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Lack of Interaction of Lopinavir Solid Drug Nanoparticles with Cells of the Immune System.

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ABSTRACT

Aims - We previously demonstrated that solid drug nanoparticles (SDNs) lopinavir dispersed into aqueous media display favourable pharmacokinetics.

Methods - The impact of lopinavir SDNs on the function and phenotype of primary human T cells and macrophages (primary sites of HIV replication), was investigated.

Results - Lopinavir significantly increased IL-1β (9-fold higher than untreated cells; P=0.045) and TNFα (6-fold higher than untreated cells; P=0.018) secretion from monocyte-derived macrophages, whereas lopinavir SDNs did not elicit these responses at comparable drug concentrations. Lopinavir SDNs were demonstrated to be immunologically inert to human T cells and monocyte-derived macrophages.

Conclusion – The lopinavir SDN was demonstrated to exhibit comparable, or favourable behaviour compared to a lopinavir aqueous solution in the employed biocompatibility assessments.

INTRODUCTION

Antiretroviral therapy has significantly improved the morbidity and mortality associated with HIV infection, but an estimated 20-30% of patients initiating therapy still discontinue treatment within two years, the majority being toxicity related but also a significant number due to virological failure [1]. Toxicity and drug failure are costly as toxicity results in significant morbidity and subsequent regimens are associated with higher pill burden and a higher expensive to healthcare providers. The introduction of HIV protease inhibitors (PIs) in the 1990s, significantly reduced morbidity and mortality and prolonged the lifespan of patients [2]. However, there are a number of side effects associated with these drugs such as; dyslipidaemia, insulin resistance, lipodystrophy and hepatotoxicity [2-5]. Although the underlying mechanisms of these side effects is yet to be fully elucidated, a number of possibilities have been demonstrated such as; induction of IL-6 and TNFα secretion [6], activation of the unfolded protein response [7], impairment of protein synthesis and activation of AMP-activated protein kinase (AMPK) [8]. PIs also exhibit incomplete absorption and rapid systemic clearance, resulting in a requirement for pharmacoenhancement by co-administration of ritonavir or cobicistat as a pharmacokinetic "booster". Despite pharmacoenhancement, pharmacokinetics are highly variable within populations and the class has attracted interest by many investigators exploring nanotechnology-enabled drug delivery [9-11].

Many nanomaterials platforms are being investigated for their potential to augment drug delivery. Unlike nanocarrier systems (e.g. lipid-based, polymer-based or inorganic materials), solid drug nanoparticles (SDNs) rely upon advanced formulation tools to generate nanoparticles that are composed of the drug itself. To date, the most commercially successful SDN manufacturing platform has been provided by nanomilling technologies [12]. The overwhelming majority of SDN formulations have been developed for oral dosing and are thought to release drug prior to absorption such that particulates do not enter the systemic circulation. However, recent work has illustrated that intact particles are able to traverse intestinal monolayers [13]. Moreover, recent

success of parenterally administered SDNs as long-acting depot formulations [14, 15], along with recent work exploring intravenous delivery of SDNs [16], has resulted in the need for a more robust understanding of their safety. Therefore, the current work focused on assessing the putative immunological consequences of direct SDN exposure.

Lopinavir SDNs were produced using a previously reported emulsion-templated freeze-drying (ETFD) technique [17] and were shown to exhibit similar pharmacokinetics to a conventional preclinical preparation of lopinavir in a rodent model [18]. In HIV therapy, delivery of antiretrovirals to T cells and macrophages is vital since these are the primary sites of HIV replication *in vivo* [19]. However, a prerequisite for this as a valid strategy depends upon the absence of unwanted immunogenic or immunosuppressive effects such as those described for other nanoparticle materials [20-22]. The purpose of this work was to assess the impact of lopinavir aqueous solution and lopinavir SDNs on the function of human T cells and macrophages *ex vivo*.

MATERIALS AND METHODS

Preparation and physical analysis of lopinavir SDNs

Samples are prepared using a 70 $mgmL^{-1}$ stock solution of Lopinavir (LPV) in chloroform, a 22.5 $mgmL^{-1}$ of poly(vinyl alcohol) (MW = 9500 g/mol, PVA) in water and a 22.5 $mgmL^{-1}$ stock solution of α-tocopherol polyethylene glycol succinate (TPGS) in water. Stock solutions are added in the following proportion; 100 µl LPV; 90 µl PVA, 45 µl TPGS and 265 µl of water, therefore solid mass is 10 mg with the ratio; 70% LPV, 20% PVA and 10% TPGS in a 1:4 oil to water (O/W) mix. The mixtures are the emulsified using a Covaris S2x for 30 seconds with a duty cycle of 20, an intensity of 10 and 500 cycles/burst in frequency sweeping mode. After which, the samples were immediately cryogenically frozen and lyophilized using a Virtis benchtop K freeze-drier for 48hrs to produce off white dry porous monolith products. Samples were then sealed in individual vials until analysis. The amorphous nature of the solid monoliths were confirmed via Powder x-ray diffraction (PXRD) using a Panalytical X'Pert PRO MPD instrument with X'Pert Operator Interface (version 1.0b) software. The instrument was equipped with a high throughput screening (HTS) XYZ stage, X-ray focusing mirror and PIXcel detector, using Ni-filtered Cu K α radiation. Data were measured over the range 4–50 ° in ≈ 0.013 ° steps over 60 min in transmission mode with the solid monolith samples held on thin Mylar film in aluminum well plates. LPV SDN samples were shown to be amorphous with no crystallinity present. In order to determine the dispersed SDN particle characteristics, samples were dispersed by addition of 3.5 mL of water (therefore 1 $mgmL^{-1}$ with respect to LPV content). Z-average diameter (D_z), Zeta potential (ζ) and polydispersity index (PdI) were determined by dynamic light scattering (DLS) at a temperature of 25˚C using a Malvern Zetasizer Nano ZS equipped with a 4 mW He-Ne, 633 nm laser and using plastic disposable cuvettes. Malvern Zetasizer software version 6.20 was used for data analysis. ζ measurements were also carried out at 1 $mgmL^{-1}$, 25˚C, and an initial pH of 6.5, using disposable capillary zeta cells. D_z, ζ and polydispersity measurements were obtained as an average of

3 individual measurements and were obtained using the instrument's automatic optimisation settings.

Detection of endotoxin using ELISA-based assays

Endotoxin was measured in lopinavir aqueous stock solutions (0.5% DMSO) and lopinavir SDN preparations using the ENDOlisa kit (Cambridge Biosciences, UK). Briefly, reagents were resuspended as instructed by the manufacturer and a serial dilution of prepared lipopolysaccharide (LPS, Invivogen, UK) was made, ranging from 0.005-500 EU/mL. Samples were diluted 1:5 in endotoxin free water and a spiked sample was prepared as a control for interference with the assay by the drug or nanoparticles. Samples were added to wells, followed by binding buffer, and plates were then protected from light and incubated at 37°C for 90 minutes with continual shaking. Wells were then washed twice with wash buffer prior to addition of assay reagent. Immediately following addition of assay reagent a zero-time point was recorded on the plate reader. Plates were then incubated at 37°C for a further 90 minutes and read again. Data were corrected for the zero-time point and a 4-point logistic curve was used to interpolate unknown concentrations.

Isolation of peripheral blood mononuclear cells from peripheral blood samples

Healthy volunteer blood was collected via venepuncture under ethics approval from the University Physical Interventions sub-committee (Reference RETH000563). Informed consent was given and accepted by the healthy volunteers for use of whole blood in subsequent assays. Peripheral Blood Mononuclear Cells (PBMC) were isolated as described previously [22]. Blood was layered over Ficoll and centrifuged at 800xg for 30 minutes (4°C). The PBMC interface was then transferred to a fresh universal tube prior to three washes in phosphate buffered saline solution (PBS). PBMC were then counted using a Nucleocounter and cell densities adjusted to the required number for subsequent experiments, as described in relevant sections below.

