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Authors	O'Neill, Martin J.;Guo, Jianfeng;Byrne, Colin;Darcy, Raphael;O'Driscoll, Caitríona M.
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Mechanistic studies on the uptake and intracellular trafficking of novel cyclodextrin transfection complexes by intestinal epithelial cells

Martin J. O' Neill[1], Jianfeng Guo[1], Colin Byrne[2], Raphael Darcy[2], and Caitriona M. O' Driscoll[1].

[1]Pharmacodelivery group, School of Pharmacy, University College Cork, Ireland.

[2]Centre of Synthesis and Chemical Biology, Conway Institute, University College Dublin, Ireland

Corresponding author: O' Driscoll, C.M. caitriona.odriscoll@ucc.ie,

Telephone: +353 21 490 1396

Fax: +353 21 490 1656

ABSTRACT

Oral delivery of gene therapeutics would facilitate treatment of local intestinal disease, including colon cancer and inflammatory bowel disease, thus avoiding invasive surgery. The aims of this study were to investigate; if the orientation of the lipid tail on the cyclodextrin (CD) influenced the efficacy of a novel poly-6-cationic amphiphilic CD to transfect intestinal enterocytes; the endocytotic uptake pathway(s), and, the intracellular trafficking of the CD.DNA complexes. Inhibitors of clathrin- and caveolae- mediated endocytosis and macropinocytosis were used to determine the mechanism(s) of CD.DNA uptake by both undifferentiated and differentiated Caco-2 cells. Cell surface heparan sulphate proteoglycans were involved in the association of CD.DNA complexes with undifferentiated Caco-2 cells. Complexation of pDNA with CD facilitated significant levels of pDNA uptake and gene expression (comparable to PEI) in both undifferentiated and differentiated Caco-2 cells. Disruption of intracellular vesicular trafficking reduced transfection activity. CD was also capable of transfecting the more physiologically relevant differentiated Caco-2 model. Macropinocytosis was responsible for the uptake of CD.DNA transfection complexes by both undifferentiated and differentiated Caco-2 cells. The ability of this novel CD to transfect differentiated intestinal cells indicates the potential of this vector for oral gene delivery.

Keywords: Non-viral gene therapy, cyclodextrins, intestinal delivery, uptake pathways, intracellular trafficking.

1. INTRODUCTION

The intestine is a compelling target for local delivery of gene therapeutics. Indeed, the intestinal tract is accessible by oral, rectal, as well as endoscopic means; thus avoiding invasive surgery. Local expression of therapeutic proteins may have therapeutic benefit in the treatment of inflammatory bowel disease, intestinal cancer, and the intestinal symptoms of cystic fibrosis (O' Neill *et al.* 2011) (Page and Cudmore 2001). Furthermore, the intestine can potentially provide a route of entry into the bloodstream for locally expressed therapeutic proteins such as Factor VIII in haemophilia patients (Bowman *et al.* 2008), or indeed for gene therapies to distant disease sites.

However, if the intestinal delivery of gene therapeutics is to be successful, a number of distinct challenges must be overcome. While the harsh intestinal environment presents a number of distinct extracellular barriers (degradative enzymes, mucus), barriers at the cellular level are also significant. Plasmid DNA (pDNA) is a high molecular weight, negatively charged, hydrophilic material, which is generally poorly taken up by cells. Therefore, the design of gene delivery vectors (GDVs) that can effectively complex pDNA and mediate significant levels of cellular uptake and transgene expression is key to achieving effective delivery. Cyclodextrins (CDs) are macrocyclic oligosaccharides and are interesting core molecules for the development of non-viral GDVs. They are non-immunogenic, have well researched pharmaceutical profiles, and are chemically versatile in that they can be modified on either face with multiple functional groups such as poly(ethylene glycol) (PEG), cationic groups, or lipid chains to facilitate optimal pDNA complexation and transfection (Cryan *et al.* 2004) (McMahon *et al.* 2008). Therefore, a vast library of modified CDs with distinctive properties (e.g. neutral, cationic, anionic, amphiphilic)

can be synthesised. In addition, the unique inclusion complex forming properties of the hydrophobic cavity of CDs can facilitate functionalisation with additional groups (e.g. PEG) by non-covalent means if they are conjugated to hydrophobic moieties capable of inclusion into the CD cavity (e.g. adamantane-PEG) (Davis 2009). This further demonstrates the inherent flexibility associated with using CDs as GDVs. The β-CD utilised in this work, the synthesis of which has been described previously (Byrne *et al.* 2009), is modified on its primary face at the 6-positions with cationic amino groups and at the 2-positions on its secondary face with lipid chains (Figure 1b,c). Selective modification of the 2-positions yields CDs with 7 lipid chains, and avoids lipid attachment at the 3-positions which would give an excess of hydrophobicity and low water solubility (Byrne *et al.* 2009). The positioning of these cationic and lipophilic side chains is the inverse of previous CD based GDVs developed by our group (Figure 1a), which were shown to be successful transfection agents (Cryan *et al.* 2004).

In terms of cellular targets, the absorptive cells of the intestine, enterocytes, represent the major cell type, comprising approximately 90% of the intestinal epithelium (Jung *et al.* 2000). Enterocytes present useful targets for certain gene therapy strategies, since, given the underlying rich blood supply, transfected enterocytes could be utilised as "protein factories" where the expressed therapeutic protein is secreted into the bloodstream (Page and Cudmore 2001). Furthermore, locally expressed proteins may have therapeutic potential in the treatment of intestinal diseases (e.g. inflammatory bowel disease). The uptake pathway(s) by which GDV.DNA complexes enter cells is an important consideration and can have a major bearing on transfection efficiency (Douglas *et al.* 2008). Endocytosis refers to the energy dependent vesicular uptake of extracellular material through invagination and

pinching off of sections of the plasma membrane; and represents the major entry route of GDV.DNA complexes (Khalil *et al.* 2006). Endocytosis can be classified into 2 broad categories- phagocytosis and pinocytosis. Pinocytosis can be subdivided further into clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME), clathrin- and caveolae-independent endocytosis, and macropinocytosis. These differ in terms of the size of vesicles formed, the mechanism of vesicle formation and the proteins involved (Khalil *et al.* 2006). Importantly, the intracellular fate of endocytosed complexes differs depending on the route of entry, and therefore the uptake pathway can have a major impact on gene expression. The endocytotic mechanism(s) by which GDV complexes enter cells depends on both the cell type and the physiochemical characteristics of the complexes. Using models of enterocytes (such as Caco-2 cells) and other polarised epithelia (such as MDCK cells), a number of endocytotic processes have been implicated as uptake pathways for delivery systems and nanoparticles in enterocytes (Kitchens *et al.* 2007) (Ma and Lim 2003) (Ragnarsson *et al.* 2008) (Roger *et al.* 2009) (Gao *et al.* 2008).

Knowledge regarding the means of transfection complex entry can potentially allow for more rational design of CDs with increased activity. Therefore, here we examine the uptake and transfection of undifferentiated Caco-2 cells (human colon carcinoma) by novel water soluble CD.DNA complexes and elucidate the means of cellular association and entry. These results are compared to those achieved in differentiated Caco-2 cells as the latter represents a more physiological model of gut enterocytes.

2. MATERIALS AND METHODS

2.1. Materials

The poly-6-cationic amphiphilic cyclodextrin (CD) was synthesised as previously described (Byrne *et al.* 2009). Its full name is heptakis[6-(2-amino-ethylthio)-6-deoxy-2-*O*-octyloxycarbonylethylsulfanylpropyl]-β-cyclodextrin hepta-*N*-trifluoroacetate. Lipofectamine™ 2000 (Lf2000) and jetPEI™ (PEI) were supplied by Invitrogen and Polyplus-transfection, respectively. Caco-2 cells (human colon adenocarcinoma) were obtained from the European Collection of Animal Cell Cultures (ECACC). Unless otherwise stated all other materials were sourced from Sigma.

2.2. Caco-2 cell culture

Caco-2 cells (passage 26-50) were cultured in DMEM supplemented with 10% foetal bovine serum (FBS) and 2 mM L-glutamine, and were maintained in a humidified 37^{o}C incubator with 5% CO_2. For undifferentiated cell studies, cells were seeded in 24-well plates at a density of 100,000 cells/cm^2 24 hours prior to transfection. For differentiated cell studies, Caco-2 cells were seeded on 24 or 12 mm diameter Transwell™ plates at a density of 63,000 cells/cm^2 (Cryan and O'Driscoll 2003). Fresh cell culture medium was placed in the apical and basolateral chambers every 48 hours, and cells were cultured for 19-21 days to facilitate differentiation.

