***In vitro* characterisation of solid drug nanoparticle compositions of efavirenz in a brain endothelium cell line**

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**Abstract**

**Background:** The antiretroviral drug efavirenz displays many desirable pharmacokinetic properties such as a long half-life enabling once daily dosing but suffers from CNS safety issues. Various nanotechnologies have been explored to mitigate some of the limitations with efavirenz. While there has been progress in increasing the bioavailability there has been no attempt to assess the impact of increased exposure to efavirenz on CNS safety. **Methods:** The uptake of aqueous and solid drug nanoparticle (SDN) formulations of efavirenz was assessed in the hCMEC/D3 brain endothelial cell line. The mechanisms of uptake were probed using a panel of transport and endocytosis inhibitors. **Results:** The cellular accumulation of an efavirenz aqueous solution was significantly reduced by amantadine but this was not observed with SDNs. The uptake of efavirenz SDNs were reduced by dynasore, but concentrations of the efavirenz aqueous solution were not affected. **Conclusions:** These data indicate that efavirenz is a substrate for transporters in brain endothelial cells (amantadine is an inhibitor of organic cation transporters, OCT1 and OCT2), and formation of SDNs may bypass this interaction in favour of a mechanism involving dynamin-mediated endocytosis.

**Introduction**

The non-nucleoside reverse transcriptase inhibitor (NNRTI) efavirenz (EFV) has been used in first-line HIV therapy for over 15 years ([Raffi et al., 2014](#_ENREF_27)). EFV displays potent activity against wild type HIV-1, with an IC50 of 0.51 ng/mL, inhibition constant (Ki) 2.93 nmol/L and a long plasma half-life of 40-76 hours, enabling once daily dosing ([Best et al., 2011](#_ENREF_4), [Adkins and Noble, 1998](#_ENREF_1)). Despite these favourable properties, EFV has very poor water solubility (<10µg/mL) and bioavailability can be poor resulting in highly variable plasma exposure after oral administration ([Siccardi et al., 2015](#_ENREF_31)).

Recently, we reported an EFV solid drug nanoparticle (SDN) formulation manufactured using an emulsion-templated freeze-drying (ETFD) approach ([McDonald et al., 2014](#_ENREF_21)). SDNs containing 70 wt% drug relative to polymer and surfactant excipients were successfully generated and displayed augmented transcellular permeation across Caco-2 cells with reduced cytotoxicity. Moreover, *in vivo* pharmacokinetic studies performed in rats demonstrated an increase in plasma EFV concentrations of approximately 4-fold following a single oral dose ([McDonald et al., 2014](#_ENREF_21)). Using SDNs, manufactured using ETFD, but composed of fluorescence resonance energy transfer (FRET) dyes, intact particles were demonstrated to traverse an intact Caco-2 monolayer ([McDonald et al., 2012](#_ENREF_22)).

If intact SDNs enter the systemic circulation after oral administration, a differential passage across the blood brain barrier (BBB) may be predicted. Importantly, this may influence EFV-associated neurocognitive adverse events such as depression, anxiety and abnormal dreams ([Sanchez Martin et al., 2013](#_ENREF_28)), which are known to negatively impact treatment ([Leutscher et al., 2013](#_ENREF_18)).

One of the major obstacles to drug permeation into the brain is the battery of transport proteins within the brain endothelium ([Al-Ghananeem et al., 2013](#_ENREF_2), [Yilmaz et al., 2012](#_ENREF_35)). Many antiretrovirals are substrates for one or more drug transporters, which limit or completely extrude them from the brain ([Ene et al., 2011](#_ENREF_9)); however, the interaction of EFV with transporters has not been thoroughly characterised.