Lymphocyte activation via CD2/CD3/CD28 conjugated MACSiBead particles

MACSiBead particles (Miltenyi Biotec, UK) were prepared following the manufacturers guidelines. MACSiBead particles (2.5 x 10^6) were added to a sterile universal tube with complete culture media (RPMI-1640, 10% FCS). MACSiBead particles were then centrifuged (450xg) for 5 minutes, the supernatant fraction was removed and the MACSiBeads were resuspended in complete culture media (RPMI-1640, 10% FCS). PBMC densities were adjusted to 5 x 10^6 cells per mL. PBMC and MACSiBeads preparations were then combined and incubated in a humidified incubator, at 37°C for 24 hours. In addition to untreated controls and MACSiBead positive controls, PBMC were treated with lopinavir aqueous solution (10µM) or lopinavir SDNs (10µM) to assess potential for lymphocyte activation. Additionally, PBMC were co-cultured with MACSiBead particles and lopinavir aqueous solution (10µM) or lopinavir SDNs (10µM) to assess potential inhibition or enhancement of activation via CD2/CD3/CD28. Finally, PBMC were cultured with only lopinavir (10µM) or lopinavir SDNs (10µM) for 24 hours prior to activation with MACSiBead particles, to assess direct effects on the system.

Preparation and activation of primary monocyte-derived macrophage (MDM) from healthy volunteers.

CD14+ positive cells were isolated from crude PBMC preparations via magnetic bead based cell separation (MACS beads, Miltenyi Biotec, UK). CD14+ cells were then cultured for 10 days in Iscove's Modified Dulbecco's medium (Sigma, UK) containing human serum (20%) and Macrophage colony stimulating factor (M-CSF, 10ng/mL, Miltenyi Biotec, UK). Following differentiation into MDM, cells were incubated in the presence of lipopolysaccharide (LPS, 1µg/mL), conventional lopinavir (10µM) or lopinavir SDNs (10µM) for 24 hours. Cell culture supernatant fractions were then harvested for cytokine analysis.

Measurement of cytokine concentrations in activated PBMC and MDM cultures

Aliquots of culture supernatant fractions (100µL) were taken for analysis of cytokine secretion following 24-hour incubation. Cytokine concentrations were measured via multiplex cytokine assays conducted using the Bioplex 200 system (Biorad, UK). IL-2, IL-10 and IFNγ were measured for PBMC stimulation and IL-1β, IL-6, IL-8 and TNFα were measured for MDM stimulation. Briefly, coupled beads (50µL) were added to every well on a 96 well plate. Plates were prepared per manufacturer's instructions. Cell culture supernatants were added to the plate alongside multiplexed standard curves for the measured cytokines. Incubations were carried out at room temperature, on a plate shaker. Detection antibodies were added for 30 minutes following three washes. Plates were again washed three times prior to the addition of streptavadin-PE antibodies (50µL) and incubation on a plate shaker for 10 minutes. Plates were then washed for a final three times and assay buffer (125µL) added to each well. Plates were then analysed on a Bioplex 200 analyser using the recommended gating settings.

Flow cytometric measurement of activation markers in CD4+ and CD8+ T cells

Prior to analysis of activation marker expression by flow cytometry, MACSiBeads were removed from cell cultures per manufacturer's instructions using magnetic separation. PBMC samples were then stained with either CD4-FITC or CD8-FITC conjugated antibodies (1:11, Miltenyi Biotec, UK) in buffer for 30 minutes prior to washing three times (800xg, 5 minutes) in ice cold Phosphate Buffered Saline (PBS) to enable gating of CD4+ and CD8+ T cells along with a combination of antibodies (Miltenyi Biotec, UK) against either CD25-PE, CD44-APC, CD69-APC or CD95-APC. Samples were then washed three times (800xg, 5 minutes) in ice cold Phosphate Buffered Saline (PBS) before analysis on a BD FACS CantoII flow cytometer. The PBMC population was gated using linear forward and side scatter.

Leukocyte proliferation, in response to nanoparticles, measured by incorporation of ^{3}H-thymidine

PBMC number was adjusted to 2.5 x 10^6 cells per ml and 25,000 cells per well were added to a 96 well round bottomed plate. Phytohaemagglutinin (PHA, Sigma, UK) (20µg/mL) was then added to each well followed by the addition of either medium or medium containing drug (lopinavir or lopinavir SDNs, 10µM) taking into account the resultant dilution. Plates were then cultured for 48 hours (37°C; 5% CO_2 in air), the final 16 h with 1µCi [^{3}H]-thymidine (Moravek, USA) per well. Cells were then harvested onto a filtermat using a tomtec harvester 96 and sealed in a sample bag with melt on scintillation cocktail. Incorporated radioactivity was counted on a Perkin-Elmer MicroBeta detector.

Impact of nanoparticles on phagocytosis in primary, human, monocyte-derived macrophages

CD14+ cells were isolated from PBMC samples by magnetic bead separation and incubated in Iscove's Modified Dulbeccos' Media (IMDM) containing macrophage colony stimulating factor (M-CSF) (10ng/mL) for 12 days replacing the media every three days to differentiate into monocyte-derived macrophages (MDM). Following differentiation, MDM were treated with lopinavir or the lopinavir SDNs (10µM) for 24 hours. After the incubation period, phagocytic activity was assessed using pHrodo reagent (Molecular probes, UK). MDM were plated at 100,000 cells per well in a black walled plate. pHrodo™ BioParticles® were prepared by suspending 2 mg of lyophilized product in 2mL of uptake buffer (Hanks Balanced Salt Solution [HBSS], 20 mM HEPES, pH 7.4) and briefly vortexed to completely suspend the particles. The positive control for inhibition of phagocytosis was Cytochalasin B (10µM, Sigma, UK)). Culture media was aspirated from each well and replaced with the pHrodo bioparticle solution. The plate was covered and transferred to an incubator at 37°C without CO_2 to prevent artificial acidification of the uptake buffer thereby minimising background signal. Plates were read using a plate reader with an excitation of 550nm and emission of 600nm.

Statistical analysis

Distribution of the data was assessed using a Shapiro-Wilk test. For comparisons between datasets either an unpaired t-test or a Mann-Whitney test was used for normally and non-normally distributed data respectively. Stats Direct software (version 3.0.171) was used for data analysis and a P value < 0.05 was considered statistically significant.

RESULTS

Physical characteristics of lopinavir solid drug nanoparticles

3.5 mL of deionised water was added to the LPV ETFD monolith, thus creating 1 $mgmL^{-1}$ SDN dispersion with respect to LPV content. Hydrodynamic diameter (Z-average D_z), PdI and zeta potential were assessed using dynamic light scattering (DLS) (representative DLS traces can be seen in supplementary information figure 1). D_z was recorded as 566 ± 26 nm, PdI at 0.37 ± 0.02 and ζ at -12 ± 2 mV. Lopinavir SDN were stable at a range of pH and over an extended period of time (supplementary information figure 2 & 3 respectively)

Quantification of endotoxin in lopinavir and lopinavir nanoparticle preparations

The presence of endotoxin in drug and nanoparticle samples was assessed using ELISA-based techniques. Following interpolation form a standard curve the level of endotoxin in the lopinavir solution and lopinavir SDN samples was 0.008 EU/mL and 0.063 EU/mL, respectively. In order to ensure nanoparticles did not interfere with the assay, samples of lopinavir and lopinavir SDNs were also spiked with 5 EU/mL of endotoxin. Recovery of endotoxin was 5.32 EU/mL and 6.13 EU/mL for the lopinavir and lopinavir sold drug nanoparticles, respectively.