2.3. Preparation of transfection complexes

The pCMV*luc* plasmid contains the reporter gene that encodes the firefly luciferase enzyme under the control of the cytomegalovirus (CMV) promoter/enhancer. This was obtained from Bath University, UK. It was propagated in DH5α *E. coli* and isolated using EndoFree Plasmid Mega kits in accordance with the manufacturer's instructions (Qiagen). For uptake studies, pCMV*luc* was

fluorescently labelled with fluorescein using *Label IT*® Nucleic Acid Labelling Kits (Mirus) according to manufacturer's instructions. A *Label* IT® Tracker™ Reagent to pDNA volume:weight (v:w) ratio of 0.5:1 was used, and the labelling reaction was performed for 2 hours at 37^{o}C. Unreacted *Label* IT® reagent was subsequently removed from the labelled plasmid by ethanol precipitation. The CD was weighed using a microbalance and dissolved in chloroform to create a 1 mg/ml solution. Subsequent to this a stream of nitrogen was applied to remove the solvent. The remaining CD film was rehydrated with water (1 mg/ml CD solution), followed by sonication for size reduction. A solution of pDNA was then mixed with CD to achieve a CD:pDNA mass ratio of 10, and 15-20 minutes were allowed for complexation. PEI and Lf2000.DNA transfection complexes were prepared as per manufacturer's instructions. Briefly, PEI.DNA complexes were prepared at a nitrogen/phosphate (N/P) ratio of 5 in 150 mM NaCl; while Lf2000 based transfection complexes were prepared in serum free Opti-MEM® media (Invitrogen) at a v:w ratio of Lf2000 to pDNA of 3.

2.4. Particle size and zeta potential measurement

The Z-average size and zeta potential of CD.DNA complexes were determined using a Zetasizer Nano ZS instrument (Malvern) at 25^{o}C in water. Scattered light was measured at an angle of 170^{o} (Z-average size) and 12.8^{o} (zeta potential).

2.5. CD.DNA-Cell binding studies

Undifferentiated Caco-2 cells were incubated with fluorescein labelled CD.DNA complexes (1 µg pDNA/well) for 1 hour at 4^{o}C. Normal serum-containing growth medium was added to 0.2% trypsin-0.1% EDTA or 0.1% EDTA treated cells,

which were subsequently centrifuged at 1,000 rpm for 7 minutes at room temperature. The supernatant was carefully removed and cells were washed and re-suspended in phosphate buffered saline (PBS). The fluorescence associated with 10,000 cells was measured with a BD FACSCalibur flow cytometer and data was analysed using Cell Quest Pro software.

To assess the contribution of heparan sulphate proteoglycans to CD.DNA binding and uptake, undifferentiated Caco-2 cells were treated for 1 hour at 37^{o}C with 10 unit/ml of heparinase III in a 0.1% bovine serum albumin (BSA), 0.1% glucose, PBS solution containing calcium. Cells were subsequently washed and serum free DMEM was added along with CD.DNA complexes. The level of CD.DNA binding and uptake was assessed 2 hours later. For uptake studies, uninternalised complexes were removed prior to trypsinisation by washing cells with PBS (containing Ca^{2+} and Mg^{2+}) and incubation with CellScrub™ buffer (Genlantis) for 10-15 minutes at room temperature. In a separate competition based experiment, soluble heparin (5 and 10 µg/ml) was included in the transfection media and uptake levels were determined 2 hours later.

2.6. Uptake and transfection studies

Fluorescently labelled pDNA complexed with GDVs was incubated with undifferentiated Caco-2 cells (1 µg pDNA/well=0.5 µg pDNA/cm^2) for 1 hour at 4^{o}C to saturate cell surface binding. Subsequently, cells were incubated at 37^{o}C for 0, 0.25, 1, 2, and 3 hours. Uninternalised complexes were removed with CellScrub™, and the number of fluorescent positive cells was assessed by flow cytometry.

Reporter gene expression levels were considered by luciferase assay. Undifferentiated Caco-2 cells were incubated with unlabelled transfection complexes

for 1 hour at 4^{o}C, and subsequently at 37^{o}C for 3 hours. Uninternalised complexes were removed, and cells incubated for 24 hours to facilitate maximal luciferase expression. 20 µl of cell lysate was added to 100 µl of luciferase assay substrate (Promega) and luminescence was measured after 10 seconds using a Junior LB 9059 luminometer. Luciferase gene expression was expressed in terms of relative light units (RLUs)/µg cellular protein as determined using a BCA (bicinchoninic acid) assay as per manufacturers instructions (Pierce). For differentiated Caco-2 cells, transfection complexes were administered apically (1.28 µg pDNA/cm^{2}). Complexes were incubated with cells for 4 hours at 37^{o}C, after which time cells were rinsed twice with serum free DMEM, and incubated for a further 24 hours under normal growth conditions to facilitate luciferase expression. For uptake studies, uninternalised complexes were removed 4 hours post complex addition with CellScrub™ and pDNA uptake was assessed by flow cytometry.

For endocytosis inhibition experiments, undifferentiated Caco-2 cells were pre-treated with a number of inhibitor treatments at concentrations based on literature values (Table 1). Inhibition of CME was achieved through hypertonic media and cytosol acidification treatments as previously described (Hansen *et al.* 1993). Cells were subsequently incubated in the presence of inhibitor and CD.DNA complexes for 90 (mβCD, hypertonic media, and cytosol acidification) or 120 minutes (wortmannin, LY294002, genistein, chloroquine, and nocodazole) prior to uninternalised complex removal with CellScrub™ buffer. The level of uptake was assessed immediately by flow cytometry, while luciferase levels were determined 24 hours later. Inhibition of macropinocytosis with wortmannin and LY294002 was confirmed by measuring the uptake of the macropinocytosis marker, tetramethylrhodamine (TMR)-conjugated 10 kDa dextran (Invitrogen). Inhibition of CME by hypertonic media and cytosol

acidification treatments was ensured by measuring the uptake of AF488 labelled transferrin (Invitrogen).

For endocytosis inhibition experiments in differentiated Caco-2 cells, all inhibitor treatments and CD.DNA complexes were applied to the apical compartment. Differentiated Caco-2 cells were pre-treated with a number of inhibitors at defined concentrations (Table 1) prior to addition of CD.DNA complexes. Cells were incubated in the presence of inhibitor and complexes for 4 hours prior to removal of uninternalised complexes with CellScrub™ buffer and uptake analysis by flow cytometry.

2.7. Toxicity of endocytosis inhibitor treatments

The toxicity of endocytosis inhibitors was assessed in undifferentiated Caco-2 cells by MTT assay (Table 1). Caco-2 cells were seeded in 96-well plates at a density of 35,000 cells per well (Cryan and O'Driscoll 2003). 24 hours post seeding, cells were incubated with the different inhibitors for up to 3 hours, after which time 100 µl of normal inhibitor-free growth media was added to cells along with 20 µl of a 5 mg/ml MTT solution. Samples were incubated for 4 hours at 37^{o}C. Subsequently, the reaction product was solubilised with 100 µl DMSO and absorbance at 590 nm was quantified. The level of cell death was expressed as % dehydrogenase activity relative to control cells.

Differentiated Caco-2 cell monolayer integrity was monitored by recording transepithelial electrical resistance (TEER) values immediately (0 hour) and up to 5 hours post inhibitor addition through the use of a volt/ohmmeter with “chopstick” electrodes. Values were expressed as % TEER (Ωcm^2) relative to 0 hour TEER readings (Table 1).

2.8. Confocal microscopy

Fluorescently labelled CD.DNA complexes (Cy™5 labelled pDNA-Mirus) were incubated with undifferentiated Caco-2 cells (grown on round glass coverslips) for 3 hours at 37^{o}C. Uninternalised complexes were removed with CellScrub™ and cells were fixed with 3% paraformaldehyde. Cell nuclei were stained with Hoechst dye (Invitrogen) and coverslips were mounted in Dako mounting medium. Images were acquired using an Olympus FV 1000 confocal microscope, and processed using Olympus Fluoview and Adobe Photoshop/Illustrator software.

