

The Use of Inhibitors to Study Endocytic Pathways of Gene Carriers: Optimization and Pitfalls

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Nonviral gene complexes can enter mammalian cells through different endocytic pathways. For efficient optimization of the gene carrier it is important to profile its cellular uptake, because this largely determines its intracellular processing and subsequent transfection efficiency. Most of the current information on uptake of these gene-delivery vehicles is based on data following the use of chemical inhibitors of endocytic pathways. Here, we have performed a detailed characterization of four commonly used endocytosis inhibitors [chlorpromazine, genistein, methyl- β -cyclodextrin (M β CD), and potassium depletion] on cell viability and endocytosis in five well-described cell lines. We found that chlorpromazine and to a lesser extent M β CD significantly decreased cell viability of some cell lines even after short incubation periods and at concentrations that are routinely used to inhibit endocytosis. Through analyzing the uptake and subcellular distribution of two fluorescent endocytic probes transferrin and lactosylceramide (LacCer) that are reported to enter cells via clathrin-dependent (CDE) and clathrin-independent (CIE) mechanisms, respectively, we showed poor specificity of these agents for inhibiting distinct endocytic pathways. Finally, we demonstrate that any inhibitory effects are highly cell line dependent. Overall, the data question the significance of performing endocytosis studies with these agents in the absence of very stringent controls.

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INTRODUCTION

Successful gene delivery relies on the development of nontoxic gene carriers or vectors that efficiently deliver foreign genetic material into target cells. Although viral vectors are quite effective, their clinical use is limited and this is mainly due to safety issues. As a safe(r) alternative, nonviral gene carriers, such as cationic liposomes or polymers, can be used. However, their low-transfection efficiency is a major limiting factor. Because the

cellular processing of the gene complexes strongly influences the efficiency of the carrier, a thorough mechanistic understanding of the pathways involved in the cellular uptake of these nonviral gene complexes may therefore help to design more effective gene carriers.

The majority of reports on cellular uptake mechanisms suggest that endocytosis is the preferred route of cell entry of nonviral gene carriers.¹ As shown in **Supplementary Figure S1**, endocytosis can be classified in two broad categories: phagocytosis and pinocytosis.² Phagocytosis is typically restricted to specialized cells whereas pinocytosis occurs in all cell types. The study of different pinocytotic pathways is still an evolving field and no current classification system is completely satisfactory. Currently, pinocytosis is subdivided into macropinocytosis, clathrin-dependent (CDE), and clathrin-independent (CIE) endocytosis. During macropinocytosis, membrane protrusions, often called ruffles, are formed which can subsequently fuse with each other or with the plasma membrane to enclose large volumes.³ Macropinocytosis is a nonselective endocytic mechanism for internalizing suspended macromolecules and the internalized vesicles or macropinosomes can have sizes up to 5 μ m.⁴ CDE is the best characterized endocytic pathway and is said to be the preferred pathway for microspheres up to 200 nm in size.⁵ Certain macromolecules preferentially associate with this pathway and are specifically taken up in clathrin-coated vesicles. Examples are the low-density lipoprotein receptor, the transferrin receptor, and the epidermal growth factor receptor. Human transferrin (hTF) is considered to be a good specific marker for CDE, as in its iron bound form it binds to the transferrin receptor and is taken up via CDE.^{6,7} Although the details of the CIE mechanism have not been completely elucidated, its current subclassification is based on the role of dynamin and several small GTPases.⁸ These include uptake from lipid rafts in caveolae or via a flotillin-dependent pathway. Rafts represent liquid ordered environments present in biological membranes enriched in unsaturated phospholipids, cholesterol, glycosphingolipids, and certain proteins.⁹ Caveolae-mediated endocytosis is probably the best characterized CIE pathway. Caveolae are small (50–80 nm), smooth, flask-shaped invaginations of the plasma membrane that are characterized by the presence of caveolin-1.¹⁰