Impact of lopinavir and lopinavir solid drug nanoparticles on T Cell cytokine secretion

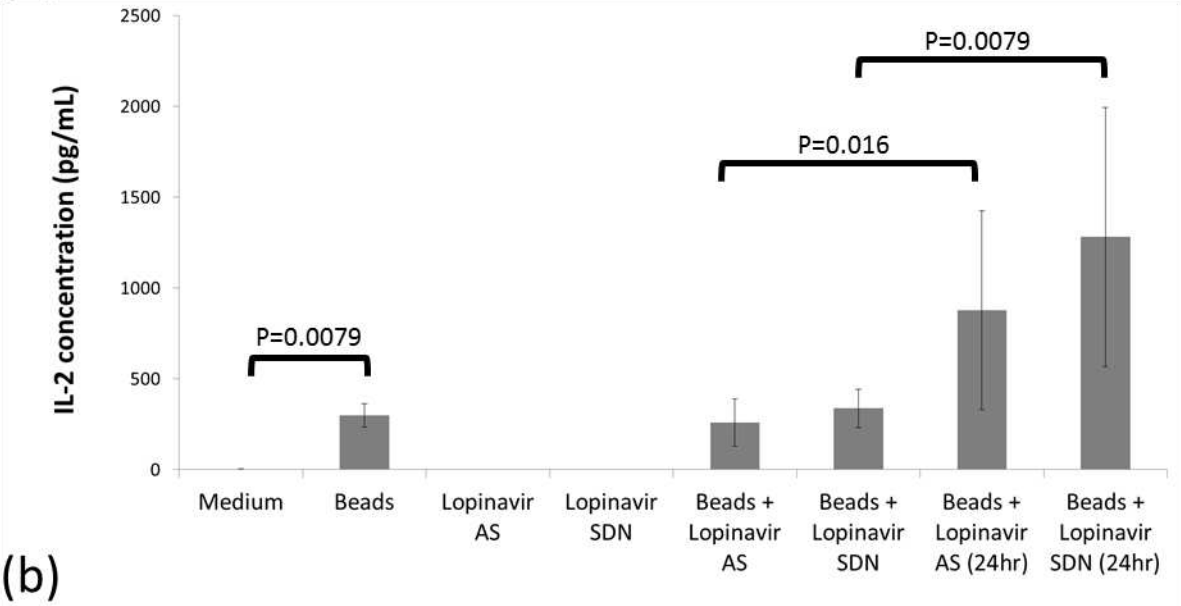
Anti-CD2, CD3 and CD28 beads were used to stimulate T cells in the PBMC population. Secretion of IL-2 (figure 1a) from PBMC treated with beads was significantly higher than that of untreated cells (148 fold higher; P=0.0079). Treatment of PBMC with lopinavir or lopinavir SDNs resulted in 65% and 74% lower secretion of IL-2, respectively, although this was not statistically significant (P=0.095 & P=0.071, respectively). Coincubation of PBMC with beads and either lopinavir or lopinavir SDN did not result in significantly different secretion of IL-2 compared to bead treated cells (P=0.54 & P=0.69, respectively). PBMC were also treated with either lopinavir of lopinavir SDNs for 24 hours prior to stimulation with beads. Preincubation with lopinavir (3-fold greater; P=0.016) or lopinavir SDN (4-

fold greater; P=0.0079) significantly increased bead stimulated IL-2 secretion. However, there were no differences in the stimulation between cells pre-treated with lopinavir or lopinavir SDN for 24 hours (P=0.42).

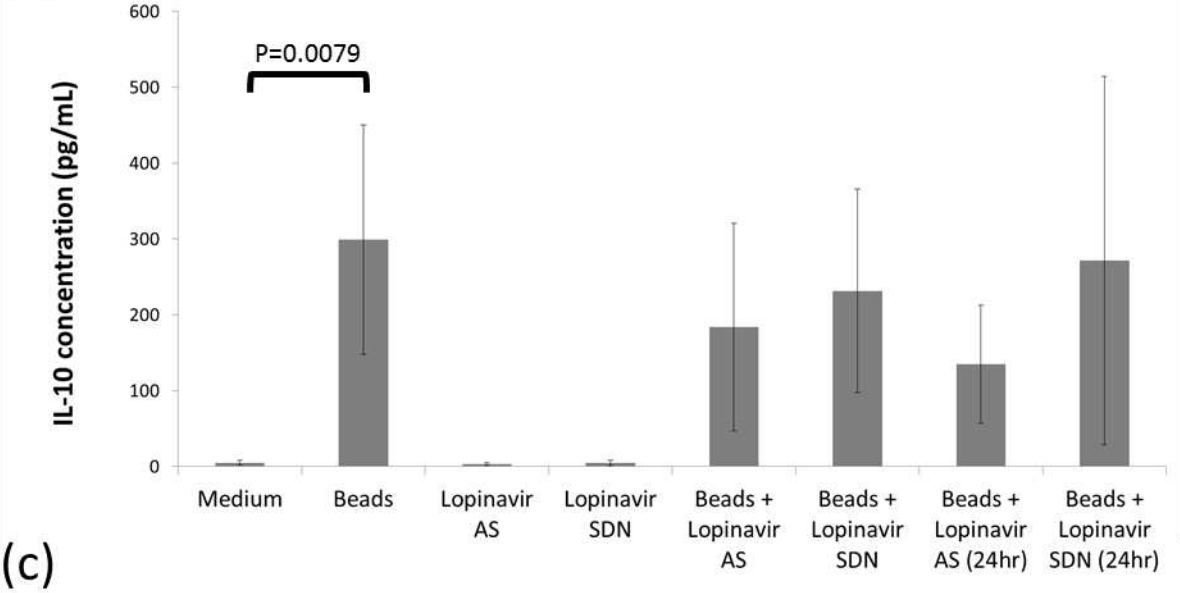
Bead treatment similarly increased IL-10 secretion (figure 1b) compared to unstimulated cells (58-fold increase; P=0.0079). When compared to unstimulated controls, cells incubated with lopinavir or lopinavir SDNs did not secrete significantly different concentrations of IL-10 (P=0.31 & P=0.84, respectively). Additionally, coincubation of cells with beads and either lopinavir or lopinavir SDNs did not result in significantly different concentrations of IL-10 compared to bead stimulated cells (P=0.15 & P=0.42 respectively). Pre-treatment of PBMC with lopinavir SDNs for 24 hours prior to stimulation with beads did not result in significantly different IL-10 secretion compared to bead stimulated cells (P=0.84). However, there was a trend towards lower IL-10 secretion from cells pre-treated with lopinavir for 24 hours prior to bead stimulation (54% lower: P=0.056). No significant differences were observed between lopinavir and lopinavir SDN treatments for any of the experimental conditions (P>0.1 for each).

Bead treatment also resulted in significantly higher IFNγ secretion (figure 1c) from PBMC than unstimulated cells (41-fold higher; P=0.0079). Treatment with both lopinavir and lopinavir SDNs resulted in a decrease in IFNγ concentrations below the limit of detection (6.4pg/mL). No significant difference was observed between bead stimulated PBMC and those stimulated with beads and co-incubated with lopinavir (P=0.42) or lopinavir SDNs (P=0.84). Similarly, no significant difference was observed for cells pre-treated with lopinavir (P=0.22) or lopinavir SDNs (P=0.31) for 24 hours prior to stimulation with beads.

(a)


IL-2 concentration (pg/mL)
2500
2000
1500
1000
500
0
P=0.0079
P=0.016
P=0.0079
Medium
Beads
Lopinavir AS
Lopinavir SDN
Beads + Lopinavir AS
Beads + Lopinavir SDN
Beads + Lopinavir AS (24hr)
Beads + Lopinavir SDN (24hr)

(b)


IL-10 concentration (pg/mL)
600
500
400
300
200
100
0
P=0.0079
Medium
Beads
Lopinavir AS
Lopinavir SDN
Beads + Lopinavir AS
Beads + Lopinavir SDN
Beads + Lopinavir AS (24hr)
Beads + Lopinavir SDN (24hr)

(c)