Although there have been genetic associations between ABCB1 polymorphisms with EFV pharmacodynamics ([Fellay et al., 2002](#_ENREF_10)) *in vitro* evidence indicates EFV is not a substrate for P-gp ([Leschziner et al., 2007](#_ENREF_17), [Janneh et al., 2009](#_ENREF_14)). Some evidence indicates EFV may be a substrate for BCRP (ABCG2) and an *ex vivo* model showed increased mucosal to serosal permeation of EFV in everted gut sacs ([Peroni et al., 2011](#_ENREF_25)). EFV may also be a substrate for one or more solute carrier organic anion transporters (SLCO), since its cellular accumulation was reduced by montelukast and estrone-3-sulphate ([Janneh et al., 2009](#_ENREF_14)). However, convincing data regarding substrate affinity of EFV for active transport systems have remained elusive.

Although the size of a nanoparticle may preclude interactions with transport proteins, other methods of cellular uptake may affect them. Endocytosis includes multiple mechanisms such as clathrin-mediated endocytosis, calveolae-mediated endocytosis and micropinocytosis, that mammalian cells have developed for the uptake of molecules from the extracellular environment ([Mukherjee et al., 1997](#_ENREF_23)). Macropinocytosis is typically involved in uptake of larger particles (<2µM), whereas clathrin-mediated (<300nm) and caveolae-mediated (<80nm) endocytosis may predominate for smaller particles ([Canton and Battaglia, 2012](#_ENREF_5)).

This study investigated differential uptake of EFV SDNs relative to an aqueous solution in the hCMEC/D3 cell line *in vitro*, which is derived from human microvascular brain endothelial cells ([Weksler et al., 2013](#_ENREF_33), [Dickens et al., 2012](#_ENREF_8), [Alfirevic et al., 2015](#_ENREF_3)). The hCMEC/D3 was selected for its frequent application as an *in vitro* model and characterisation as an immortalised BBB cell line. The HCMEC/D3 cell line exhibits many of the characteristics of the BBB, such as expression of protein necessary for tight junction formation, polarized expression of multiple transporter proteins (including P-gp, BCRP, organic cation transporters) ([Sekhar et al., 2017](#_ENREF_30), [Poller et al., 2008](#_ENREF_26)) and endocytic processes ([Ilina et al., 2015](#_ENREF_12)). In order to investigate the putative mechanisms, uptake was assessed in the presence and absence of broad-spectrum transport (influx and efflux) and endocytosis inhibitors.

**Methods**

**Materials**

Pharmaceutical grade α-tocopherol polyethylene glycol succinate (TPGS) was purchased from BASF (Royal Tunbridge Wells, UK). Pharmaceutical grade polyvinyl alcohol (PVA grade 4-88, MW 57-77,000, was purchased from Merck KGaA (Darmstadt, Germany). Chloroform and dichloromethane were purchased from Fisher Scientific (Loughborough, UK). Efavirenz was purchased from LGM Pharma (Chicago, US) and donated by CIPLA (Mumbai, India). EBM-2 media was purchased from Lonza (Slough, UK), Penicillin-Streptomycin, Chemically Defined Lipid Concentrate and HEPES were purchased from Invitrogen (Paisley, UK). Fetal Bovine Serum (FBS) gold were purchased from PAA, the Cell Culture Company (Cambridge, UK). The hCMEC/D3 cell line was a kind gift from Pierre-Olivier Couraud, (INSERM, Paris, France). All other consumables were purchased from Sigma Aldrich (Dorset, UK).

**Manufacture and physical characterisation of efavirenz solid drug nanoparticles**

**Preparation of Emulsion-Templated Freeze-Dried Monoliths Containing 70% Efavirenz (EFV).** Stock solutions of EFV (70 mg/mL in chloroform (CHCl3), poly(vinyl alcohol) (PVA, 22.5 mg/mL in water), and α-tocopherol polyethylene glycol succinate (TPGS, 22.5 mg/mL in water) were prepared. The three stock solutions were added to a sample tube in the ratio 100:90:45 (lμL) (EFV:PVA:TPGS) plus 265 μL water. The final solid mass ratio was therefore 70% EFV: 20% PVA: 10% TPGS (total solid mass 10 mg) in a 1:4 CHCl3 to water mixture (total volume 0.5 mL). The sample was emulsified using a Covaris S2x acoustic homogenization system for 30 s with a duty cycle of 20, an intensity of 10 and 500 cycles/burst in frequency sweeping mode. Immediately after emulsification, the sample was cryogenically frozen and lyophilized using a Virtis benchtop K freeze-drier for 48 h. The white dry porous product was stored at ambient temperature prior to analysis.