2.9. Statistical analysis

A student's t-test (two tailed, α=0.05) was used to test the significance between two mean values.

3. RESULTS

3.1. Poly-6-cationic amphiphilic CD

The modified CD used in this study contains amino groups on the primary-hydroxyl face and lipid chains on the secondary-hydroxyl face (Figure 1b and c). Rendering cationic CDs amphiphilic through conjugation of lipid chains has previously been shown to increase transfection activity (Cryan *et al.* 2004) (McMahon *et al.* 2008). This CD was capable of complexing pDNA as assessed by a gel retardation assay (data not shown) and formed cationic nanoparticulate complexes (154 ± 12 nm; 48.3 ± 5.5 mV) at a CD:pDNA mass ratio of 10 (determined by dynamic light scattering measurements). The size and charge of these complexes was similar to transfection complexes prepared from previous CDs (Figure 1a) (Cryan *et*

al. 2004) (McMahon *et al.* 2008) and indicates that the side chain orientation does not have a major bearing on the physicochemical characteristics of the complexes. The cellular association, uptake and processing of this novel inverted CD were examined in an effort to aid design of CD vectors with improved transfection activity.

3.2. Association of CD.DNA complexes with Caco-2 cells

Caco-2 cells were used for all cell based studies as these are a commonly used model of gut enterocytes. To determine if cell surface proteins were involved in CD.DNA complex binding to Caco-2 cells, cells were pre-incubated with these fluorescent complexes for 1 hour at $4^{o}C$ to saturate cell surface binding, and subsequently detached with trypsin-EDTA or EDTA alone, prior to analysis by flow cytometry. Trypsin cleaves the protein links by which adherent cells attach to the surface of tissue culture plates. However, it also cleaves a range of other membrane surface proteins, and therefore complexes associated with trypsin cleavable surface proteins. In contrast, EDTA-mediated cell detachment is due to its cation-chelating properties and hence complexes associated with surface proteins remain attached to the cells. Trypsinisation resulted in a significant drop ($P<0.05$) in the number of fluorescent positive cells of approximately 70% as compared with EDTA-detached cells (Table 2) and indicates that CD.DNA binding to Caco-2 cells is mediated by trypsin-cleavable cell surface proteins.

Proteoglycans are a group of cell surface proteins commonly associated with non-viral GDV binding to cells (Wiethoff and Middaugh 2003). To examine whether heparan sulphate proteoglycans are involved in the binding of CD.DNA complexes to Caco-2 cells, Caco-2 cells were pre-treated with the glycosaminoglycan lyase heparinase III (Table 2), which specifically cleaves heparan sulphate proteoglycans.

Heparinase III treatment resulted in significant drops in both the cellular association (80%) and uptake (25%) of CD.DNA complexes ($P<0.05$) (Table 2). Furthermore, inclusion of exogenous soluble heparin in the transfection medium led to significant reductions ($P<0.05$) in CD-mediated pDNA uptake (Table 2) and reporter gene expression (data not shown) further supporting the hypothesis of proteoglycan involvement in CD.DNA-cell binding.

3.3. Uptake kinetics of transfection complexes in undifferentiated Caco-2 cells

The uptake kinetics of CD.DNA complexes were compared with those for two of the most commonly used GDVs, polyethyleneimine (PEI) and Lipofectamine™ 2000 (Lf2000) (Figure 2a). CD.DNA uptake was significantly higher than that of the other vectors after 0.25 and 1 hour incubations with Caco-2 cells ($P<0.05$). CD and Lf2000 complex uptake was comparable after 2 and 3 hour incubations with Caco-2 cells, and significantly higher than that observed for PEI.DNA complexes ($P<0.05$). The number of cells positive for CD and Lf2000.DNA complexes was approximately 5 times higher than that achieved by PEI. The uptake of CD.DNA complexes by Caco-2 cells was also confirmed by confocal microscopy (Figure 2b).

The transfection activity of CD.DNA complexes was compared with that mediated by PEI and Lf2000 (Figure 2c). Lf2000 proved the most efficient GDV demonstrating approximately 2-fold higher luciferase expression than either PEI or CD ($P<0.05$). Previous CDs with lipid modified primary faces and cationic secondary faces achieved comparable transfection levels to the commercial vectors DOTAP and Lipofectin (Cryan *et al.* 2004) (McMahon *et al.* 2008). Therefore, the similar transgene expression levels achieved here relative to PEI indicate that CDs with inverted side chain positioning retain their transfection activity.

3.4. Endocytosis of CD.DNA complexes by undifferentiated Caco-2 cells

To assess whether endocytosis and/or passive mechanisms are involved in the uptake of CD.DNA complexes, transfection experiments were carried out at 4 and 37^{o}C. CD.DNA uptake and transfection (Figure 3a and b) were reduced by >90% when cells were incubated at 4^{o}C ($P<0.05$) indicating that endocytosis was the principal route of entry.

In an effort to elucidate the specific endocytotic uptake pathway(s) utilised by CD.DNA complexes in undifferentiated Caco-2 cells, a number of endocytosis inhibitor treatments were used (Table 1). The effect of inhibitor treatments on CD.DNA uptake was assessed by flow cytometry (Figure 3a). However, given that successful uptake does not necessarily translate into successful transgene expression, the relationship between the method of CD.DNA uptake and transfection was also examined (Figure 3b). Pretreating Caco-2 cells with mβCD resulted in a significant drop ($P<0.05$) in both CD.DNA uptake (~80%) and transfection (>90%) (Figure 3a & b). MβCD treatment has been reported to inhibit a range of endocytotic processes including CME (Zuhorn *et al.* 2002) and CvME (Lamaze and Schmid 1995) as well as macropinocytosis (Grimmer *et al.* 2002) and was used here as a general endocytosis inhibitor. Both wortmannin and LY294002 treatments resulted in significant drops ($P<0.05$) in the level of CD.DNA uptake (~40%) and transfection (~60%). This indicates that macropinocytosis is involved in the uptake and successful transfection of CD.DNA complexes by Caco-2 cells. Conflicting results were observed post CME inhibition treatments. Treatment with 0.45 M sucrose (hypertonic media) resulted in significant drops ($P<0.05$) in both CD.DNA uptake and transfection of ~90%. This suggested that CME may have a major role in CD.DNA transfection. However, cytosol acidification treatment did not result in any decrease in

the level of CD.DNA uptake or transfection. Indeed, a slight increase in uptake and transfection was observed, although this was not statistically significant. Genistein treatment led to a significant reduction ($P<0.05$) in both CD.DNA uptake and transfection of approximately 40% (Figure 3a & b) implying a possible role for CvME.

The effects on uptake and transfection are unlikely a result of inhibitor-induced toxicity since inhibitor treatments were well tolerated by undifferentiated Caco-2 cells as assessed by MTT assay, with cell viability levels of >80% (Table 1). However, EIPA and filipin were toxic in undifferentiated Caco-2 cells over the concentration ranges tested and were not used (Table 1).

3.5. Intracellular trafficking of CD.DNA complexes in undifferentiated Caco-2 cells

In addition to assessing the mode of CD.DNA internalisation, the intracellular trafficking of CD.DNA complexes was assessed using two pharmacological inhibitors, namely chloroquine and nocodazole.

Chloroquine is a weak hydrophobic base which increases pH in endosomes and lysosomes preventing endosomal maturation and subsequent lysosomal degradation of genes (Khalil *et al.* 2006). Although chloroquine is generally believed to exert its effect on GDV efficacy by affecting intracellular trafficking, some studies have reported that it can also affect particle uptake (Huth *et al.* 2006). Therefore, the impact of chloroquine on CD.DNA entry was assessed (Figure 3a). Chloroquine treatment did not significantly affect the level of CD.DNA uptake by undifferentiated Caco-2 cells. However, CD-mediated transfection was significantly reduced ($P<0.05$) indicating altered intracellular trafficking (Figure 3b).

The microtubule depolymerising agent nocodazole has been used previously to assess the involvement of microtubules in the processing of non-viral GDVs by cells (van der Aa *et al.* 2007) (Doyle and Chan 2007). While nocodazole treatment had no significant impact on CD.DNA uptake (Figure 3a), luciferase expression was reduced by ~60% (Figure 3b) indicating microtubule involvement in the intracellular trafficking of complexes.