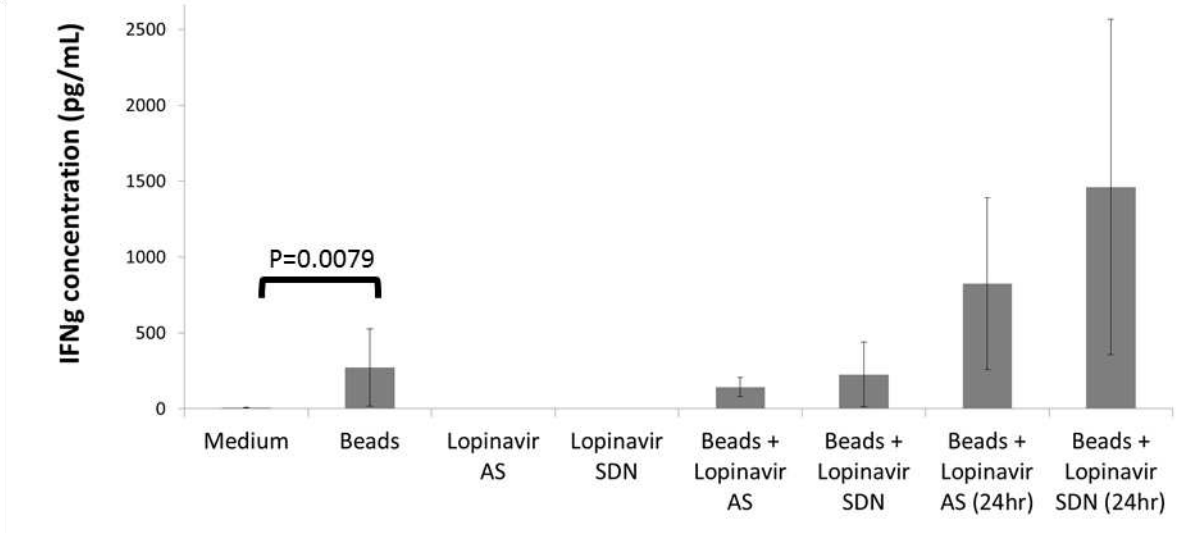

IFNg concentration (pg/mL)
2500
2000
1500
1000
500
0
P=0.0079
Medium
Beads
Lopinavir AS
Lopinavir SDN
Beads + Lopinavir AS
Beads + Lopinavir SDN
Beads + Lopinavir AS (24hr)
Beads + Lopinavir SDN (24hr)

Figure 1. Analysis of cytokine secretion from peripheral blood mononuclear cells treated with lopinavir or lopinavir solid drug nanoparticles. Concentrations of IL-2 (a), IL-10 (b) and IFNγ (c) were measured in culture supernatant 24 hours post incubation with lopinavir or lopinavir SDNs. Anti-CD2, CD3 and CD28 beads were used as a positive control. Data presented as mean ± SD, N=6.

Impact of lopinavir and lopinavir nanoparticles on T lymphocyte activation markers

Expression of classic markers of activation were determined in CD4+ and CD8+ T cells (figure 2a & 2b, respectively). In CD4+ T cells (figure 2a) stimulation with beads resulted in a significantly higher expression of CD44 (1.3-fold higher; P=0.0159) and CD69 (10-fold higher; P=0.0079). CD25 (2.2-fold higher) and CD95 (1.15-fold higher) expression was higher in bead treated cells but the differences were not statistically significant. Similarly, in CD8+ T cells (figure 2b) bead stimulation resulted in significantly higher expression of CD25 (2.9-fold higher; P=0.045), CD44 (1.3-fold higher; P=0.035) and CD69 (4.6-fold higher; P=0.032) but not CD95 (1.9-fold higher; P=0.055). There was no significant difference in expression of activation markers when cells were treated with lopinavir or lopinavir SDNs. Similarly, lopinavir and lopinavir SDNs did not significantly affect stimulation of cells with beads.

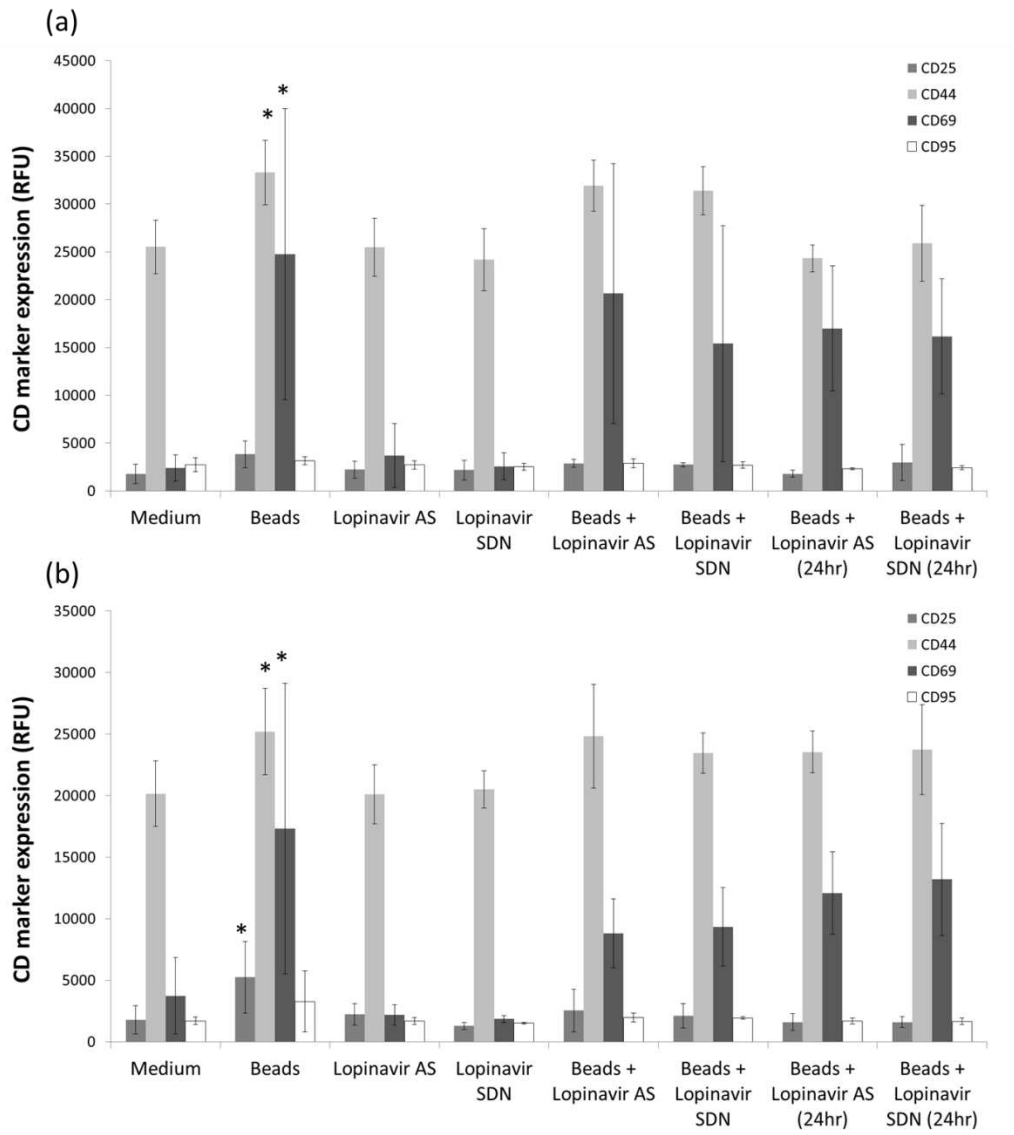


Figure 2. Analysis of markers of activation in CD4+ and CD8+ T cells treated with lopinavir or lopinavir solid drug nanoparticles. Levels of expression of CD25, CD44, CD69 and CD95 were determined by multiparameter flow cytometry in CD4+ (a) and CD8+ (b) T cells from PBMC 24 hours post incubation with lopinavir or lopinavir SDNs. Anti-CD2, CD3 and CD28 beads were used as a positive control. Data presented as mean ± SD, N=6. When compared to unstimulated cells *=$P<0.05$.