**Preparation of Emulsion-Templated Freeze-Dried Monoliths Containing 69% Efavirenz (EFV) labelled with 1% of the dye 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt (DID).**

Preparation followed the same procedure as described above, however a sock solution of 70 mg/mL DID was prepared in CHCl3. The four stock solutions were added to a sample tube in the ratio 97:3: 90:45 (μL) (EFV:DID:PVA:TPGS) plus 265 μL water. The final solid mass ratio was therefore 69% EFV: 1% DID: 20% PVA: 10% TPGS (total solid mass 10 mg) in a 1:4 CHCl3 to water mixture (total volume 0.5 mL). The blue dry porous product was stored at ambient temperature prior to analysis.

**Preparation of Emulsion-Spray-Dried Powders Containing 70% Efavirenz (EFV).**

Stock solutions of EFV (280 mg/mL in dichloromethane (DCM), poly(vinyl alcohol) (PVA, 50 mg/mL in water), and α-tocopherol polyethylene glycol succinate (TPGS, 50 mg/mL in water) were prepared. The three stock solutions were added to a sample tube in the ratio 16:25.6:12.8 (mL) (EFV:PVA:TPGS). The final solid mass ratio was therefore 70% EFV, 20% PVA: 10% TPGS (total solid mass 6.4 g) in a 1:2.4 DCM to water mixture (total volume 54.4 mL). The sample was emulsified using a Hielscher UP400S ultrasonic processor equipped with H7 Probe at 70% output (50 W) for 90 seconds. Immediately after emulsification, the sample was spray dried on a benchtop spray dryer (BUCHI Mini-290) using an air-atomizing nozzle and compressed air as the drying gas. Spray drying process conditions were 5 mL/min solution flow rate and 65oC outlet temperature. The white powder was collected and stored at room temperature before analysis.

**Characterization of Aqueous EFV Nanodispersions**

Dynamic Light Scattering (DLS).Immediately prior to analysis, samples were dispersed by addition of water (1 mg/mL with respect to EFV content, therefore 7 mL for every 10 mg total solid mass) and vortex mixed to generate a uniform dispersion. Z-average diameter (*Dz*), zeta potential (ζ), polydispersity Index (PDI) and number average diameter (*Dn*) were determined by 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 7.03 was used for data analysis. ζ measurements were also carried out at 1 mg/mL, 25 °C, and an initial pH of 6.5, using disposable capillary zeta cells. Dz , ζ, PDI and *Dn* measurements were obtained as an average of three individual measurements and were obtained using the instrument’s automatic optimization settings.

Scanning Electron Microscopy (SEM): SEM images were recorded using a Hitachi S-4800 field emission instrument (FE-SEM) at 3 kV. Dry samples were placed onto aluminium stub with carbon tabs. The samples were gold coated for 2 minutes at 15 mA using a sputter-coater (EMITECH K550X) prior to imaging.

**Routine hCMEC/D3 culture**

hCMEC/D3 were cultured in EBM-2 media supplemented with FBS gold 5%, penicillin-streptomycin 1%, hydrocortisone 1.4μM, ascorbic acid 5μg/mL, chemically defined lipid concentrate 1/100, HEPES 10mM and bFGF 1ng/mL. All culture flasks and plates were coated with rat collagen type 1 for 1 hour prior to use. Cells were cultured at 37°C in 5% CO2. Cells were passaged every 3-4 days when confluent.