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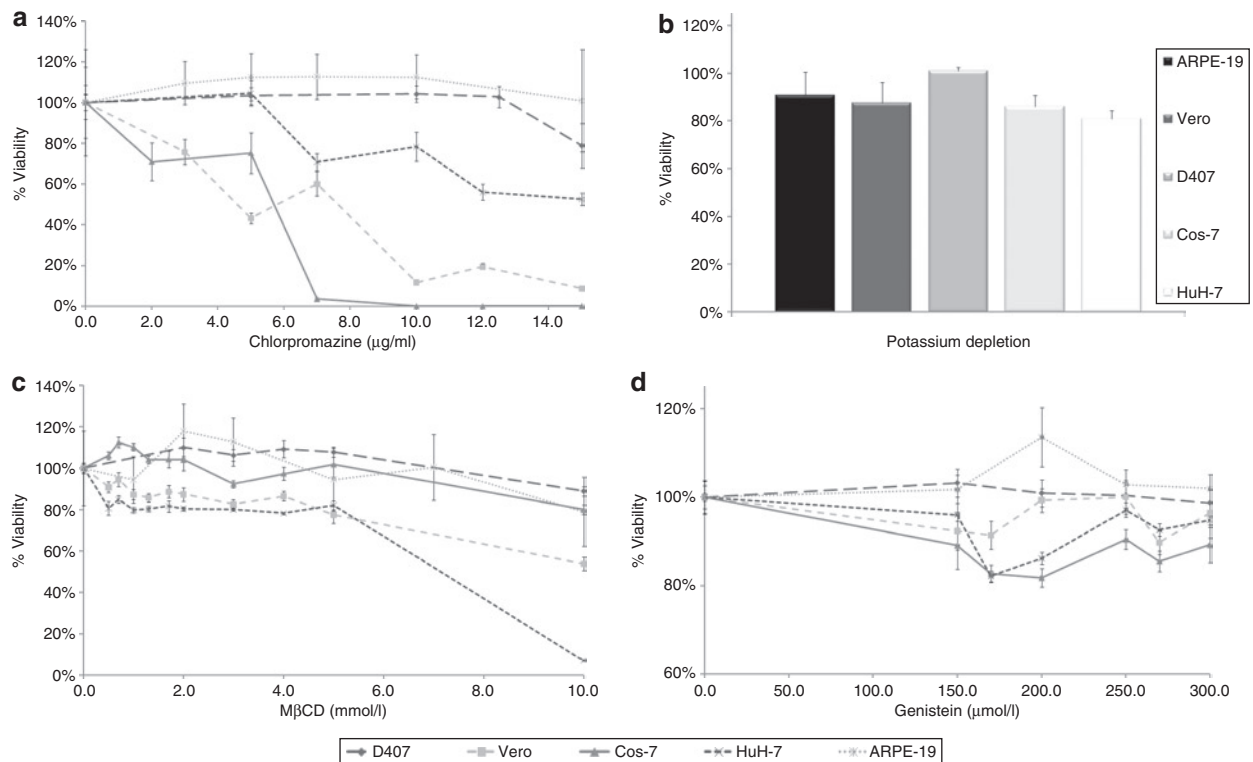


Figure 1 *In vitro* cytotoxicity of inhibitors. *In vitro* viability of D407, Vero, COS-7, HuH-7, and ARPE-19 cells incubated for 2 hours with endocytosis inhibitors (a) chlorpromazine, (b) K⁺-depletion buffer, (c) MβCD, and (d) genistein. Cell viability was assessed with an MTT-based assay. Values are given as means + SD of quadruplicates. MβCD, methyl-β-cyclodextrin.

Certain pathogens, like SV40 virus and cholera toxin subunit B specifically use this pathway to enter cells and the latter is often used as a marker for this pathway.^{10,11} However, a significant fraction of cholera toxin subunit B is also taken up in clathrin-coated vesicles.¹² An alternative marker for CIE is lactosylceramide (LacCer), a glycosphingolipid that resides preferably in lipid rafts and the uptake from which is CIE but dependent on dynamin and caveolin-1.^{13–15} LacCer has previously been used as a marker for caveolae-mediated endocytosis.^{5,16} Nonetheless, because there is evidence that caveolin-1 is not necessary for endocytosis of LacCer, we preferably define LacCer as a marker for CIE endocytosis. Flotillin-dependent endocytosis was recently characterized as a dynamin-2, caveolin-1, and clathrin independent process.¹⁷ Most cells also have the capacity to constitutively internalize fluid via less well-characterized pathways and it is likely that other pathways remain to be discovered and characterized.

A number of endocytic pathways have been demonstrated to be involved in the uptake of DNA complexes,^{18–21} and this is highly dependent on cell type,^{22,23} the nature of the gene carrier^{24,25} and the particle size.⁵ This variability stresses the necessity of studying the internalization of each of the different types of nonviral gene carriers in an appropriate cell line model. A quantitative assessment of the contribution of each endocytic pathway to the overall cellular uptake is essential for elucidating the corresponding intracellular pharmacokinetic models. Specifically this may allow one to relate the endocytic pathway of a gene complex to its subsequent intracellular processing and transfection efficiency.²⁶ Much of the data on the cellular uptake of gene-delivery vectors comes from studies

using chemical inhibitors of endocytosis. A number of these are deemed to be specific for some of the described pathways but an increasing volume of evidence suggests that they lack specificity and may give misleading results.²⁷ Although there are several tools available for elucidating the endocytic pathways involved in the uptake of gene complexes, the present study focuses on the use of these endocytosis inhibitors.