Impact of lopinavir and lopinavir solid drug nanoparticles on lymphocyte proliferation

To determine the impact on lymphocyte proliferation and the response of lopinavir and lopinavir SDN treated PBMC to known mitogens, incorporation of ^{3}H-thymidine was used as a marker of cellular proliferation. Treatment with PHA resulted in a 51-fold higher proliferation of cells (P=0.02) than that of unstimulated PBMC (figure 3). Lopinavir (P=0.46) and lopinavir SDNs (p=0.27) did not result in any significant effect upon proliferation compared to unstimulated cells and there was no difference between lopinavir and lopinavir SDN treated cells (P=0.12). Similarly, co-incubation of PBMC with lopinavir or lopinavir SDNs and PHA did not impact the proliferative response compared to PHA only treated cells (P=0.91 and P=0.61, respectively). Finally, there was no difference observed between cells co-incubated with PHA and lopinavir and that of PHA and lopinavir SDNs (P0.48).

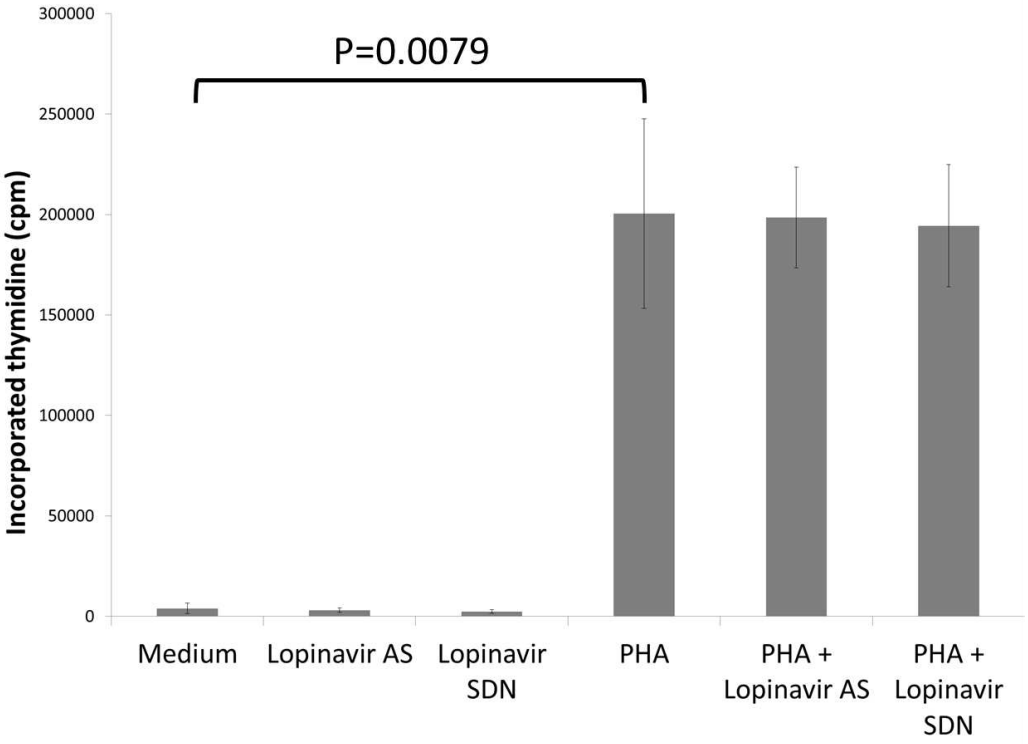


Figure 3. Analysis of proliferation of PBMC in response to treatment lopinavir or lopinavir solid drug nanoparticles in the absence and presence of PHA. Measurement of incorporated ^{3}H-

276 thymidine was determined by liquid scintillation counting in PBMC 24 hours post incubation with
277 lopinavir or lopinavir SDNs. PHA was used as a positive control. Data presented as mean ± SD, N=6.

Impact of lopinavir and lopinavir nanoparticles on secretion of cytokines from Monocyte-Derived Macrophages

MDM were generated from primary human monocytes and treated with either lopinavir or lopinavir SDNs for 24 hours (figure 4). Lipopolysaccharide (LPS) was used as a positive control for stimulation of macrophages. Treatment with LPS resulted in a 32-fold higher secretion of IL-1β (P=0.008), 1360-fold higher secretion of IL-6 (P=0.0079), 158-fold higher secretion of IL-8 (P=0.0079) and a 458-fold higher secretion of TNFα (P=0.0066). Aqueous lopinavir treatment resulted in a significantly higher secretion of IL-1β (9-fold higher; P=0.045) and TNFα (6-fold higher; P=0.018) than untreated cells, whereas treatment of MDM with lopinavir SDNs did not result in significantly different cytokine secretion compared to controls.

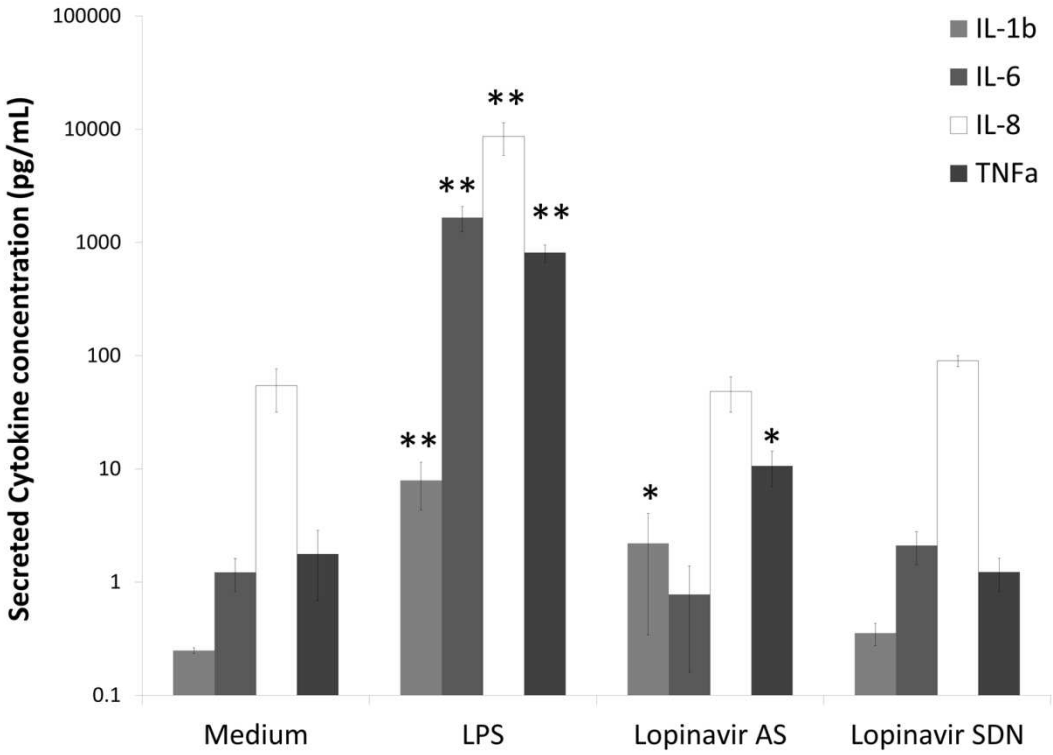


Figure 4. Analysis of cytokine secretion from monocyte derived macrophages treated with lopinavir or lopinavir solid drug nanoparticles. Concentration of cytokines in cell culture milieu 24 hours post treatment with either lopinavir or lopinavir SDNs were determined by multiplex

suspension array. Lipopolysaccharide (100ng/mL) was used as a positive control. Data presented as mean ± SD, N=6. When compared to unstimulated cells *=P<0.05 & **=P<0.01

Impact of lopinavir and lopinavir nanoparticles on phagocytosis by monocyte-derived macrophages

Phagocytosis in MDM was assessed using fluorescent bioparticle uptake into MDM. MDM were treated with lopinavir or lopinavir SDNs for 24 hours prior to the assessment of bioparticle uptake. Cytochalasin was used as a known inhibitor of phagocytosis and bioparticle uptake was shown to be 3.5-fold lower (P=0.035) in MDM treated with cytochalasin (figure 5). Treatment of MDM with either lopinavir or lopinavir SDNs did not significantly alter the uptake of bioparticles in MDM (figure 5).