**MTT cytotoxicity assay**

Cells were seeded on plates pre-coated with collagen at 100µl of 1x105 cells/mL. The plates were then incubated for 24 hours at 37°C in 5% CO2 to allow cell adherence. Following incubation, the media was replaced with 100 μl of fresh media containing the drug at desired concentration (plus vehicle, 0.5% DMSO). Positive and negative controls were represented by no cells (representing 100% cell death) and by cells cultured in the presence of a vehicle control (representing 100% cell viability) respectively. The cells were then incubated for 1 hour. Following incubation with the drug, the media was removed and replaced by 20 μl of MTT reagent (5 mg/mL solution 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in Hanks balanced salt solution [HBSS]). The cells were incubated for 2 hours in the MTT reagent. Following incubation, 100 uL of lysis buffer (50% N-N-dimethylformamide in water containing 20% sodium dodecyl sulfate, 2.5% glacial acetic acid and 2.5% HCl, pH 4.7) was added to each well. Cells were incubated overnight at 37°C in 5% CO2 to allow complete cell lysis. Following incubation, the absorbance of each well was read using the using the TECAN GENios plate reader, with filters set to 560 nm.

**Impact of inhibition of drug transporters on efavirenz cellular accumulation**

Cells were seeded on pre-collagenated 6 well plates at a density of 2x106/mL and allowed to adhere overnight. Media was aspirated and replaced with 1mL fresh media containing 10μM (0.014μCi) of an aqueous solution of EFV or EFV SDNs (in the presence or absence of transporter inhibitors shown in Table 1) ([Dickens et al., 2012](#_ENREF_8)). Cells were incubated at 37°C in 5% CO2 in the presence of the drugs for 1 hour. Following incubation, 100µl of media was aspirated and added to scint vials with 4mL goldstar scintillation fluid (extra cellular drug content). Cells were then washed with ice-cold HBSS x3. Following washes, 1mL of trypsin was added to the cells then incubated at 37°C in 5% CO2 for 15 minutes. The trypsin was then aspirated and added to scint vials with 4mL goldstar scintillation fluid (intracellular content). Cellular accumulation ratios (CAR) were calculated using the following formula (where DPM = disintegrations per minute):

Cellular volumes were determined using the ScepterTM cell counter 2.0 (Merck Millipore, Billerica USA). Cell volumes were taken from a mean of 3 replicates, hCMEC/D3 volume 2.27pl.

**Impact of endocytosis inhibition on efavirenz accumulation**

Cells were seeded on pre-collagenated 6 well plates at a density of 2x106/mL and allowed to adhere overnight. Media was aspirated and replaced with 1mL fresh media containing dynasore (100µM), indomethacin (100µM) or cytochalasin B (5µM) and incubated for 30 minutes at 37°C, 5% CO2 (Table 1) ([Kee et al., 2004](#_ENREF_15), [Sato et al., 2009](#_ENREF_29)). Following incubation, the media was aspirated and replaced with fresh media containing 10μM of EFV aqueous solution formulation EFV or EFV SDNs. Cells were incubated at 37°C in 5% CO2 in the presence of the drugs for 1 hour. Following 1 hour incubation, 1mL of media was aspirated and added to 1.5mL eppendorf tubes (extra cellular content). Cells were then washed with ice-cold HBSS x3. Following washes, 1mL of trypsin was added to the cells then incubated at 37°C in 5% CO2 for 15 minutes. The trypsin was then aspirated and added to 1.5mL eppendorf tubes (intracellular content). Samples were stored at -80°C until analysis via LC-MS/MS.

Cellular volumes were determined using the ScepterTM cell counter 2.0 (Merck Millipore, Billerica USA). Cell volumes were taken from a mean of 3 replicates, hCMEC/D3 volume 2.27pl.