3.6. Transfection of differentiated Caco-2 cells

In order to better predict the applicability of CD vectors for intestinal gene delivery, a fully differentiated intestinal cell culture model was employed. Differentiated Caco-2 cells are used as a model of the small intestinal tract for drug transport studies, and more recently in screening GDVs, as they provide a more physiologically relevant system than undifferentiated cells. When cultured on semi-permeable membranes over 18-21 days, Caco-2 cells differentiate to form mature enterocyte-like cells complete with tight junctions and apical microvilli (Uduehi *et al.* 1999) (Cryan and O'Driscoll 2003).

CD.DNA demonstrated approximately 50 fold higher transfection than naked pDNA alone, and comparable transfection to PEI (Figure 4). Lf2000 was the most efficient GDV achieving significantly higher transfection than CD and PEI. This trend was similar to that observed for undifferentiated Caco-2 cells (Figure 2c), but with much lower levels indicating the increased resistance of differentiated cells to transfection.

3.7. Endocytosis of CD.DNA complexes by differentiated Caco-2 cells

The decreased activity of GDVs in differentiated Caco-2 cells is believed to be due, at least in part, to a decrease in the level of cell association and uptake (Cryan and O'Driscoll 2003). This reduction in uptake may also be due to a change in the mechanism of particle uptake. To first assess whether CD.DNA uptake by differentiated Caco-2 cells is mediated by endocytosis, cells were incubated with complexes at 4 or 37^{o}C for 4 hours. Uptake was reduced by approximately 80% when cells were incubated at 4^{o}C ($P<0.05$) (Figure 5). It should be noted that < 2% of the total cell population were positive for CD.DNA uptake in control samples (37^{o}C), demonstrating the low endocytotic activity of differentiated enterocytes.

Effects of endocytosis inhibitor treatments were also assessed in differentiated Caco-2 cells (Figure 5). Hypertonic media and cytosol acidification treatments were again used to inhibit CME. Sucrose at 0.2 M was used in addition to 0.45 M sucrose as hypertonic media, since this lower concentration has been used previously with differentiated Caco-2 cells (Kitchens *et al.* 2008) and is less likely to cause toxicity. In the literature, the majority of studies use amiloride or its analogs such as EIPA to inhibit macropinocytosis in differentiated Caco-2 cells (des Rieux *et al.* 2007) (Keita *et al.* 2006) (Mathot *et al.* 2007), and therefore EIPA was the inhibitor of choice for differentiated Caco-2 cell studies. Similarly, filipin is the most commonly used inhibitor of CvME in differentiated Caco-2 cells (Ma and Lim 2003) (Kitchens *et al.* 2008) and was therefore utilised.

EIPA treatment resulted in a reduction in CD.DNA uptake to background levels; and sucrose hypertonic treatments resulted in significant reductions of about 70%. Cytosol acidification did not significantly affect the level of CD.DNA uptake, nor did filipin. These results indicate a role for macropinocytosis and possible roles for CME in the uptake of CD.DNA complexes by differentiated Caco-2 cells.

To ensure that the observed effects on CD.DNA uptake in differentiated cells were not due to inhibitor associated cytotoxic effects, the TEER value of each well was recorded during the inhibitor incubation (Table 1). TEER values were significantly reduced after incubation with 0.45 M sucrose (~80% drop) and 1 µg/ml filipin (~20% drop). Other treatments did not result in significant toxicity, therefore, with the exception of 0.45 M sucrose treatment, the observed effects of the inhibitors are unlikely due to inhibitor induced toxicity.

3.8. The specificity of CME inhibitors and genistein

As all macropinocytosis inhibition treatments led to significant reductions in CD.DNA uptake in both undifferentiated (wortmannin & LY294002) and differentiated (EIPA) Caco-2 cells, it was apparent that macropinocytosis was involved in CD.DNA uptake. However, given that hypertonic media and cytosol acidification treatments had dissimilar effects on CD.DNA uptake in both undifferentiated (Figures 3) and differentiated cells (Figure 5), their specificity as CME inhibitors was assessed. To determine whether these treatments were specific for CME, their effect on TMR-dextran (macropinocytosis marker) uptake was examined in undifferentiated cells, as non-specific inhibition of macropinocytosis may explain reduction by hypertonic media treatment. Also given that the CvME inhibitor genistein reduced CD.DNA uptake and its specificity has been referred to as "doubtful" (Khalil *et al.* 2006), its effect on TMR-dextran uptake was assessed.

Treatment with hypertonic media (0.45 M sucrose) resulted in a significant decrease ($P<0.05$) in TMR-dextran uptake of about 80% (Figure 6). In contrast, cytosol acidification treatment produced a considerable increase in uptake. This is not fully understood but it has been reported that inhibition of endocytotic pathways can

lead to induction of compensatory endocytotic processes (Damke *et al.* 1995), in our case possibly an increased level of macropinocytosis. Indeed, an increased level of macropinocytosis was believed responsible for the increased uptake of cationic particles by HeLa cells upon CME and CvME inhibition (Harush-Frenkel *et al.* 2007). This data explains why a drop in CD.DNA uptake/transfection was observed with hypertonic media but not with cytosol acidification treatment (Figure 3 & 5), and indicates that CME is not involved in CD.DNA uptake in Caco-2 cells. In addition, genistein treatment reduced TMR-dextran uptake ($P<0.05$) by approximately 60% (Figure 6) indicating that CvME may not be involved in CD.DNA uptake. This non-specific inhibition of macropinocytosis by CME and CvME inhibitors demonstrates the importance of ensuring the specificity of inhibitors.

4. DISCUSSION

The CD gene vector used in these studies is the first successful cationic amphiphilic CD-based GDV of this structural class (cationic primary face, lipophilic secondary face). It formed cationic nanoparticulate complexes with pDNA and retained the ability to transfect relative to CDs of the opposite orientation. The route used in its synthesis was high-yielding (Byrne *et al.* 2009) and provides us with increased flexibility in synthesis. These findings highlight the structural flexibility of CDs as GDVs and illustrated that attachment of functional groups to the CD core is possible on both primary and secondary sides effectively doubling the library of modified CDs possible.

Heparinase III binding and uptake studies helped identify that heparan sulphate proteoglycans are responsible for CD.DNA cellular association. As proteoglycans are anionic in nature, the interaction between them and the cationic

CD.DNA complexes likely occurs largely through ionic interactions. Heparan sulphate proteoglycans have been implicated in the binding of a number of viruses, cationic polymers (including non-amphiphilic polycationic CDs), and lipids by cells (Wiethoff and Middaugh 2003) (Cryan *et al.* 2004). In addition, proteoglycans have been shown to be cleaved by trypsin treatment of cells (Vogel 1978), explaining the decreased level of CD.DNA binding to cells post trypsin treatment. Similarly, heparinase treatment of Caco-2 cells has been reported to decrease the level of cellular association of chitosan nanoparticles (Behrens *et al.* 2002).

The incubation time of GDV.DNA complexes with cells is an important parameter that can dictate the level of transfection (Legendre and Szoka 1992) (van der Aa *et al.* 2007). A significant level of CD.DNA uptake was detectable after 15 minute incubation with undifferentiated Caco-2 cells. This is in agreement with a previous study by our group that reported uptake of a cationic amphiphilic CD by HepG2 cells within 15 minutes as determined by confocal microscopy (McMahon *et al.* 2008). In our studies, the level of CD.DNA uptake was equivalent to that of Lf2000 and significantly higher than that of PEI after 3 hours. Despite this high level of uptake, CD-mediated luciferase expression was significantly lower than Lf2000 and comparable with PEI. Therefore, rapid/high uptake does not necessarily lead to high transfection. This is in agreement with Cryan *et al*. (2004) who reported higher levels of non-amphiphilic cationic CD uptake compared with the commercial cationic lipid DOTAP but significantly less transgene expression (Cryan *et al.* 2004). There are a number of possible explanations for this phenomenon. It may be that Lf2000 and PEI.DNA complexes enter cells by different endocytotic pathway(s) to CD.DNA complexes, which are more favourable to transfection. Douglas *et al*. for example reported significant uptake of alginate-chitosan nanoparticles by both 293T and CHO

cells (Douglas *et al.* 2008). Whereas 293T cells were successfully transfected, no significant transgene expression was detected in CHO cells. They deduced that CME was responsible for uptake by 293T cells while CvME was responsible for uptake by CHO cells. Therefore, it is clear that different endocytotic pathways can yield different transfection efficiencies.