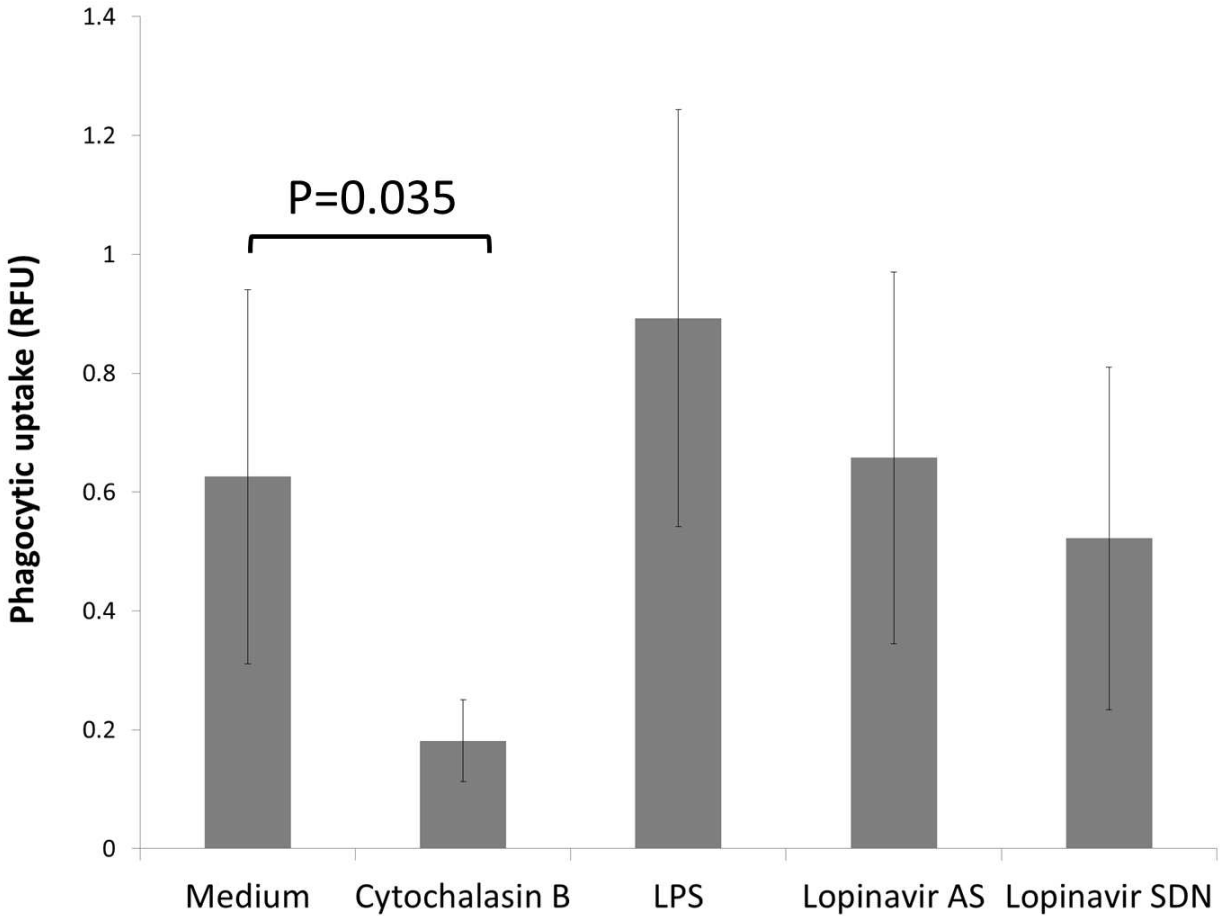


Figure 5. Impact of lopinavir and lopinavir solid drug nanoparticles on the uptake of fluorescent bioparticles in monocyte derived macrophages as a measure of phagocytosis. Bioparticle uptake was measured by fluorescence spectroscopy 24 hours post incubation with lopinavir of lopinavir SDNs. Cytochalasin was used as a positive control. Data presented as mean ± SD, N=6.

DISCUSSION

Determining the interaction of nanomaterials with cells of the immune system is key to understanding potentially limiting safety issues. This is particularly relevant in conditions where the primary target for the active pharmaceutical ingredient is within cells of the immune system, such as in the treatment of HIV. This is the first study to investigate the impact of SDNs on the function of primary human T cells and monocyte-derived macrophages, despite over 25 SDN-based medicines being approved for use in humans. This work formed part of a putative safety assessment of LPV SDNs, driven by the ambition to explore the potential for improved accumulation within these cell types (to supplement potential benefits in terms of pharmacokinetics).

Using an emulsion-templated freeze-drying approach [17, 18, 23], lopinavir SDNs were produced with reproducible physico-chemical characteristics and previously shown to be bioequivalent to a conventional preclinical preparation of lopinavir in a rodent model. Importantly, the lopinavir SDNs are capable of dispersion in water thereby overcoming the issues of current paediatric dosing formats, which contain a high content of organic solvent [18]. The presence of endotoxin in nanoparticle samples can result in potentially false positive results in studies of immunogenicity [24] and it is therefore important to determine the concentration of endotoxin in nanomaterial preparations before embarking on such studies. The concentration of endotoxin in both the aqueous lopinavir solution and lopinavir SDNs was very low and unlikely to interfere with immunological assays. Additionally, using samples spiked with a known amount of endotoxin, lopinavir aqueous solution and the lopinavir SDNs do not interfere with recovery of endotoxin. Indeed, the results from endotoxin spiked samples were well within the 50-200% recovery acceptable by the USA and EU pharmacopoeia.

Previous reports within the literature have shown that nanoparticles can stimulate T cells and, depending on their physico-chemical properties, can result in differential activation of either Th1 or Th2 profiles. The potential for lopinavir and/or lopinavir SDNs to stimulate T cells was investigated

and neither aberrantly stimulated T cells to produce Th1 or Th2 cytokines. However, when cells were pre-treated with lopinavir or lopinavir SDNs for 24 hours prior to control stimulation the secretion of IL-2 was significantly higher than when cells were stimulated with beads and material simultaneously. This suggests an enhancement of the stimulatory effects of the beads by the lopinavir, which is independent of SDN formation. It has been shown previously that lopinavir can increase the amount of reactive oxygen species in a number of cell types [25, 26]. Reactive oxygen species are well known as mediators of inflammation and it is possible that this enhanced stimulation is a result of lopinavir eliciting endoplasmic reticulum stress. Importantly, lopinavir SDNs did not differ significantly in their impact on stimulation from that of a lopinavir solution. Additionally, the expression of cell surface receptors associated with T-cell activation [27-30] was monitored in response to incubation with the lopinavir SDNs or a lopinavir aqueous solution. No differences in expression between SDNs and aqueous solution and no differences in the response to anti-CD3/antiCD28 beads were observed in either CD4+ or CD8+ T cells. Finally, no difference in proliferation of PBMC from healthy volunteers were observed between SDNs and aqueous solution, and neither interfered with proliferation in response to the known mitogen, PHA.

Secretion of cytokines from macrophages in response to treatment with lopinavir or lopinavir SDNs was also assessed. LPS treatment resulted in significantly higher secretion of IL-1β, IL-6, IL-8 and TNFα from MDM, which is in line with previously published observations [31, 32]. Aqueous lopinavir treatment resulted in significantly higher IL-1β and TNFα secretion from MDM compared to unstimulated cells. This is also in agreement with previous reports that have shown lopinavir induces the secretion of IL-6 and TNFα in rat peritoneal macrophages [6]. It is possible that subtle differences between rodent and human intracellular signalling can explain why IL-6 secretion was not significantly different in the current study. However, further work may be required to confirm this and clarify the underlying mechanisms. Lopinavir SDNs did not significantly alter cytokine secretion from MDM compared to untreated cells. This is particularly interesting and further work is required to elucidate why this difference between solution and SDNs was evident. However, the observation

potentially represents an attractive feature of this particular type of nanoparticle as it appears to have reduced a possibly unintentional effect of lopinavir. The possible consequences of this differential induction of IL-1β and TNFα now warrant further investigation to determine additional effects. The impact of IL-1β and TNFα in HIV infection are still under debate; elevated concentrations of these proinflammatory cytokines have linked to aging of the immune system [33] and therefore lower levels of these cytokines induced by lopinavir SDN may ameliorate the effects of standard formulations of lopinavir on the aging of the immune system. IL-1β and TNFα have been shown to play a major role in neuronal death (and subsequent associations with HIV associated dementia) as well as increasing the permeability of the blood-brain barrier to allow HIV infected monocytes to enter the brain [34]. Lower levels of these cytokines induced by lopinavir SDN may also prevent subsequent side effects but these issues need to be assessed in clinical trials. Finally, the impact of lopinavir and lopinavir SDNs on phagocytosis in MDM was assessed. Previous reports in the literature have shown the primary route of uptake into professional antigen presenting cells to be phagocytosis [35, 36]. Given the possibility of interference with this vital mechanism in MDM the impact of lopinavir and lopinavir SDNs on the uptake of fluorescent bioparticles was assessed. Cytochalasin was used as a known inhibitor of phagocytosis and a significantly lower uptake of bioparticles was observed in treated MDM. Lopinavir and lopinavir SDNs again did not significantly affect the uptake of bioparticles into MDM suggesting no interference with this mechanism.

