Higher Cell Accumulation and Antiviral Activity of Lopinavir/Ritonavir Nanoparticles

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Introduction

- Lopinavir (LPV) is a human immunodeficiency virus (HIV) protease inhibitor (PI), highly bound to plasma proteins (98-99%) and extensively metabolized by the hepatic cytochrome P450 (CYP) system.
- When administered alone LPV has poor bioavailability and is therefore routinely co-administered with ritonavir (RTV) as a pharmaco-enhancer to increase its pharmacokinetic (PK) exposure.
- RTV inhibits proteins including CYP3A4 and P-glycoprotein (P-gp) increasing bioavailability and reducing clearance.
- Although RTV is widely used in second line combination antiretroviral therapies it is associated with numerous toxicities and any strategy that reduces RTV dose while maintaining "boosting" efficacy is of clear importance

Aim

- To assess combination LPV:RTV solid drug nanoparticles (at 4:1, 8:1 and 40:1 ratios) on antiretroviral activity (cell free and cell based systems), cellular accumulation and cytotoxicity in human primary immune cells.

Methods

Solid Drug Nanoparticle Synthesis: Combination LPV/RTV SDNs were synthesised using a previously described technology (McDonald *et al.*,2014) and consisted of 70 wt% of total active pharmaceutical ingredients at either 4:1. 8:1 or 40:1 LPV:RTV ratio, 20 wt% polymer (poly(vinyl alcohol) (PVA)) and 10 wt% surfactant (α-tocopherol poly(ethylene glycol) succinate (Vit-E-PEG)). Where radio-labelled drug was required, tritiated LPV (LPV; 0.1μCi H^3) was utilised. Characterisation of SDNs were determined by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS and associated software

Cytotoxicity: MT4 cells or cryopreserved primary human immune cells (CD4+, CD56+, CD14+ and CD14+ monocyte derived macrophages (MDM)) were incubated for 24 hours with a range of concentrations (0.01, 0.1, 1, 10, 100, 1000μM), as determined by mass of LPV, of combination SDNs or the equivalent aqueous solutions at 4:1. 8:1 or 40:1 LPV/RTV ratios. With the exception of MT4 cells where methyltetrazolium (MTT) assay was utilised, cytotoxicity was determined using ATP cell viability assay (CellTiter-Glo® (Promega, USA)) and absorbance or luminescence measured using XFluor4 GENios (Tecan) plate reader.

Cellular Accumulation: Immune cells were incubated with 10μM (mass of LPV (LPV; 0.1 μCi H^3)) of either combination SDNs or equivalent aqueous combination solutions at 4:1, 8:1 or 40:1 ratios of LPV:RTV for 60 minutes and an aliquot of media (extracellular) was removed. Cells were then washed twice, resuspended and lysed (intracellular). A cellular accumulation ratio was then calculated as the ratio of intracellular/extracellular concentrations determined by scintillation counting (Perkin Elmer 3100TS scintillation counter).

Cell Free HIV-1 Protease Inhibition: HIV-1 protease inhibition was assessed across a range of concentrations (100nM to 0.78nM) of SDNs or aqueous combination solutions at 4:1, 8:1 or 40:1 ratio LPV/RTV as per manufacturers' instructions (SensoLyte HIV-1 Protease; AnaSpec, UK). Absorbance was measured at 405 nm using FLUOstar Omega plate reader (BMG Labtech, UK) and readings used to determine inhibitory concentrations causing half maximal response (IC_{50}).

Inhibition of HIVIIIB replication: MT4 cells were infected with $HIVIII_B$ then seeded at 2 x 10^4 cells per well of a 96 well plate then incubated for 5 days with either SDNs or equivalent aqueous solutions at 1:4, 1:8 or 1:40 LPV:RTV. Inhibition was assessed by methyltetrazolium assay prior to calculation of IC_{50}.

Statistical analysis.: Normality was determined using Shapiro-Wilk and unpaired t-test was used to determine statistical significance. All data are given as mean ± standard deviation of at least 4 independent experiments conducted in duplicate in StatsDirect software . IC_{50} were generated using GraphPad Prism 3.0.