Endocytosis is generally considered as the main mechanism of GDV uptake by cells (Khalil *et al.* 2006). In agreement with this we found that CD.DNA complexes were taken up by undifferentiated and differentiated Caco-2 cells by endocytosis. Inhibitor data indicates that macropinocytosis is the principal endocytotic pathway by which CD.DNA complexes enter both undifferentiated and differentiated cells. CME inhibition with hypertonic media and cytosol acidification treatments however yielded conflicting results. Indeed, our data indicates that the hypertonic media induced decrease in CD.DNA uptake was due to non-specific inhibition of macropinocytosis. Hypertonic media has been previously reported to inhibit the uptake of the fluid phase endocytosis marker horseradish peroxidase (Carpentier *et al.* 1989) and a number of other studies have commented on its lack of specificity (Perumal *et al.* 2008). Therefore, CME is unlikely to be involved in the uptake of CD.DNA complexes by Caco-2 cells. The CvME inhibitor genistein also inhibited TMR-dextran uptake indicating that it was not a selective CvME inhibitor. Indeed, genistein's specificity as a CvME inhibitor has previously been questioned (Khalil *et al.* 2006). The most commonly used CvME inhibitor, filipin, did not significantly reduce CD.DNA uptake in differentiated Caco-2 cells. Interestingly, CvME was implicated in a recent study in the uptake of poly(amido amine) dendrimers by undifferentiated Caco-2 cells (Goldberg *et al.* 2010). However, in the same study it was apparent that CvME was not involved in their uptake by differentiated Caco-2

cells. In addition, the expression of the caveolae associated protein, caveolin-1, in Caco-2 cells is thought to be quite low. Indeed, some studies have reported that caveolin-1 is not expressed in Caco-2 cells and reported little evidence of caveolar structures (Vogel *et al.* 1998) (Lim *et al.* 2009). However, other studies reported detectable levels of caveolin-1 in Caco-2 cells indicating that a low level of expression may be present (Field *et al.* 1998). Notably, when Caco-2 cells were stably transfected with caveolin-1, caveolae were only detected in the basolateral membrane (Vogel *et al.* 1998). Similarly, in another model of polarised epithelia, caveolae were localised exclusively at the basolateral membrane (Vogel *et al.* 1998). Therefore, it is unlikely that CvME will play a major role in CD.DNA uptake by enterocytes. A recent study on the uptake of a highly lipid-modified CD (14 lipid side chains) by Vero African green monkey kidney epithelial cells indicated that CME and CvME are the major routes of uptake for this particular CD (Diaz-Moscoso *et al.* 2010). This is in contrast to the results obtained in the current study and may be explained by structural variations between the CDs and cell type dependent effects.

Increased transfection may be accomplished by increasing the level of uptake by macropinocytosis as has been done in the case of cell penetrating peptides (Wadia *et al.* 2004) (Khalil *et al.* 2006b). However, our data demonstrates that high uptake does not necessitate high levels of transgene expression. Therefore, improving the intracellular trafficking properties of CD.DNA complexes is a necessity for future success. There is a paucity of data regarding the intracellular trafficking of GDV complexes taken up by macropinocytosis. However, the pH of macropinosomes has been reported to decrease in cells (Khalil *et al.* 2006). Indeed, it appears that acidification of macropinosomes is necessary for maximal CD.DNA transfection given that chloroquine reduced transfection levels. CD.DNA escape may be more

efficient at lower pH due to an increase in CD.DNA surface charge, allowing fusion with the macropinosomal membrane. Furthermore, inhibition of endosome acidification has been reported to inhibit the breakdown of endocytosed heparan sulphate proteoglycans by heparinases (Yanagishita and Hascall 1984). Given that dissociation of cell penetrating peptides from heparan sulphate is important for their endosomal escape (Magzoub *et al.* 2005), dissociation of CD.DNA complexes from heparan sulphate proteoglycans may be necessary for efficient CD.DNA escape from macropinosomes. Similarly, Harashima's group reported that inhibition of macropinosome acidification by bafilomcin A treatment reduced transfection by octarginine liposomes (El-Sayed *et al.* 2008). Notably, the authors reported that octarginine residues improved the macropinosomal escape of GDVs internalised by macropinocytosis and so this approach might improve CD activity by improving intracellular trafficking (El-Sayed *et al.* 2008).

Our study is among the few that have assessed the impact of differentiation on GDV uptake pathways. Low levels of complex uptake in differentiated cells are certainly a factor in the reduction in activity of transfection complexes in differentiated Caco-2 cells (Cryan and O'Driscoll 2003). Similarly, Boucher's group reported that well differentiated airway cells had reduced levels of lipoplex endocytosis relative to poorly differentiated cells (Matsui *et al.* 1997). Notably, heparan sulphate proteoglycans are initially present along the entire surface of undifferentiated Caco-2 cells, but during differentiation redistribute to the basolateral membrane (Esclatine *et al.* 2001). Given the presence of tight junctions in differentiated Caco-2 cells, it is unlikely that vectors can reach the basolateral proteoglycans resulting in a reduction in cell association and uptake via proteoglycans. The low levels of transfection in differentiated Caco-2 cells is also

likely a consequence of their low mitotic activity (Zauner *et al.* 1999). The poor nuclear accessibility of pDNA in differentiated cells is a significant obstacle and will need to be overcome through strategies such as nuclear localisation signal conjugation, or the use of siRNAs as alternative therapeutic nucleic acids.

5. CONCLUSIONS

The means of cellular association and uptake of CD.DNA complexes by Caco-2 cells were elucidated. Heparan sulphate proteoglycans are responsible for binding of complexes to undifferentiated Caco-2 cells, while macropinocytosis is responsible for the uptake of CD.DNA complexes by both undifferentiated and differentiated Caco-2 cells. Microtubules are implicated in the intracellular trafficking of complexes and endosomal acidification is necessary for maximal gene expression. This information will be used to develop optimised CD based transfection complexes for intestinal delivery with enhanced cellular uptake and intracellular trafficking properties.

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TABLES

Table 1: Inhibitor concentrations and % cell viability post treatment in undifferentiated and differentiated Caco-2 cells as assessed by MTT assay and TEER values respectively. Data represents mean ± S.D. (n=3) expressed as a % of control values. * indicates statistical significance (P<0.05).

Treatment	Concentration	Effect	% Dehydrogenase activity (undifferentiated cells)	% TEER (differentiated cells)
Low temperature (4°C), 1 hour pre-incubation	N/A	Endocytosis inhibition	105 ± 4	104.5 ± 2.8
Methyl-β-CD (mβCD), 15 minute pre-treatment	10 mM	Endocytosis inhibition	103.6 ± 3.9	N/A
Wortmannin, 1 hour pre-treatment	50 nM	Macropinocytosis inhibition	98.9 ± 8.8	N/A
LY294002, 1 hour pre-treatment	50 µM	Macropinocytosis inhibition	97.3 ± 1.2	N/A
5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA), 1 hour pre-treatment	50 µM	Macropinocytosis inhibition	5.98 ± 1.66*	77.5 ± 8.7
Hypertonic medium, 30 minute pre-treatment	0.45 M sucrose	Clathrin mediated endocytosis (CME) inhibition	94.1 ± 3.6	22.7 ± 2.5*
Hypertonic medium, 30 minute pre-treatment	0.2 M sucrose	CME inhibition	N/A	95.7 ± 20.7
Cytosol acidification, 10 minute pre-treatment	10 mM acetic acid (pH 5)	CME inhibition	86.5 ± 2.4*	98.8 ± 5.9
Genistein, 1 hour pre-treatment	200 µM	Caveolae mediated endocytosis (CvME) inhibition	95.1 ± 5.1	N/A
Filipin, 1 hour pre-treatment	1 µg/ml	CvME inhibition	23.7 ± 6.5*	79.7 ± 2.5*
Nocodazole, 1 hour pre-treatment	10 µM	Disrupts microtubules	100.7 ± 2.5	N/A
Chloroquine, 15 minute pre-treatment	100 µM	Disrupts endosomes and lysosomes	88.8 ± 1.8*	N/A

Table 2: Involvement of Caco-2 (undifferentiated) cell surface proteins in the binding and/or uptake of CD.DNA complexes.