Our putative immunological safety assessment uncovered no obvious issues, but additional investigation in other cells of the immune system is now warranted to confirm biocompatibility. The formation of lopinavir SDNs may have the potential to mitigate unwanted effects whilst improving the bioavailability of lopinavir. This lopinavir SDN formulation, given its bioequivalence and comparative safety to conventional lopinavir preparations, is a viable option for pharmaceutical scalable manufacture, has been manufactured to GMP standards, and is currently undergoing assessment in a healthy volunteer clinical trial (EudraCT number 2013-004913-41). Given that SDNs are being investigated as intravenous, intramuscular and subcutaneous administration formats,

these data bode well for the direct administration of such materials. However, similar work with SDNs composed of other drug molecules is required to confirm the appropriateness of generalising these observations across this class of nanomaterial.

Future perspective

Assessing the biocompatibility of novel, engineered, nanomaterials is an ongoing challenge in the field of nanomedicine. A number of points must be considered including, but not limited to; standardisations of the techniques used in biocompatibility assessment to more easily compare results between researchers, a more complete analysis of the healthy volunteers samples that are used in these studies to understand potential inter-individual variability and comprehensive physical characterisation of the materials under investigation to clearly identify relationships between nanoparticle characteristics and biological effect.

Executive Summary

Background

- Solid drug nanoparticles of Lopinavir have previously been demonstrated to have a number of pharmacological benefits for use in paediatric patients by mitigating the need for organic solvents and/or augmenting bioavailability.
- The formulation described in this paper is currently undergoing assessment in human healthy volunteers.
- The interaction of nanomaterials with immunological systems is a developing field of research but, to date; solid drug nanoparticles have not been extensively studied. Therefore we assessed the impact of these nanoparticles on T cell and macrophage function

Results

- Endotoxin was present in the studied formulations however; it was present at very low levels unlikely to induce an immunological response.

- Solid drug nanoparticles did not induce the same immunogenic response as conventional lopinavir.
- No other interactions with T cells or monocyte-derived macrophages were observed. In these *ex vivo* analyses, lopinavir SDNs were demonstrated to be immunologically inert on exposure to human T cells and monocyte-derived macrophages.

Conclusion

- Lopinavir was shown to induce the secretion of proinflammatory cytokines however further clarification of the impact of this on disease progression, and treatment, in HIV patients requires further clarification.
- The Lopinavir solid drug nanoparticles did not interfere with normal responses of T cells and macrophages within this study. This suggests that their accumulation within these cells should not raise any particular issues.

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Author contributions

NJ Liptrott conceived and carried out the experiments and wrote the manuscript. M Giardiello prepared the solid drug nanoparticles, performed physical characterisation and reviewed the manuscript. TO McDonald and S Rannard reviewed the manuscript prior to submission. A Owen is principal investigator for the experimental investigation and reviewed the manuscript.