In this study, chlorpromazine and K⁺-depletion are evaluated as inhibitors of CDE. Chlorpromazine is a cationic amphiphilic drug which is believed to inhibit clathrin-coated pit formation by a reversible translocation of clathrin and its adapter proteins from the plasma membrane to intracellular vesicles.²⁸ Potassium depletion is said to inhibit the formation of clathrin-coated pits by dissociating the clathrin lattices at the inner leaflet of the plasma membrane.²⁹ Methyl-β-cyclodextrin (MβCD) and genistein are evaluated as inhibitors of CIE. MβCD is a cyclic oligomer of glucopyranoside that inhibits cholesterol-dependent endocytic processes by reversibly extracting the steroid out of the plasma membrane.³⁰ MβCD is regularly used to determine whether endocytosis is dependent on the integrity of lipid rafts. Genistein is a tyrosine-kinase inhibitor and this agent causes local disruption of the actin network at the site of endocytosis and inhibits the recruitment of dynamin II, both known to be indispensable events in the caveolae-mediated uptake mechanism.^{31,32} Here, we evaluate the usefulness of these four well-characterized inhibitors of endocytosis in five different cell lines. We initially investigate the cytotoxicity of each of the inhibitors and subsequently, by using a combination of flow cytometry and confocal fluorescence

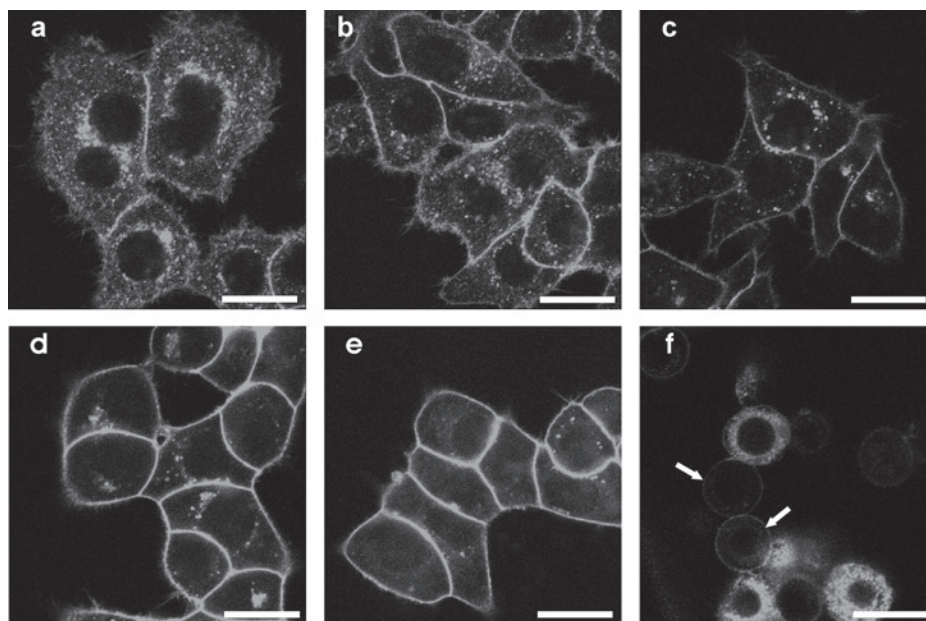


Figure 2 Optimization of the concentration of M β CD to inhibit CIE in D407 cells. Optimization of the concentration of M β CD to inhibit CIE in D407 cells. Cells were incubated with LacCer following preincubation with increasing concentrations of M β CD for different incubation times. (a) Positive control, (b) 30 minutes 2.5 mmol/l M β CD, (c) 1 hour 2.5 mmol/l M β CD, (d) 2 hours 3 mmol/l M β CD, (e) 2 hours 5 mmol/l M β CD, and (f) 2 hours 10 mmol/l M β CD. The presence of fluorescently labeled vesicles in the interior of the cell is a measure of endocytic uptake of LacCer. Arrows are pointing out cells which suffered the M β CD treatment, resulting in rounding up and lifting of the cells from the growth surface. All experiments were performed at least three times and representative images are shown. Bars = 20 μ m. CIE, clathrin-independent endocytosis; LacCer, lactosylceramide; M β CD, methyl- β -cyclodextrin.

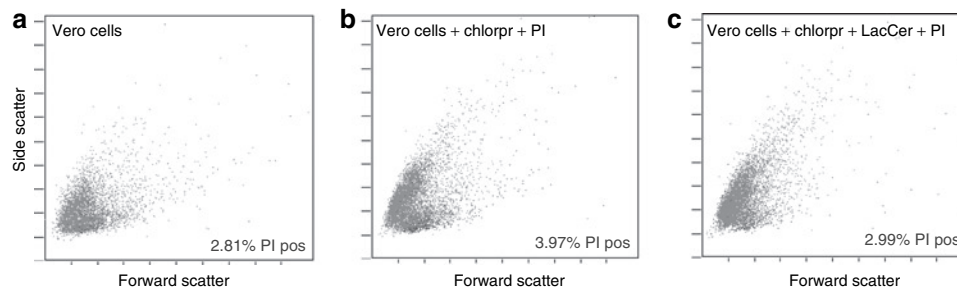


Figure 3 Effect of chlorpromazine on morphology of Vero cells. The diagram shows side and forward scattering histograms for Vero cells as measured by flow cytometry. In the scatterplots, each spot represents a cell and the x- and y-values are related with size (forward scatter) and granularity (side scatter). Scatterplot of (a) normal untreated Vero cells, (b) Vero cells treated for 2 hours with 10 μ g/ml chlorpromazine and stained for dead cells with PI, (c) Vero cells treated with chlorpromazine and incubated with LacCer and back exchanged with defatted BSA before PI staining. The percentage of PI positive cells is shown in the right lower corner of the diagrams. BSA, bovine serum albumin; chlorpr, chlorpromazine; LacCer, lactosylceramide; PI, propidium iodide.