Treatment	Relative % CD.DNA bound cells	Relative % CD.DNA cellular uptake
Trypsinisation	32.25 ± 6.12	N/A
Heparinase III	20.95 ± 4.22	74.81 ± 1.96
Soluble heparin (5 µg/ml)	N/A	7.4 ± 2.8
Soluble heparin (10 µg/ml)	N/A	0.96 ± 0.4

Data represents mean ± S.D. (n=3) relative to control treated cells. All data are significantly different to control (100%) values ($P<0.05$). CD.DNA binding post trypsinisation is expressed as a % of EDTA treated cells. Uptake post heparinase III and heparin treatment is expressed as a % of cells not incubated with these treatments i.e. buffer alone.

LEGENDS TO FIGURES

Figure 1: Amphiphilic cationic cyclodextrins. The CD used in this study has structure (c), represented schematically by (b). Previously tested CDs had structure (a).

Figure 2: Uptake of CD.DNA complexes by undifferentiated Caco-2 cells and resulting transfection efficiency. a) Uptake kinetics of CD.DNA complexes over time compared with PEI and Lf2000. Caco-2 cells were incubated with complexes for 1 hour at 4^{o}C and subsequently at different times at 37^{o}C. The level of complex uptake was assessed by flow cytometry. Data represents mean ± S.D. (n=4). **b)** Cells were incubated with fluorescent CD.DNA complexes for 3 hours at 37^{o}C. Uninternalised complexes were removed, and cells were fixed and subsequently stained for the nucleus with HOECHST dye, and imaged using a confocal microscope. **c)** Undifferentiated Caco-2 cells were incubated with transfection complexes for 1 hour at 4^{o}C, and subsequently at 37^{o}C for 3 hours. Complexes were removed, and cells incubated for 24 hours. Luciferase expression was assessed and expressed as RLU/μg protein. Data represents mean ± S.D. (n=3).

Figure 3: Effect of various endocytosis inhibitor treatments on CD.DNA a) uptake and b) transfection in Caco-2 cells. Caco-2 cells were pre-treated with mβCD (15 minutes), wortmannin, LY294002, genistein (1 hour), hypertonic media (30 minutes), and cytosol acidification buffer (10 minutes) for the indicated period of time prior to addition of CD.DNA complexes. Cells were incubated in the presence of inhibitor and CD.DNA complexes for 90 (mβCD, hypertonic media, and cytosol acidification) or 120 minutes (wortmannin, LY294002, and genistein) prior to complex removal with CellScrub™ buffer. Uptake was assessed by flow cytometry

and expressed as % uptake relative to control (a). Luciferase expression was assessed 24 hours later and expressed as % RLU/µg protein of control treated cells (b). All data represents mean ± S.D. (n=3). * indicates statistical significance relative to control treated cells ($P<0.05$).

Figure 4: Transfection of differentiated Caco-2 cells. Transfection complexes were incubated with differentiated Caco-2 cells (day 21) at 37^{o}C. Media was removed and replaced with serum supplemented DMEM 4 hours later and cells were incubated for a further 24 hours. Luciferase expression was assessed and expressed as RLU/µg protein. Data represents mean ± S.D. (n=3).

Figure 5: Effect of endocytosis inhibitors on CD.DNA uptake by differentiated Caco-2 cells. Differentiated Caco-2 cells were pre-treated with 50 µM EIPA, 1 µg/ml filipin (1 hour), 0.2 and 0.45 M sucrose (30 minutes), and cytosol acidification buffer (10 minutes) for the indicated period of time prior to addition of CD.DNA complexes. Cells were incubated in the presence of inhibitor and CD.DNA complexes for 4 hours prior to washing with CellScrub™ buffer and analysis by flow cytometry. Data represents mean ± S.D. (n=3) relative to control treated cells (*$P<0.05$). * indicates statistical significance relative to control treated cells ($P<0.05$).

Figure 6: The effect of hypertonic media, cytosol acidification, and genistein treatment on the uptake of TMR-dextran by undifferentiated Caco-2 cells. Cells were incubated with inhibitor treatments prior to addition of TMR-dextran (1 mg/ml) and the level of dextran uptake was assessed 2 hours later by flow cytometry and

expressed as a % of control treated cells. Data represents mean ± S.D. (n=3). * indicates statistical significance relative to control treated cells ($P<0.05$).

Figure 1

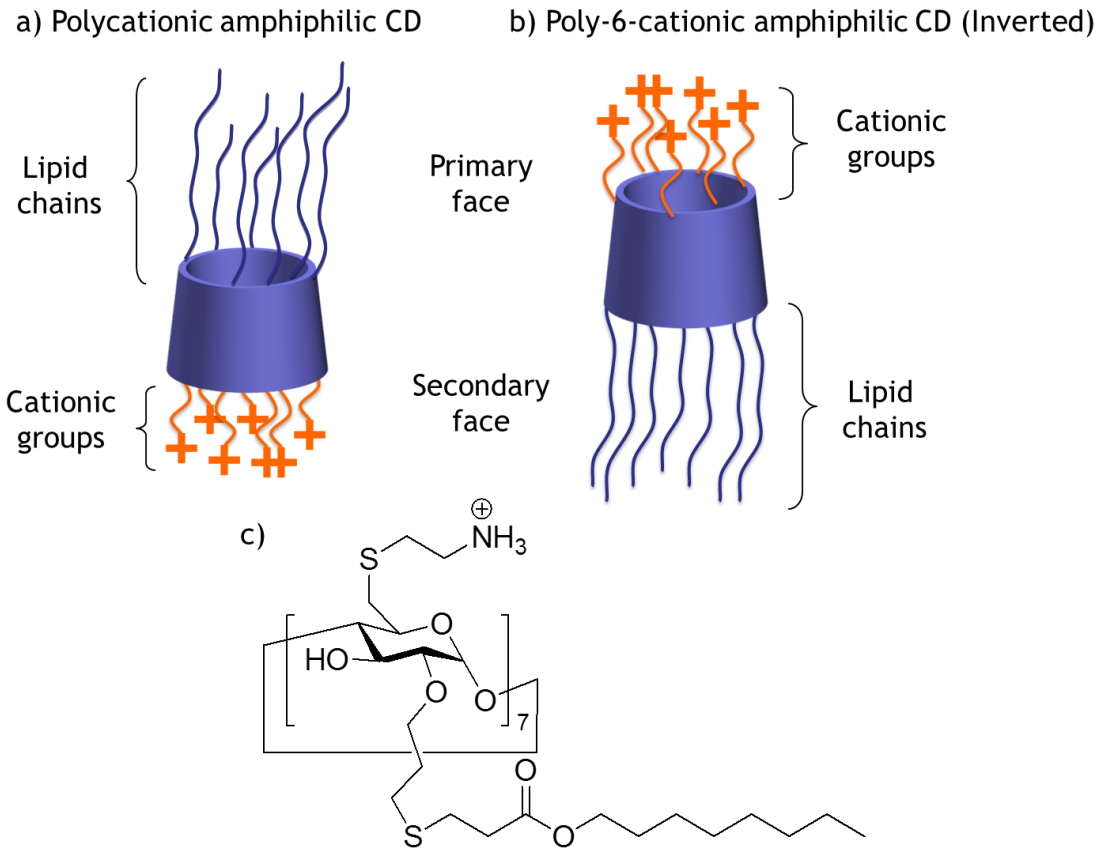


Figure 2 (a)