**Sample treatment and Quantification of Efavirenz via LC-MS/MS**

Efavirenz was extracted by protein precipitation. 20µl of internal standard (lopinavir 1000ng/mL) was added to 100µl of sample (20% acetonitrile [ACN] was added to cell culture medium to aid efavirenz dissolution), standard or QC which was then treated with 400µl of ACN. Samples were then centrifuged at 4000g for 10 minutes at 4°C. The supernatant fraction was transferred to a fresh glass vial and placed in a rotary vacuum centrifuge at 30°C to evaporate. Samples were then and reconstituted in 140μl of H2O:ACN (60:40). 100μl of the sample was then transferred into 200μl chromatography vials. 5μl of each sample was injected for analysis.

Quantification was achieved via LC-MS/MS (TSQ Endura, Thermo Scientific) operating in negative mode ([Curley et al., 2016](#_ENREF_7)). The following ions were monitored for quantification in selected reaction monitoring scan: efavirenz (m/z 315 > 242.1, 244.0 and 250.0) and internal standard, lopinavir (m/z 627 > 121.2, 178.1 and 198.1). A stock solution of 1 mg/mL efavirenz was prepared in methanol and stored at 4°C until use. A standard curve was prepared in EBM-2 cell culture medium by serial dilution from 500 ng/mLto1.9 ng/mL and an additional blank solution was also used.

Chromatographic separation was achieved using a multi step gradient with a Hypersil gold C-18 column (Thermo scientific) using mobile phases A (100% H2O, 5mM NH4HCO2) and B (100% ACN, 5mM NH4HCO2). Chromatography was conducted over 8.55 minutes at a flow rate of 300 µl/min. At the start of each run, mobile phase A was 90% until 0.1 minutes when mobile phase B was increased to 86% at 0.5 minutes. Mobile phase B was then gradually increased to 92% over 4.5 minutes. Mobile phase B was then increased to 97% at 5.1 minutes which was held until 6 minutes. Mobile phase A was then increased to 90% and held till the termination of the run at 8 minutes. Inter- and intra- assay variance in accuracy and precision were <15%.

**Assessing efavirenz solid drug nanoparticle uptake by flow cytometry**

Cells were seeded on pre-collagenated 6 well plates at a density of 2x106/mL and allowed to adhere overnight. Media was aspirated and replaced with 1mL fresh media containing dynasore (100µM), indomethacin (100µM) or cytochalasin B (5µM) and incubated for 30 minutes. Following incubation, media was aspirated and replaced with fresh media containing 10μM of EFV SDNs containing 1% DiD or control (SDN DiD particles dissolved in 50% H2O and 50% MeOH) ([Liptrott et al., 2015](#_ENREF_19)). Cells were incubated at 37°C in 5% CO2 in the presence of the drugs for 1 hour. Following 1 hour incubation, media was aspirated and cells were then washed with ice-cold HBSS x3. Following washes, 1mL of trypsin was added to the cells, then incubated at 37°C in 5% CO2 for 5 minutes. The cells were then aspirated and transferred to 1.5mL eppendorf tubes. Samples were then centrifuged at 2000rpm for 5 minutes at 10°C. The trypsin was aspirated and the cell pellet was re-suspended in 500µl of Macs buffer for analysis by flow cytometry using a MACSQuant analyser. Side scatter outcome was set to logarithmic and detected at a scattering angle of 90°. Flow rates were chosen such that less than 2000 events/s were recorded to prevent coincidence. For each measurement, a total number of 10,000 events were recorded ([Liptrott et al., 2015](#_ENREF_19)). Data were analysed using MACSQuantify software.

**Statistical analysis**

All data were assessed for normality using the Shapiro-Wilk test. Statistical analysis was performed by unpaired t-test (for normally-distributed data) or Mann-Whitney U test (for non-normally distributed data) and significance was defined as P < 0.05 (calculated in SPSS v21). All data are given as mean with standard deviation. IC50 values were calculated in Prism v6.0.