microscopy, we evaluate the capacity and specificity of the agents to interfere with the uptake of endocytic markers. As endocytic markers, we utilized two well-characterized probes: hTf for CDE and LacCer for CIE. Overall, our data suggest that extreme care should be taken when interpreting data obtained from the use of these agents and we discuss these implications on drug-delivery studies.

RESULTS

Cytotoxicity of endocytosis inhibitors is cell type dependent

To establish an optimal protocol for the use of endocytosis inhibitors for a specific cell type, it is imperative to evaluate their *in vitro*

cellular toxicity. We investigated the viability of five different cell lines, HuH-7, Vero, COS-7, ARPE-19, and D407, after exposure to four frequently used inhibitors: chlorpromazine, K⁺-depletion, genistein, and M β CD. Different concentrations of each inhibitor were tested on all cell lines and cellular toxicity was subsequently assessed with the MTT-based EZ4U test. The inhibitor was in all cases diluted in OptiMEM and incubated with the cells for 2 hours. In case of K⁺-depletion, cytotoxicity was determined following treatment with the different buffers as described in Materials and Methods section. As positive and negative controls, cells were exposed for 2 hours to 20 mg/ml phenol solution in OptiMEM and in OptiMEM alone, respectively. Cytotoxicity, expressed as a percentage of cell viability, was calculated from the

measured absorbance values normalized to the negative control (100% viability) and taking into account the correction for the positive control (0% viability). Each experiment was carried out in quadruplicate, the results of which are shown in **Figure 1**.

Unlike HuH-7, ARPE-19, and D407 cells that were relatively insensitive to <10 µg/ml chlorpromazine, COS-7, and Vero cells, both African green monkey kidney cells, were found to be sensitive to this agent (**Figure 1a**). All cell lines retained >85% viability upon K⁺-depletion (**Figure 1b**). MβCD exhibited only very slight cytotoxicity to ARPE-19, D407, and COS-7 cells at 10 mmol/l,

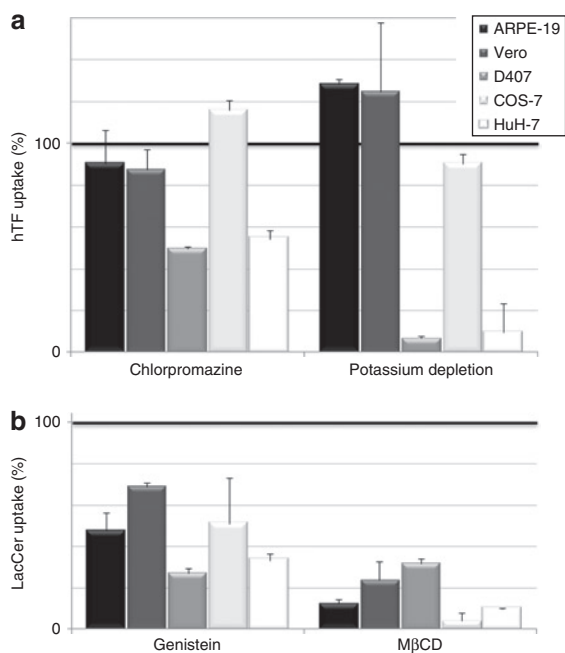


Figure 4 Efficacy of endocytosis inhibitors. Quantification of **(a)** hTF and **(b)** LacCer uptake by flow cytometry in ARPE-19, Vero, D407, COS-7, and HuH-7 cells after incubation with **(a)** chlorpromazine or potassium depletion buffer and **(b)** genistein or MβCD. Values are given as means + SD of triplicates. hTF, human transferrin; LacCer, lactosylceramide; MβCD, methyl-β-cyclodextrin.

whereas Vero and especially HuH-7 cells were clearly more sensitive to the effects of this agent (**Figure 1c**). Finally, none of the tested genistein concentrations had a noticeable effect on the viability of the five cell lines tested (**Figure 1d**). Based on these results, we conclude that some of these cell lines do show significant toxicity after treatment with some of these inhibitors at concentrations that are routinely used for endocytosis studies.