Figure 1. Schematic of the production of combination LPV/RTV loaded solid drug nanoparticles using a non-attrition method.

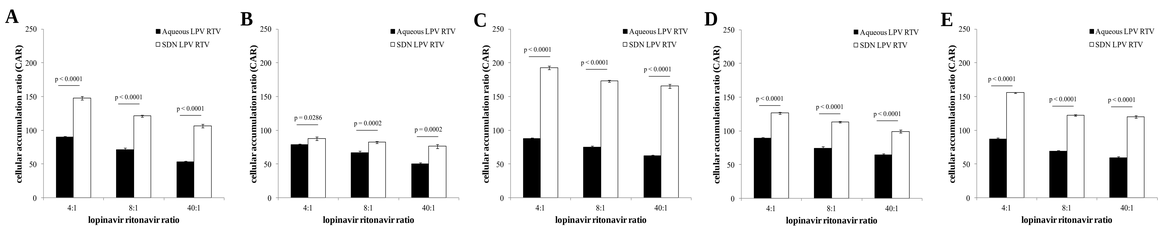


Figure 3. Cellular accumulation of a combined aqueous solution of LPV (radio-labelled LPV (H^3)) and RTV (ratio 4:1, 8:1 or 40:1) or combined solid drug nanoparticle of LPV (radio-labelled LPV(H^3) and RTV (4:1, 8:1 or 40:1) in primary CD4+ T cells (A), CD14+ T cells (B), Monocyte derived macrophages cells (C), Natural killer CD56+ cells (D) and MT4 cells (E).

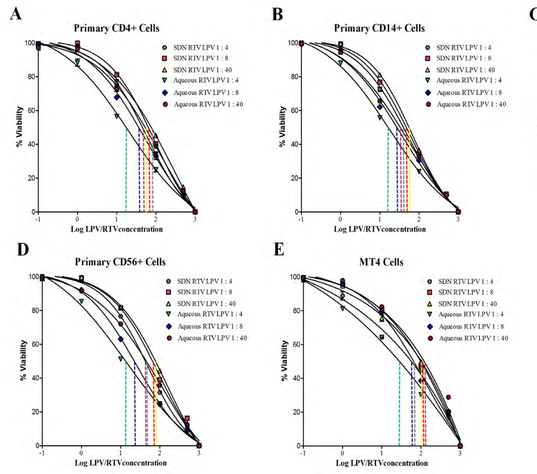


Figure 2. Cytotoxicity of LPV/RTV SDNs compared to aqueous LPV/RTV solution in primary CD4+ T cells (A), CD14+ T cells (B), Monocyte derived macrophages cells (C), Natural killer CD56+ cells (D) and MT4 cells

Table 1. Quantitative description for Figure2

Ratio RTV LPV 1:4	Aqueous IC_{50}(μM)	SDN IC_{50}(μM)	Ratio RTV LPV 1:40	Aqueous IC_{50}(μM)	SDN IC_{50}(μM)
CD4+	12.7 ± 1.1	38.2 ± 1.6	CD4+	30.8 ± 2.7	57.2 ± 4.1
CD14+	11.9 ± 0.9	32.2 ± 2.6	CD14+	21.0 ± 1.3	40.1 ± 2.8
MDM	22.8 ± 2.3	80.2 ± 5.8	MDM	35.4 ± 2.9	102.4 ± 6.3
CD56+	10.3 ± 0.8	36.9 ± 3.4	CD56+	18.3 ± 1.1	57.1 ± 2.8
MT4	18.2 ± 1.5	50.8 ± 4.2	MT4	35.7 ± 2.9	70.1 ± 3.6
Ratio RTV LPV 1:8	**Aqueous IC_{50}(μM)**	**SDN IC_{50}(μM)**			
CD4+	54.4 ± 3.6	89.0 ± 6.2			
CD14+	27.1 ± 1.3	47.6 ± 2.1			
MDM	53.1 ± 4.3	146.3 ± 8.7			
CD56+	48.7 ± 2.8	80.3 ± 5.4			
MT4	61.4 ± 23.2	96.7 ± 8.7			