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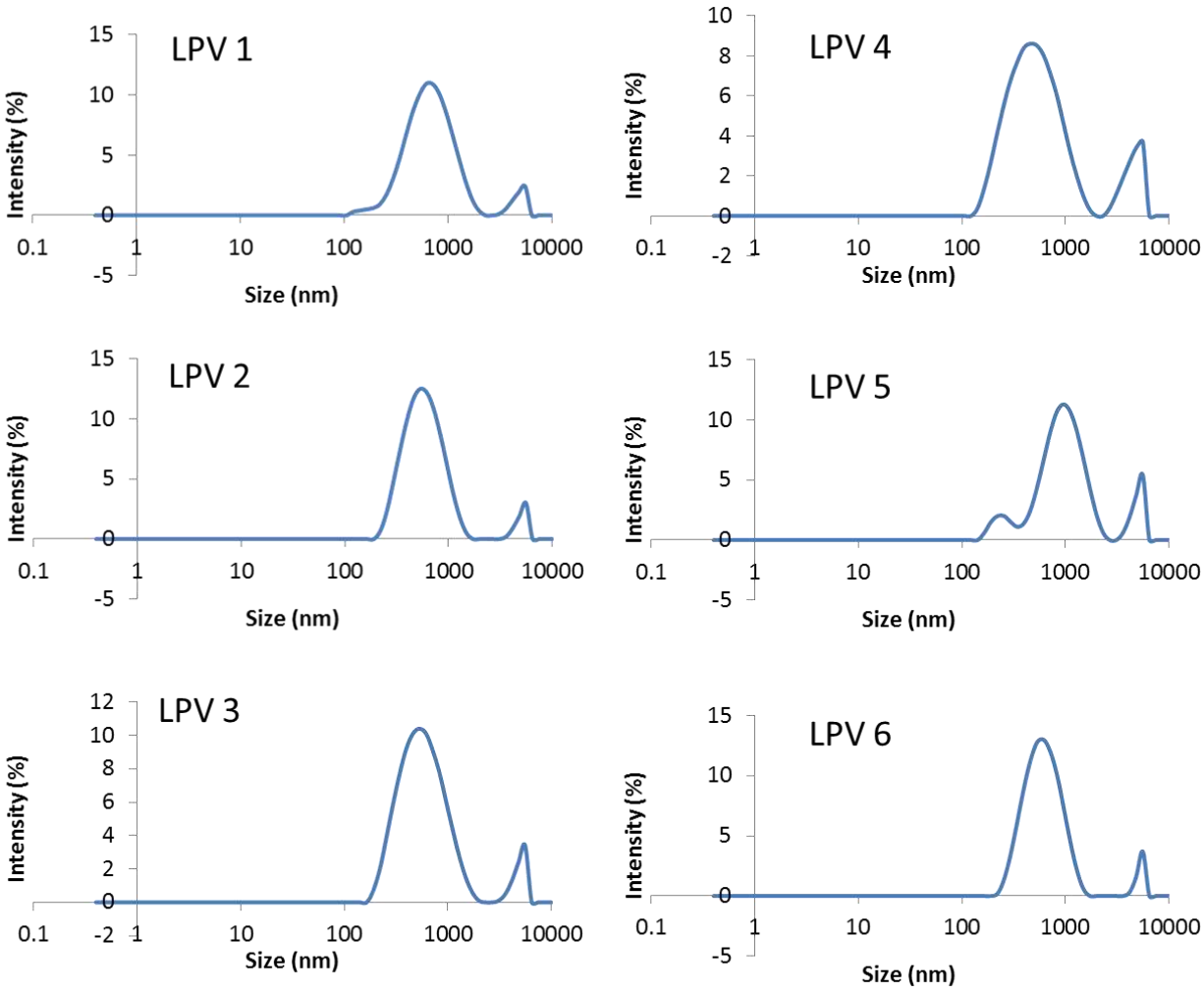
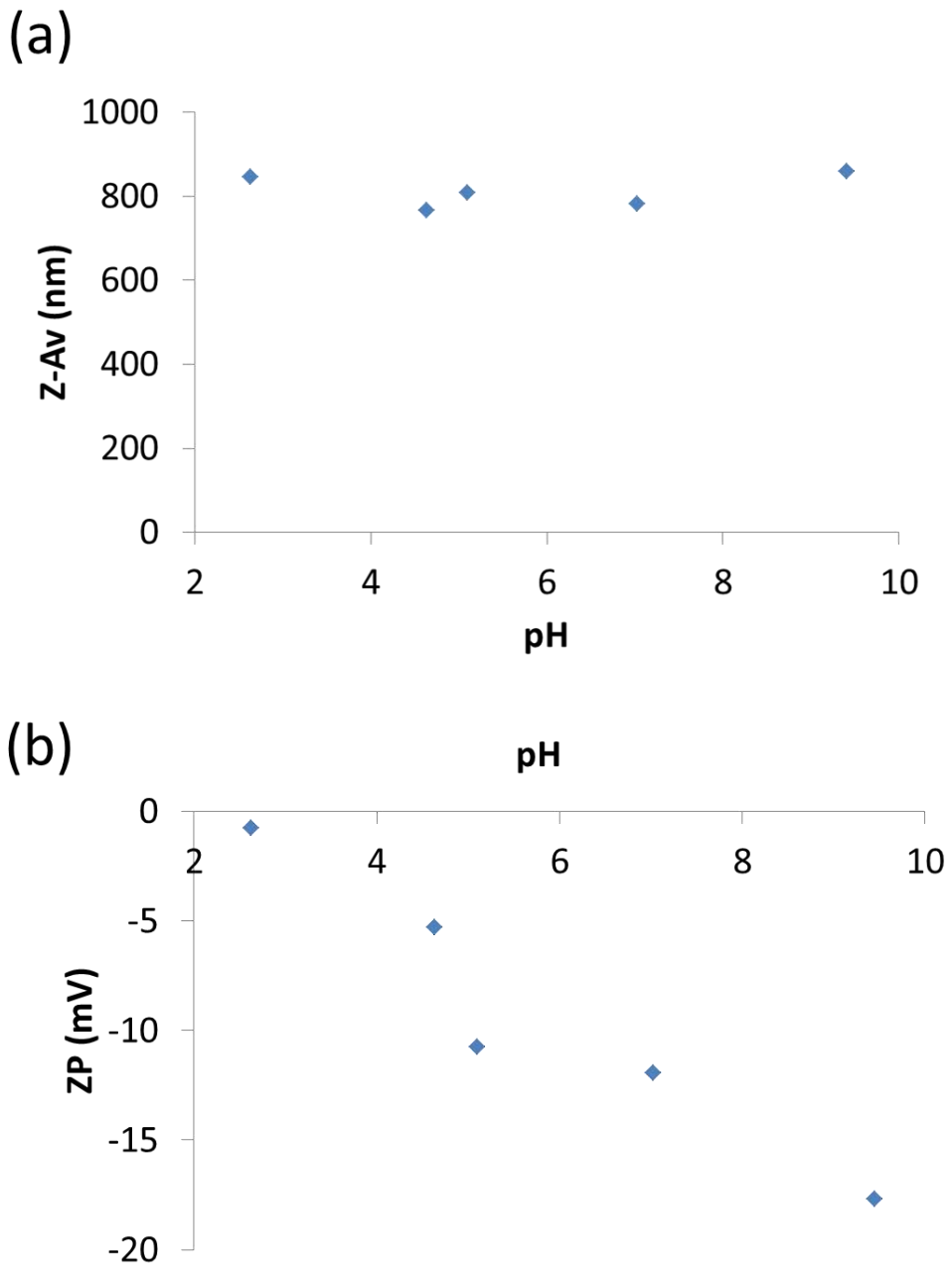
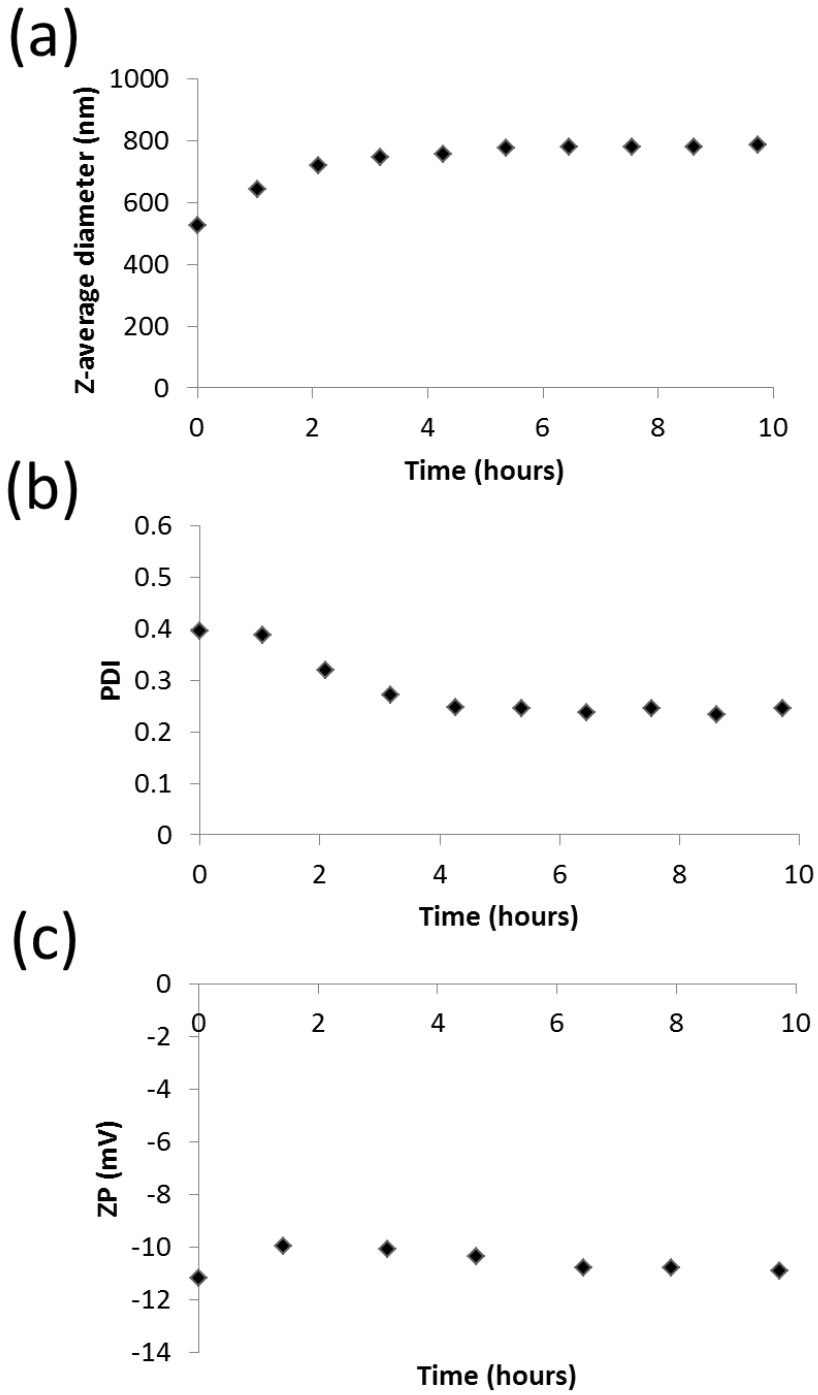


Supplementary figure 1. Representative DLS traces of Lopinavir solid drug nanoparticles dispersed in water. Samples dispersed at 1 mgmL^{-1} in water at 25^{o}C.



Supplementary figure 2. Measurement of (a) z-average and (b) zeta potential of lopinavir solid drug nanoparticles over a range of pH. Samples dispersed at 1 $mgmL^{-1}$ in water at 25^{o}C.



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475 **Supplementary figure 3**. Stability of lopinavir solid drug nanoparticles as determined by
476 measurement of (a) z-average, (b) polydispersity index and (c) zeta potential over a period
477 of 35 hours. After addition of water to the emulsion-templated monolith and subsequent
478 dynamic light scattering measurements. Samples dispersed at 1 $mgmL^{-1}$ in water at 25^{o}C.

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