]; CsA encapsulated nanoparticles (nCsA); CsA encapsulated microparticles (mCsA); Sprague Dawley (SD); Ceruloplasmin (CP); Low-density lipoprotein (LDL); Blood urea nitrogen (BUN); Plasma creatinine (PC); Fibrinogen (FBG); C-Reactive Protein (CRP); Superoxide dismutase (SOD); Catalases (CAT); Haemoglobin (Hb); Red blood corpuscles count (RBC); White blood corpuscles count (WBC); Differential leukocyte count (DLC); Phycoerythrin (PE); Haemopoietic stem/progenitor cells (HSPC); Forward (FSC) and side scatter properties (SSC); Glomerular capillary tuft (CD); Bowman's capsule (BD).

### **Author Contributions**

MNVRK and JHG planned the experiments; VVP, JGH; RS; OKA conducted the experiments; TCD and JHG performed statistical analysis; MNVRK; JHG; EMA; RS; OKA and VVP wrote the manuscript.

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## **Figure legends**

### **Figure 1**

Percentage body weight of rats on repeated dosing with (a) nCsA or (b) mCsA at different doses.

### **Figure 2**

Food consumption of rats on long-term dosing with (a) nCsA and (b) mCsA at different doses.

### **Figure 3**

Serum concentration of CsA at the end of the study.

### **Figure 4**

Nephrotoxic indicators (i) BUN and (ii) PC levels of rats at the end of the study.

### **Figure 5**

CD90<sup>+</sup> cell detection in peripheral blood at the end of the study.

A-D. After red cell lysis, total mononuclear cells were analysed by flow cytometry for live cells based on forward (FSC) and side scatter properties (SSC) of viable cells [Panel A]. Doublets were then excluded (based on the ratio of the area versus width of the pulse generated by single cells) [Panel B]. Within the single cell, viable subpopulation, CD90 cell surface expression was determined [Panel C]. To determine the percentage of haemopoietic stem/progenitor cells (HSPC) specifically, the cells with high CD90 expression were back gated into FSC/SSC [Panel D]; HSPC are boxed.

E-F Representative overlaid histograms for CD90 expression and corresponding back-gated CD90<sup>high</sup> events into FSC/SSC dot plots are shown for animals treated with Neoral<sup>®</sup> (15mg/kg) (red); high dose mCsA (45mg/kg) (green); and untreated control (blue).

**Figure 6**

Renal and hepatic histology at the end of the study.

**Table Legends****Table 1**

Randomisation of rats for oral toxicity study.

**Table 2**

Plasma markers indicative of oxidative stress and inflammation markers, and antioxidant enzyme levels at the end of the study.

**Table 3**

Differential white cell counts at the end of the study.

**Table 4**

Platelet count (PTL) and mean platelet volume (MPV) at the end of the study.

## **Supplemental Information (SI)**

**SI Figure 1.** Particle size distribution of (a) nCsA and (b) mCsA.

**SI Figure 2.** Nanoparticles in the supernatants of mCsA preparation after differential centrifugation at (a) 2000, (b) 5000 and (c) 6500 g.

**SI Figure 3.** Percentage of CD90<sup>+</sup> cells in rat peripheral blood at the end of the study. Results are mean  $\pm$  standard deviation.

**SI Figure 4.** Haematoxylin and eosin (H&E) stained histology sections of heart and lung at the end of the study.

**SI Figure 5.** Haematoxylin and eosin (H&E) stained histology sections of spleen and testis at the end of the study.

**SI Table 1** Absolute white cell counts at the end of the study.

**SI Table 2** Erythrocyte numbers at the end of the study.

**SI Table 3** Percent relative organ weight at the end of the study.