Selection of suitable inhibitor concentrations based on confocal microscopy and cytotoxicity data

In addition to the cytotoxicity data, which define the upper concentration limit for a particular inhibitor and a particular cell line, we also evaluated the effect of the inhibitors at different concentrations in order to find the lower concentration limit at which the inhibitor is still active. In this way, we determined suitable concentrations of these agents allowing sufficient inhibition of specific endocytic pathways with acceptable toxicity. We evaluated by confocal microscopy the remaining internalization of two fluorescently labeled endocytic markers, hTF and LacCer, that are known to be specifically internalized by CDE and CIE, respectively, after incubation with different inhibitor concentrations. K⁺-depletion and chlorpromazine are reported to specifically inhibit CDE, whilst MβCD and genistein have been extensively used to inhibit CIE. As an example, the effect of D407 cell exposure to different incubation times and concentrations of MβCD on LacCer uptake is shown in **Figure 2**. **Figure 2a** shows control cells after incubation with LacCer for 15 minutes and the probe is shown to label the plasma membrane and intracellular vesicles in the perinuclear region. Incubating the cells with MβCD before LacCer addition significantly reduced its uptake in a time- and concentration-dependent manner (**Figure 2b-f**). However, longer incubation times (>2 hours) and higher concentrations (>5 mmol/l) had major morphological effects and the cells tended to detach from the growing surface during the experimental procedure. **Figure 2f** already shows that MβCD can have pronounced effects on cellular morphology. Like MβCD, other inhibitors also induce strong effects on cellular morphology, which could also be observed from flow cytometry analysis. Based on the flow cytometry scatter plot, one can observe for example that treatment with

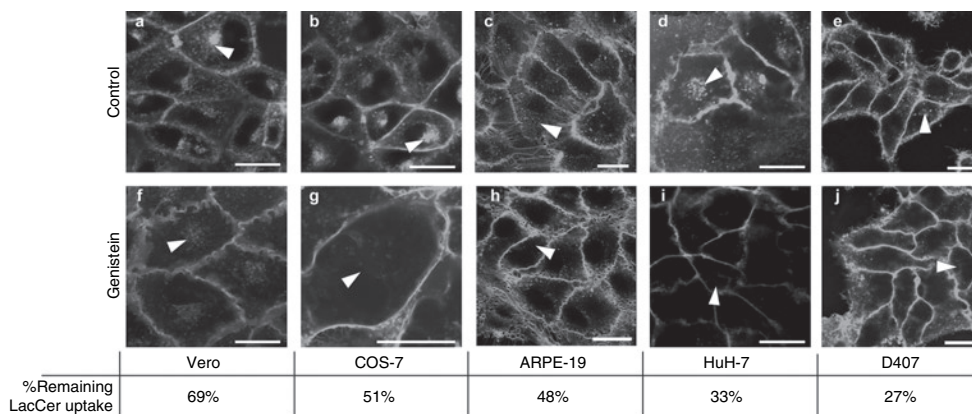


Figure 5 Intracellular distribution of LacCer after genistein treatment is dependent on cell type. Evaluation of LacCer uptake in **(a,f)** Vero, **(b,g)** COS-7, **(c,h)** ARPE-19, **(d,i)** HuH-7, and **(e,j)** D407 cells in the absence and presence of genistein. **a-e**, Control, **f-j** are the corresponding cell lines incubated with 400 µmol/l genistein for 2 hours. The amount of intracellular stained vesicles is a measure for the remaining uptake of LacCer (arrowheads). The percentage of remaining LacCer uptake shown at the bottom of the figure are the corresponding values obtained by flow cytometry. All experiments were performed at least three times and representative images are shown. Bars = 20 µm. LacCer, lactosylceramide.

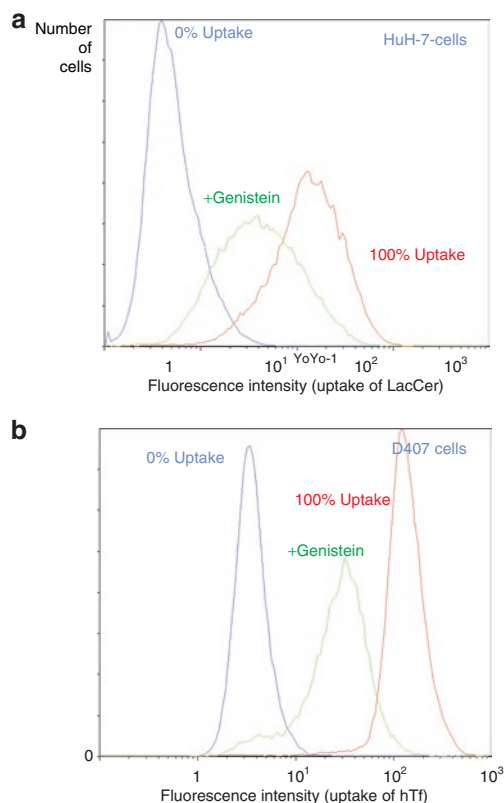


Figure 6 Distribution of fluorescence values after marker uptake as obtained with FC. **(a)** Fluorescence intensity histograms of HuH-7 cells incubated with LacCer at 4°C (blue; “0% uptake”), at 37°C (red; “100% uptake”), and cells incubated with 400 μmol/l genistein for 2 hours before LacCer addition (green; “+genistein”). **(b)** Fluorescence intensity histograms of D407 cells incubated with hTf at 4°C (blue; “0% uptake”), at 37°C (red; “100% uptake”), and cells incubated with 400 μmol/l genistein for 2 hours before hTf addition (green; “+genistein”). FC, flow cytometry; hTf, human transferrin; LacCer, lactosylceramide.