**Results**

**Production and physical characterisation of efavirenz solid drug nanoparticles**

The production of EFV SDNs was conducted using two emulsion-based techniques; ETFD and emulsion spray drying (ESD), Figure 1. ETFD utilizes the rapid cryogenic freezing of an oil-in-water emulsion containing a volatile water-immiscible solvent dispersed phase containing dissolved hydrophobic compounds, Figure 1A. The emulsion is stabilized by the presence of water-soluble polymers and surfactants within the continuous aqueous phase. Freeze-drying of the frozen emulsion leads to a porous monolithic structure, which readily disperses to yield stabilised nanoparticles of the hydrophobic compounds on addition of water ([Zhang et al., 2008](#_ENREF_37)). The incorporation of two hydrophobic compounds within the dispersed organic phase leads to multi-component nanoparticles and the inclusion of the fluorescer DiD to form EFV/DiD SDNs has been recently reported using ETFD ([Liptrott et al., 2015](#_ENREF_19), [Giardiello et al., 2012](#_ENREF_11)). Dispersed dual-component EFV/DiD nanoparticles were analysed by dynamic light scattering (DLS) and shown to have an average hydrodynamic diameter (*D*z) = 295 ± 25 nm with a polydispersity (PDI) = 0.37. Zeta potential measurements ( = -18 ± 0.5 mV) were consistent with earlier reports.

EFV SDNs were also formed using ESD which rapidly dries the emulsion, containing EFV dissolved within the dispersed organic solvent phase, after atomisation into a stream of hot air, Figure 1B. The spray dryer employed for this study utilized a two-fluid nozzle and generated spherical powder particles with sizes ranging from < 1- 10 µm, as judged by scanning electron microscopy (SEM) imaging, Figure 2.

The spray dried powder particles showed a dimpled crust morphology (Figure 2A&B) that is consistent with the external surface of the atomized droplets of emulsion drying quickly within the gas stream, followed by removal of residual volatile material (water and organic solvent) in subsequent stages (Figure 1B). In contrast, SEM analysis of ETFD monoliths showed a convoluted open morphology (Figure 2C&D) with little evidence of ice crystal growth or removal that may be seen in materials that have been freeze dried directly from aqueous solutions ([Zhang et al., 2005](#_ENREF_36)). Dispersion of ESD powders into water resulted in nanodispersions that were also studied by DLS and found to have similar values to the ETFD SDNs containing 1 wt% DiD a *Dz* value of 250 ± 25 nm, PDI = 0.26 and  = -10 ± 0.1 mV.

The conformity of size and physical properties across the ETFD and ESD techniques suggested no inherent or meaningful difference (within error) between the two samples other than the presence of DiD. The use of the two techniques was required to allow correlation of the readily-traceable EFV/DiD nanoparticles, produced by ETFD due to the prohibitive cost of DiD, with an ESD EFV nanoformulation that is progressing towards human clinical trial. Imaging of the SDN single and dual-component particles after dispersion is significantly hampered by the presence of water-soluble polymer and surfactant excipients as reported previously ([Zhang et al., 2008](#_ENREF_37)).

**Cytotoxicity of efavirenz solution, efavirenz SDNs and inhibitors in hCMEC/D3 cells**

Prior to accumulation studies, it was necessary to determine the concentrations of EFV in solution, EFV SDNs and the various inhibitors that did not affect cell viability. To assess the cellular toxicity of the compounds used in accumulation studies, a concentration range of each drug was assessed using the MTT assay.

Table 1 summarises the IC50 data for all drugs and formulations used in the study. EFV solution and EFV SDNs were assessed over the range of 0.19µM to 100µM EFV concentration. No statistically significant difference was observed in IC50 between the aqueous solution and the SDN (P = 0.49).

Transporter inhibitors were assessed over the final concentration range of 0.98µM to 500µM. The IC50 could not be generated for amantadine, cyclosporine A, naringin or corticosterone as cytotoxicity was not observed under these conditions. Endocytosis inhibitors were assessed over the range of 0.39µM to 200µM. Similarly; the IC50 could not be generated for dynasore and indomethacin, as cytotoxicity was not observed under the experimental conditions.

