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Drug- not carrier-dependent haematological and biochemical changes in a repeated dose study of cyclosporine encapsulated polyester nano- and micro-particles: Size does not matter

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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® 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.

Abbreviations: CsA, cyclosporine; PLGA, poly(lactide-*co*-glycolide); nCsA, CsA encapsulated nanoparticles; mCsA, CsA encapsulated microparticles; SD, Sprague Dawley; CPC, eruloplasmin; LDL, low-density lipoprotein; BUN, blood urea nitrogen; PC, plasma creatinine; FBG, fibrinogen; CRP, C-reactive protein; SOD, superoxide dismutase; CAT, catalases; Hb, haemoglobin; RBC, red blood corpuscles count; WBC, white blood corpuscles count; DLC, differential leukocyte count; PE, phycoerythrin; HSPC, haemopoietic stem/progenitor cells; FSC, forward scatter properties; SSC, side scatter properties; CD, glomerular capillary tuft; BD, Bowman's capsule.

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1. 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 and Ferraccioli, 2004; Sullivan et al., 2011; Colombo et al., 2013; Iyengar, 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 μm) vehicles lead to greater up-take compared to larger (5 μm) (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 (Lukas 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- (<300 nm) and micro-particles (>1 μm) 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.

2. Materials and methods

2.1. Materials

PLGA 50:50 (Resomer® RG 503H; 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® water (in house supply) was used for all experiments.

2.2. 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 8600 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 14,000 × *g* 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.

2.3. 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 5 mL glass vials and frozen at −80 °C overnight. Freeze drying process was carried out using a bench top freeze drier system (MicroModulyo®230, Thermo Electron Corporation, Ohio, USA) operating at −50 °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.

2.4. 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).

2.5. Animals, housing and feeding conditions

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

2.6. 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 1 mL of