chlorpromazine has strong effects on the ratio of forward scatter and side scatter of Vero cells (Figure 3). We see a shift in the scatter profile to cells which exhibit more side scatter (Figure 3b). The shift becomes even larger after LacCer and back exchange treatment (Figure 3c). This indicates a marked effect of the inhibitor treatment on cell morphology. However, after an additional propidium iodide staining step, it seemed that only 1.2% extra dead cells were measured after chlorpromazine treatment and even less after chlorpromazine and back exchange (Figure 3). The dead cells are probably all lost during the washing steps before flow cytometry analysis. Slight morphological effects were also observed after K^+ -depletion (not shown here). Based on the images in Figure 2 and the toxicity data (Figure 1), we determined that 2-hours incubation with 5 mmol/l M β CD was the preferential condition for subsequent analysis in case of the D407 cells. By performing similar endocytic marker uptake experiments for each endocytic inhibitor and each cell line, we have finally found the following optimal inhibitor conditions: 5 μg/ml chlorpromazine for 2 hours for Vero and COS-7 cells, 10 μg/ml chlorpromazine for 2 hours for ARPE-19, D407, and HuH-7 cells, 400 μmol/l genistein for 2 hours, 5 mmol/l M β CD for 2 hours and 15 minutes incubation with hypotonic buffer between the washing steps with K^+ -free buffer for K^+ -depletion.

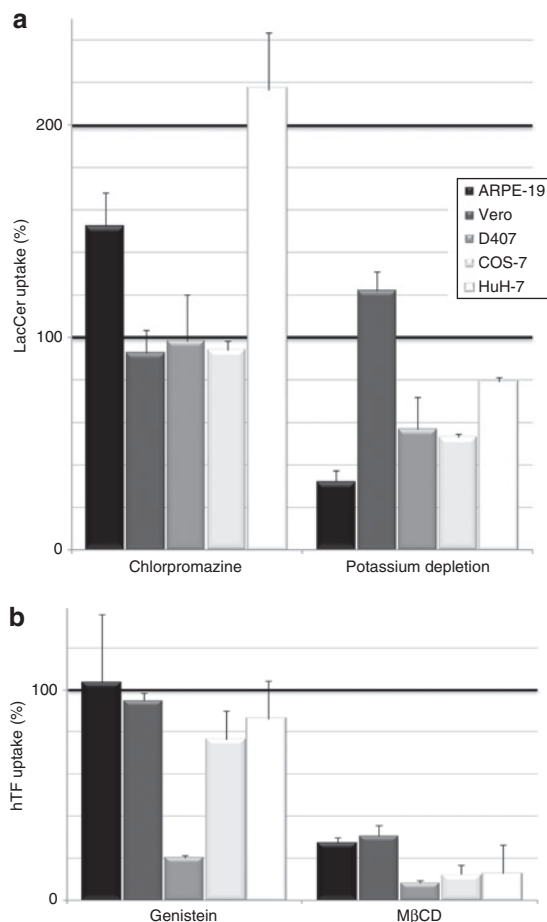


Figure 7 Specificity of endocytosis inhibitors. Quantification of **(a)** LacCer and **(b)** hTf uptake by flow cytometry in ARPE-19, Vero, RPE (D407), COS-7, and HuH-7 cells after incubation with **(a)** chlorpromazine or potassium depletion buffer and **(b)** genistein and M β CD. Values are given as means + SD of triplicates. hTf, human transferrin; LacCer, lactosylceramide; M β CD, methyl- β -cyclodextrin; RPE, retinal pigment epithelium.

Efficacy of endocytosis inhibitors is cell type dependent

Having determined suitable inhibitor conditions, the efficacy of the CDE and CIE inhibitors to inhibit the uptake of hTf and LacCer was assessed quantitatively in the different cell lines by flow cytometry. As can be seen in Figure 4a, chlorpromazine treatment inhibited the uptake of hTf by ~50% in D407 and HuH-7 cells, but showed no or little significant inhibitory capacity in ARPE-19 and Vero cells and even an enhanced effect in COS-7 cells. Although potassium depletion reduced hTf uptake by >80% in the D407 and HuH-7 cells, it had no considerable effect on the COS-7 cells and even enhanced hTf uptake in the ARPE-19 and Vero cells. Pronounced differences between cell lines were also found when studying the inhibition of CIE, as can be seen in Figure 4b. In general, both genistein and M β CD reduced LacCer uptake. We note that, although M β CD effectively inhibited LacCer uptake (>60% inhibition) in all cell lines, postincubation treatments including back exchange and trypsinisation often reduced the number of cells that were available for subsequent analysis because cells started to detach during M β CD treatment (see also Figure 2f). In the study of Rodal *et al.* and in other examples,^{30,33} M β CD was used in combination with