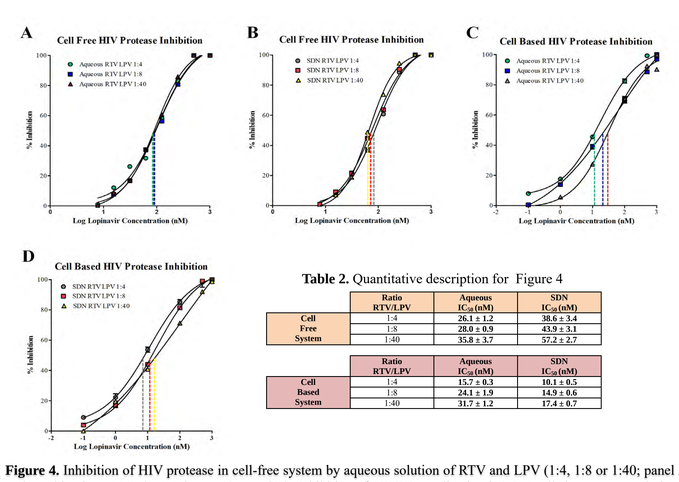


Table 2. Quantitative description for Figure 4

	Ratio RTV/LPV	Aqueous IC_{50}(nM)	SDN IC_{50}(nM)
Cell Free System	1:4	26.1 ± 1.2	38.6 ± 3.4
	1:8	28.0 ± 0.9	43.9 ± 3.1
	1:40	35.8 ± 3.7	57.2 ± 2.7
	Ratio RTV/LPV	**Aqueous IC_{50}(nM)**	**SDN IC_{50}(nM)**
Cell Based System	1:4	15.7 ± 0.3	10.1 ± 0.5
	1:8	24.1 ± 1.9	14.9 ± 0.6
	1:40	31.7 ± 1.2	17.4 ± 0.7

Figure 4. Inhibition of HIV protease in cell-free system by aqueous solution of RTV and LPV (1:4, 1:8 or 1:40; panel A) and combination SDNs (1:4, 1:8 or 1:40; panel B). Inhibition of HIVIIIB replication in MT4 cells by aqueous solution of RTV and LPV (1:4, 1:8 or 1:40; panel C) and combination SDNs (1:4, 1:8 or 1:40 panel D)

Results

- DLS analysis showed SDNs to be typically 350nm diameter,-10mV charge with 0.5 polydispersity index.
- A summary of IC_{50} values for cytotoxicity is given in Table 1. Combination SDNs were significantly less cytotoxic compared to aqueous solutions of LPV/RTV across all ratios for CD4+ (Figure 2A), CD14+ (Figure 2B), MDM (Figure 2C); CD56+ (Figure 2D), and MT4 cells (Figure 2E).
- Cellular accumulation of LPV/RTV SDNs was higher than the equivalent aqueous solution in CD4+ (Figure 3A), CD14+ (Figure 3B), MDM (Figure 3C), CD56+ (Figure 3D), and MT4 cells (Figure 3E). .
- A summary of IC_{50} values for cell-free and cell-based activity assays is given in table 2. Activity against HIV-1 protease cell free was more potent for aqueous solutions (Figure 4A) compared to SDNs (Figure 4B) across all ratios (1:4;1.5-fold; $p < 0.0001$, 1:8; 1.6-fold; $p < 0.0001$ and 1:40; 1.6-fold; $p < 0.0001$).
- Activity against $HIVIII_B$ in MT4 cells was more potent for SDNs (Figure 4D) compared to aqueous solutions (Figure 4C).across all ratios (1:4, 1.6-fold; $p<0.0001$; 1:8; 1.6-fold; $p<0.0001$; 1:40; 1.8-fold; $p<0.0001$).

Conclusion

Combination SDNs have increased cellular accumulation and enhanced antiviral activity in cell-based assays. Notably, equivalent protease inhibition *in vitro* with LPV doses requiring 10-fold less RTV was observed. Due to RTV intolerance and long-term toxicities associated with chronic exposure to RTV, strategies to reduce RTV dose are worthy of further investigation.

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