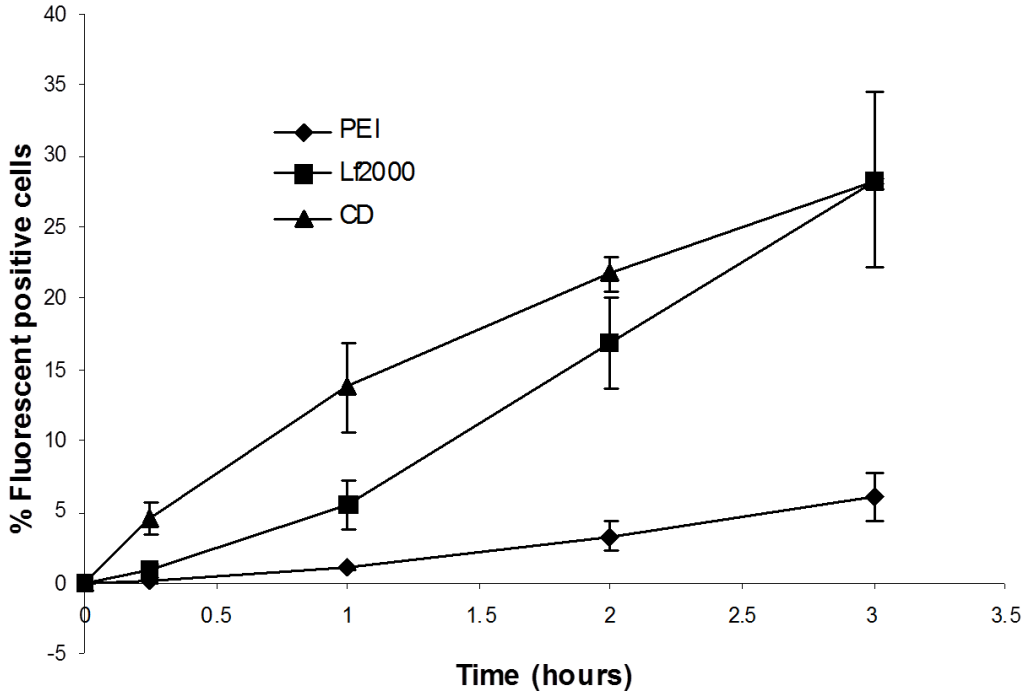


Figure 2 (b)

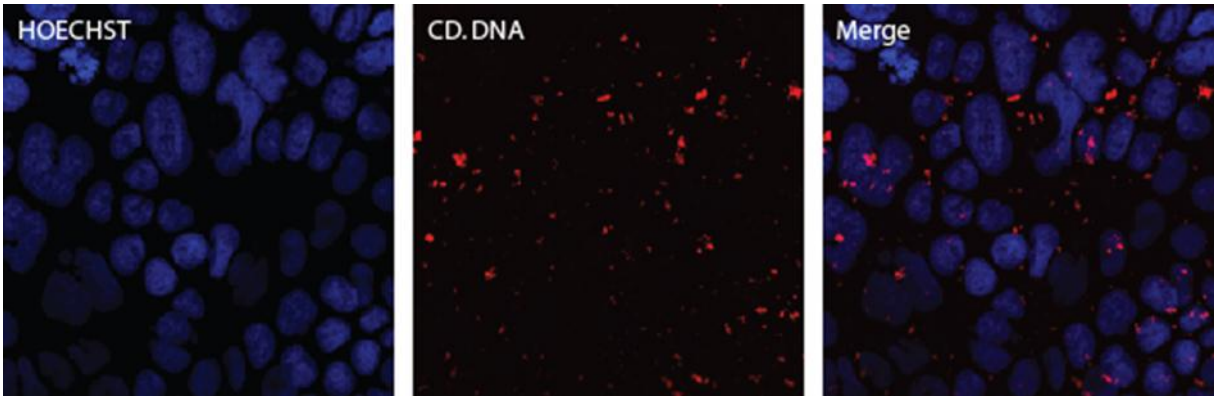


Figure 2 (c)

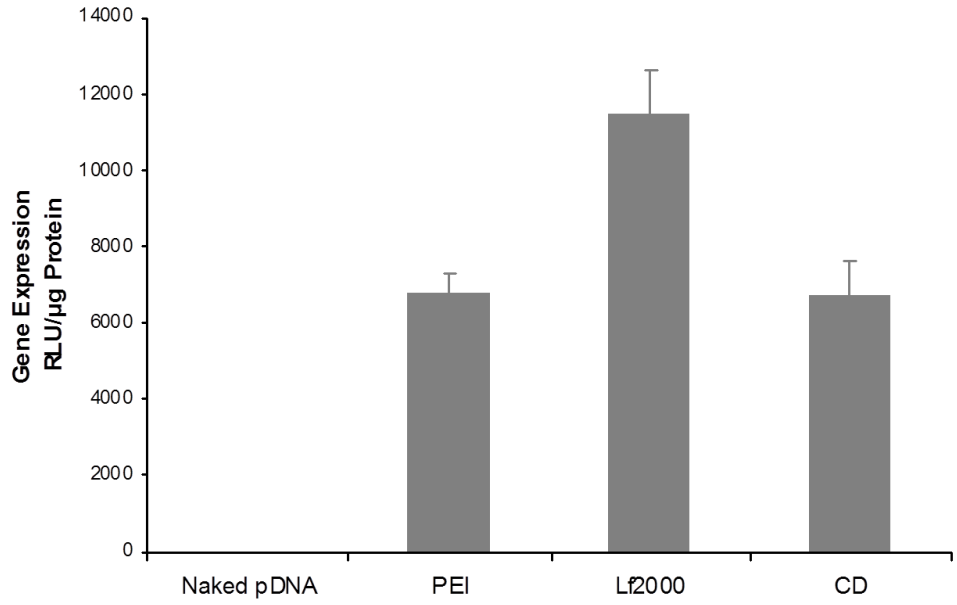


Figure 3 (a)

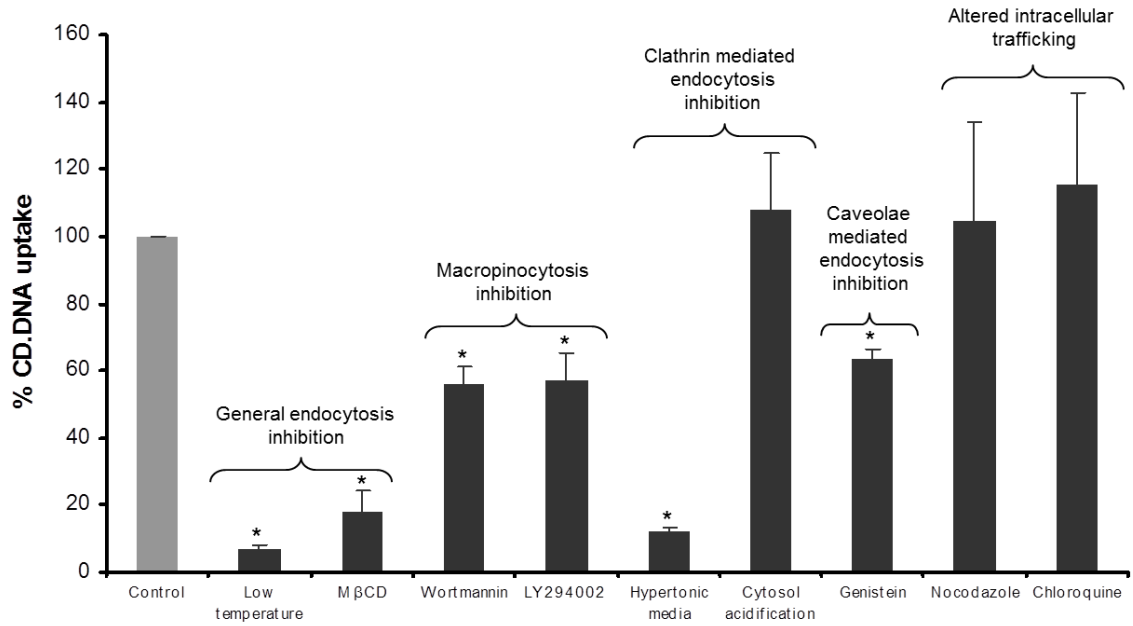


Figure 3 (b)