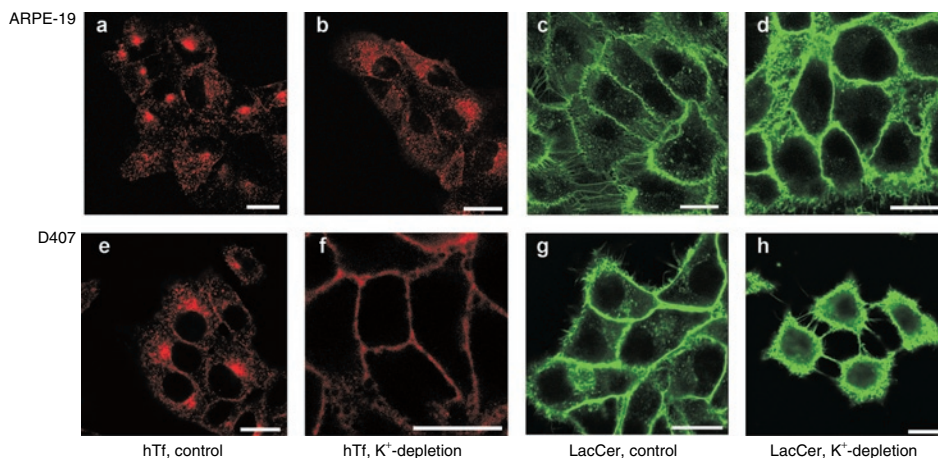


Figure 8 Intracellular distribution of endocytic markers after K^+ -depletion in RPE cells. Evaluation by confocal microscopy of hTf (red) and LacCer (green) uptake in (a–d) ARPE-19 and (e–h) D407 cells. (a,e) Control samples following incubation with hTf uptake, or (c,g) LacCer. b,f,d, and h are the corresponding cell lines following K^+ -depletion. All experiments were performed at least three times and representative images are shown. Bars = 20 μ m. hTf, human transferrin; LacCer, lactosylceramide; RPE, retinal pigment epithelium.

an inhibitor of cholesterol biosynthesis lovastatin. We therefore performed the same experiments where the preincubation periods were performed in the presence of both M β CD and lovastatin. The same inhibitory effects on endocytosis were observed in cells treated with M β CD alone (data not shown).

The flow cytometry data was visually confirmed by confocal microscopy. Images in [Figure 5](#) show the cell type dependency of genistein effects on the cellular uptake of LacCer. From left to right, the confocal images show an increase in inhibition of LacCer internalization ([Figure 5f–j](#)). At this instance, we would like to point out that care should be taken when evaluating inhibitory effects based on microscopy images alone. As is clear from the fluorescence histograms in [Figure 6](#), obtained by flow cytometry, the inhibitory effect is quite heterogeneous. The broad distributions indicate that, within the same cell population, some cells will be strongly inhibited, whereas others will not show any inhibition at all. Because microscopy images only show a very limited number of cells (typically tens of cells), we would like to suggest that they rather should be used as qualitative complementary data when studying cellular uptake to the flow cytometry data where tens of thousands of cells are analyzed quantitatively.

Lack of specificity of endocytosis inhibitors

Finally, we evaluated the specificity of the inhibitors for a particular endocytic pathway. The specificity of K^+ -depletion and chlorpromazine for inhibiting CDE was tested by looking at their effects on the uptake of LacCer. Similarly, the specificity of genistein and M β CD for inhibiting CIE was evaluated via studying the uptake of hTf. Chlorpromazine treatment had no inhibitory effect on LacCer uptake ([Figure 7a](#)) and even caused a marked increase of the internalization of this marker in ARPE-19 and HuH-7 cells. In strong contrast to chlorpromazine, but equally surprising, we found that K^+ -depletion also inhibited CIE of ARPE-19, D407, and COS-7 cells. Potassium depletion had no significant effect on CIE of Vero cells and slightly reduced LacCer uptake in HuH-7 cells. With the exception of D407 cells, genistein had little effect on hTf uptake in these cell lines. M β CD at the other hand, was highly effective at

inhibiting this probe in all cell lines, which points out the lack of specificity of M β CD treatment. Again, these results were supported by confocal microscopy data. [Figure 8](#) shows that K^+ -depletion has a contrasting effect in both retinal pigment epithelial cell lines: although the hTf internalization is completely inhibited in D407 cells, a considerable increase could be observed in ARPE-19 cells, in accordance with the flow cytometry data shown in [Figure 4a](#). The uptake of LacCer after this K^+ -depletion is strongly reduced in D407 cells and even more pronounced in ARPE-19 cells, again in agreement with the flow cytometry data ([Figure 7a](#)).