**Impact of drug transporter inhibition on efavirenz accumulation**

Cellular accumulation studies were performed in the hCMEC/D3 cell line and a panel of transporter inhibitors (Table 2) was employed to probe potential interactions with an EFV aqueous solution with transporters. Secondly the accumulation of EFV SDNs in the presence of transporter inhibitors was probed to identify any differences in cellular accumulation due to nanoformulation (Figure 3).

The screen of transporter inhibitors demonstrated no effect on the accumulation ratio of the EFV aqueous solution when in the presence of cyclosporine A (CAR = 70.3 ± 27.7, P = 0.51), naringin (CAR = 89.6 ± 8.1, P = 0.18) or corticosterone (CAR = 80.8 ± 12.5, P = 0.96). However, the accumulation ratio was reduced in the presence of amantadine (CAR 64.7 ± 6.0, P = 0.03).

The accumulation ratio of EFV after incubation with the SDN formulation was not affected by amantadine (CAR = 91. 9 ± 22.7, P = 0.40), cyclosporine A (CAR = 73.1 ± 17.2, P = 0.40), naringin (CAR = 89.1 ± 15.7, P = 0.38) or corticosterone (CAR = 77.2 ± 12.2, P = 0.57).

**The Effects of Inhibitors of Endocytosis on Efavirenz Accumulation**

In addition to transport proteins, endocytosis is a potential mechanism for cellular uptake, especially for particulates. In order to probe the impact of endocytosis on the uptake of EFV aqueous solution and EFV SDNs, a panel of endocytosis inhibitors was screened (Table 1) and the resulting data are presented in Figure 4.

The screen of endocytosis inhibitors demonstrated no effect on the accumulation ratio of either the aqueous solution (CAR = 92.7 ± 47.4) or SDNs (CAR = 118.7 ± 41.0) when in the presence of dynasore (aqueous CAR = 96.7 ± 13.1, P = 0.87, SDN CAR = 121.8 ± 18.0, P = 0.90), indomethacin (aqueous CAR = 137.6 ± 60.0, P = 0.29, SDN CAR = 119.4 ± 16.5, P = 0.98) or cytochalasin B (aqueous CAR = 96.5 ± 47.2, P = 0.91, SDN CAR = 141.4 ± 35.4, P = 0.43).

**The effects of inhibitors of endocytosis on nanoparticle uptake using flow cytometry**

The uptake of DiD-labeled EFV SDNs (4.02 ± 0.86 relative fluorescence units [RFU]) were significantly reduced (Figure 5) by dynasore (0.91 ± 0.45 RFU, P = 0.001). Indomethacin had no effect on uptake of DiD-labeled EFV SDNs (3.44 ± 0.58 RFU, P = 0.307), whereas cytochalasin B significantly increased uptake (5.40 ± 0.70 RFU, P = 0.048; Figure 6).

The uptake of dissolved DiD-labeled EFV-SDNs (8.75 ± 1.14 RFU) was significantly reduced by dynasore (0.43 ± 0.13 RFU, P = <0.001) and indomethacin (4.45 ± 0.54 RFU, P = <0.001). Cytochalasin B significantly increased the uptake of dissolved DiD-labeled EFV SDNs (12.12 ± 0.20 RFU, P = <0.001; Figure 6).

**Discussion**

The data presented here demonstrated cellular accumulation of aqueous EFV was reduced by amantadine (19.5% vs control) but this was not the case when incubating with EFV SDNs. These data indicate aqueous EFV may be a substrate for one of the OCT transporters and the SDN formulation may mitigate the influence of these transporters. Further transport studies are required to fully confirm these observations using multiple time points and a range of concentrations of substrate and inhibitor. The SDN formulation procedure generates particles with sizes of 322 ± 29nm. Particles of this size are subjected to endocytosis and nanoformulation has been used previously to reduce the impact of the transporter, BCRP ([Wong et al., 2006](#_ENREF_34), [Canton and Battaglia, 2012](#_ENREF_5)). Further studies are required to fully elucidate the interactions of both the EFV aqueous solution and SDN formulations.