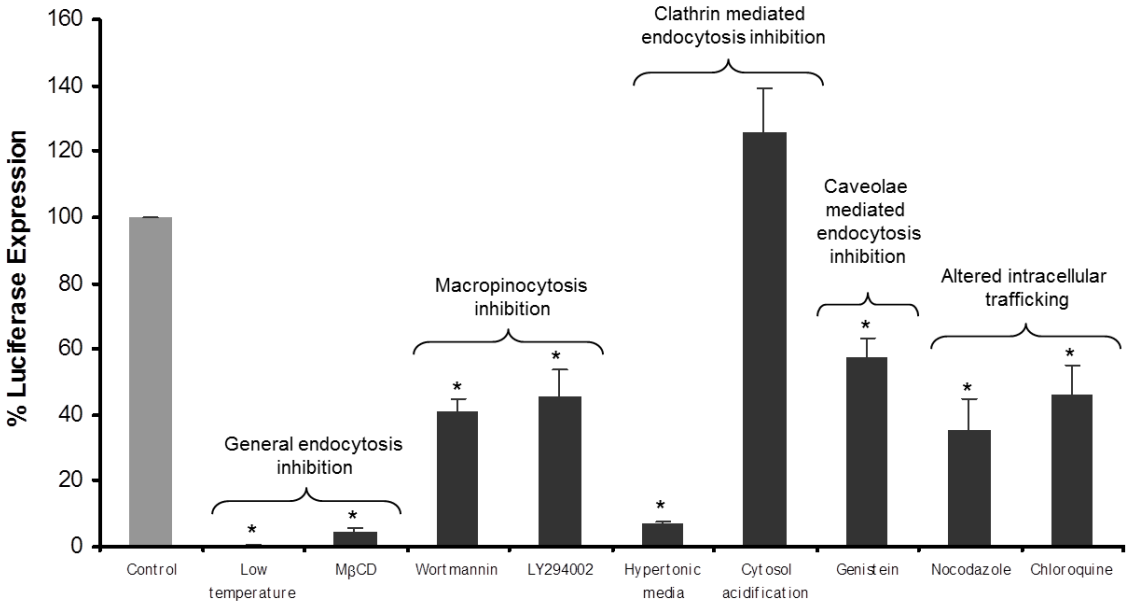


Figure 4

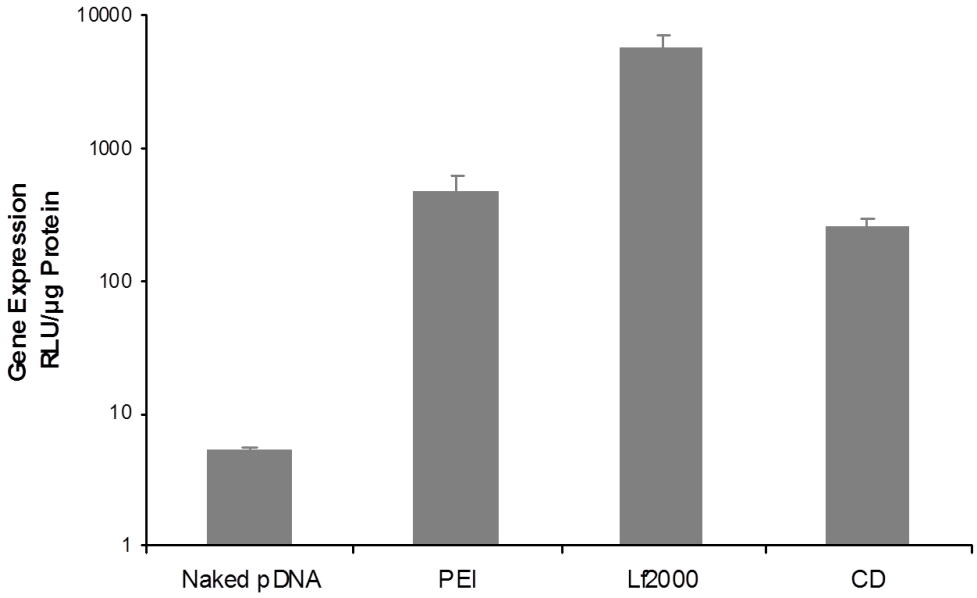


Figure 5

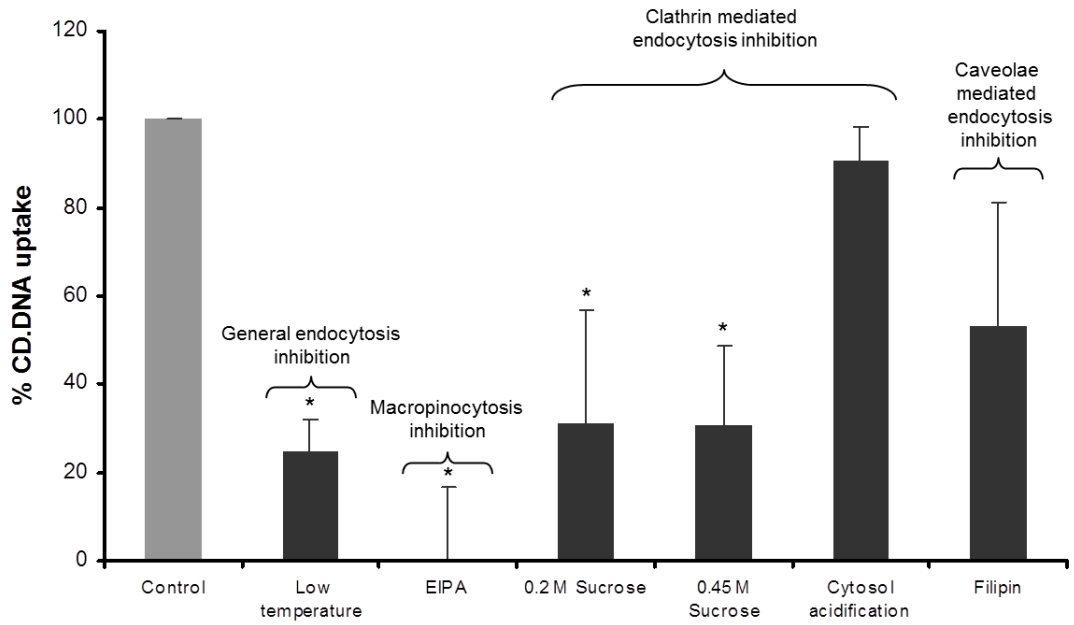
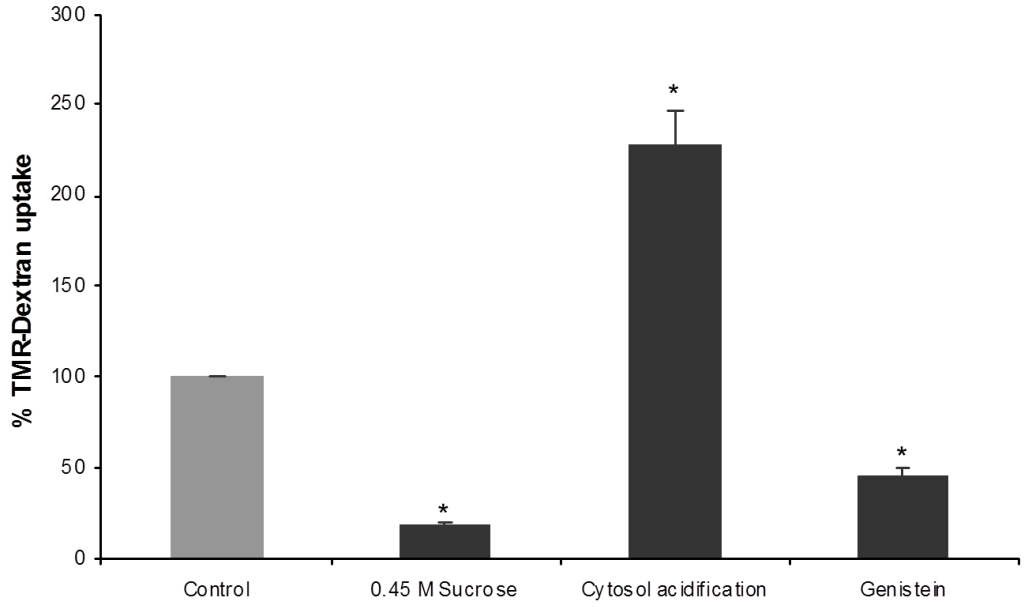


Figure 6



ABBREVIATIONS

BCA:	Bicinchoninic acid
CD:	Cyclodextrin
CME:	Clathrin mediated endocytosis
CMV:	Cytomegalovirus
CvME:	Caveolae mediated endocytosis
EIPA:	5-(*N*-ethyl-*N*-isopropyl)amiloride
Lf2000:	Lipofectamine™ 2000
MR:	Mass ratio
MTT:	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N/P:	Nitrogen/Phosphate
pDNA:	Plasmid DNA
PEG:	Poly(ethylene glycol)
PEI:	Polyethylenimine
RLU:	Relative light units
S.D.:	Standard deviation
TMR:	Tetramethylrhodamine
v:w:	Volume:weight