The data generated utilising the endocytosis inhibitors provided some contrasting data. When the drug accumulation ratio was examined, the endocytosis inhibitors had no effect on either the aqueous solution or the SDN formulation of EFV. Interestingly, uptake of the DiD-labelled SDNs was reduced by dynasore, indicating the role of dynamin-mediated uptake. However, the uptake of the dissolved DiD-labelled SDNs was reduced by both dynasore and indomethacin. This may indicate the incomplete dissolution of the SDN particles, or that the dissolution process has altered the structure of the SDN particles, enabling uptake via calveoli-dependent endocytosis. The data also demonstrated higher uptake for the dissolved DiD SDN particles. This is not entirely unexpected, as DiD is a lypophilic dye and would be expected to readily pass through the lipid cell wall. This limitation could be resolved by use of a cell impermeable dye, such as propidium iodide (fluorescence only observed when associated with intracellular nucleic acids). Propidium iodide has previously been incorporated into rhodamine B isothiocyanate–labeled silica nanoparticles to indicate cellular uptake ([Neumeyer et al., 2011](#_ENREF_24)). These data indicate the importance of measuring both nanoparticle uptake and drug uptake. Although the drug concentrations may be equal in both preparations, the mechanism of cell entry and consequently intracellular fate may be significantly different.

The hCMEC/D3 cell line has been demonstrated to express many of the proteins found in the enterocytes of the BBB, making this cell line a suitable model for probing interactions at the BBB ([Weksler et al., 2013](#_ENREF_33)). One of the limitations of the hCMEC/D3 cell line, is the formation of tight junctions ([Stanimirovic et al., 2015](#_ENREF_32)). The BBB is characterised by the presence of tight junctions, limiting paracellular transport. In order to fully replicate the presence of tight junctions, the hCMEC/D3 cell line requires technically demanding and prohibitively expensive culture conditions. It has been demonstrated to reproduce the tight junctions observed *in vivo*, but sheer stress induced by a pulsatile flow was required ([Cucullo et al., 2008](#_ENREF_6)). Although accumulation experiments are useful for identifying potential mechanisms of uptake at the BBB, they do not demonstrate permeability across the BBB.

One of the limitations of investigating transporter interactions in cell lines is the lack of specificity in pharmacological transport inhibitors. Lack of specificity is also a consideration when examining inhibitors of endocytosis ([Ivanov, 2008](#_ENREF_13)). In addition to other mechanisms of endocytosis, endocytosis inhibitors have also been shown to influence transport proteins, such as inhibition of ABCC1 by indomethacin ([Leite et al., 2007](#_ENREF_16)). Cytochalasin B was shown to disrupt actin filaments and increase the intracellular accumulation of doxorubicin. Therefore, the conclusions that are drawn here are based on the known interactions of the inhibitors used but inhibition of other, as yet unknown, processes cannot be ruled out with this strategy. Further studies utilising more specific methods (such as knock-down models, small interfering RNA and oocyte uptake experiments) may be useful to complement *in vivo* studies and further elucidate the interactions relevant to distribution.

An additional limitation of the experimental design was that all experiments were conducted at 1 hour. However, Liptrott *et al* previously demonstrated that the cellular accumulation ratio of EFV SDNs varied over 24 hours in THP-1 cells, with highest accumulation achieved within the first hour ([Liptrott, 2012](#_ENREF_20)).

The presented data indicate that EFV SDNs may not traverse the BBB via the same mechanisms as dissolved EFV molecules. Amantadine significantly reduced EFV uptake, while there was no effect observed with the SDNs. Additionally, dynasore reduced the uptake of DiD-labelled SDN particles indicating a role of dynamin-mediated endocytosis.

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