DISCUSSION

A thorough understanding of the intracellular pharmacokinetics and fate of a gene carrier complex, which is in part determined by the endocytic pathways through which they are internalized, is of great importance for optimal design of effective nonviral gene-delivery carriers. Quantitative insight into the contribution of different endocytic pathways to the cellular uptake of carriers can be very helpful to achieve this goal because it will allow correlation of the effects of specific carrier characteristics with cellular uptake, intracellular processing, and subsequent gene expression. The internalization and postendocytic trafficking of gene carrier complexes can be studied by different approaches but all have their limitations. A common approach is to use inhibitors that are deemed to affect specific cellular uptake mechanisms. Transient or stable knock out mutants of proteins required for specific pathways can also be used as biological inhibitors. Transient knock outs can be created by using the RNA interference silencing mechanism or by introducing dominant-negative proteins, resulting in silencing of crucial genes or inactivation of specific proteins, respectively and subsequent inhibition of specific pathways.^{8,34–41}

Here, we have focused on the use of chemical inhibitors⁴² to investigate their capacity to selectively block different endocytic pathways. The selected inhibitors are often used to study endocytic pathways, and in part this is because they are easy to use. When we first started using these agents, however, we found their specificity to be rather questionable, an issue which was recently brought up

by others as well.²⁷ This led us to design a series of experiments to test a number of parameters that would dictate their usefulness for these kinds of studies. We initially discovered that some inhibitors showed clear morphological effects and toxicity even with short incubation times. There was also a huge variation in the efficacy of the selected inhibitors in the different cell lines. Additionally, they generally lacked specificity for defined pathways. It is therefore important to fully characterize their effects on any particular cell line before attempting to define the uptake pathway for any particular macromolecule or nanoparticle that is deemed to enter cells by endocytosis. In the literature only a few examples exist whereby the nonspecific effects of these agents were evaluated.^{22,39,43,44}

Initially, we showed that several of the tested inhibitors exhibit cell type-dependent cytotoxic effects. For example, high-chlorpromazine concentrations had little effect on the ARPE-19 and D407 cell viability, whereas all other tested cell lines, especially the COS-7 cells, were very sensitive to this agent. Similarly, M β CD showed cell specific cytotoxicity at high concentrations that are routinely used for these kinds of studies. These cytotoxic effects complicate data interpretation as the possibility exists that the cells will not survive the combined stress of inhibitor treatment and application of potentially cytotoxic cationic gene complexes. Additionally, in several cases, we observed morphological changes of the cells after incubation with *e.g.*, M β CD, whereas MTT test did not show any cytotoxic effects. This observation is in agreement with what has been reported before, namely that the sensitivity of the MTT assay depends on the mechanism by which the agent is affecting the cells.⁴⁵ Moreover, other side effects of inhibitors have been reported, such as effects on postendocytic trafficking of gene carrier complexes,¹⁶ thus they may affect initial uptake but also downstream pathways. It is therefore clear that all knowledge on the effects of inhibitors on cells will lead to more accurate reliable interpretations and conclusions following their use.

Some of the results obtained were very unexpected and highlighted the different sensitivities of cell lines for individual inhibitors or treatments. Potassium depletion as expected inhibited the uptake of hTf in D407 and HuH-7 cells but was totally ineffective in other cell lines and even showed evidence of promoting uptake. Potassium depletion also proved to be a very effective inhibitor of LacCer uptake in four of the five tested cell lines suggesting that a fraction of LacCer is internalized via CDE in these cells or more likely that potassium depletion affects a number of endocytic pathways. The inhibitory effect of K⁺-depletion on LacCer uptake has not previously been reported, neither has the underlying mechanism by which potassium depletion inhibits CIE been elucidated. The inhibitory effects of M β CD on transferrin uptake has previously been demonstrated^{30,46} and we further demonstrate here that inhibition by this compound is not always a sign that uptake is directed via a CIE route. In our experiments, chlorpromazine showed some degree of specificity, but in some cell lines it was highly toxic. Interestingly Huth *et al.* showed that the effects of chlorpromazine on uptake of liposomes was cell line dependent and that it enhanced liposome uptake in COS-7 cells.⁴⁷ In agreement with this we show cell line specific inhibition of hTf uptake at the tested concentrations of chlorpromazine; only in D407 cells did we observe the expected effects. Moreover, to the best of our knowledge this is the first demonstration of the effects of chlorpromazine on CIE,

as chlorpromazine promoted noticeably the uptake of LacCer in ARPE-19, HuH-7 cells but also in Vero and COS-7 cells at higher concentrations (data not shown). It may be the case that inhibition of clathrin-coated uptake upregulates other pathways and we are currently pursuing this line of observation. Alternatively, because of the short timeframe wherein these compensatory mechanisms should occur, it could be, as described, that this amphipathic molecule is affecting the fluidity of the membrane in a positive way and therefore facilitating endocytosis.²⁷ Furthermore, despite the fact that ARPE-19 and D407 cells are both model cell lines for human retinal pigment epithelial cells, the effects of nearly all inhibitors differ strongly on these related cell lines. The same cell dependency can likely explain why our results on the inhibition of LacCer uptake by potassium depletion and chlorpromazine differ to some extent with the results obtained by Puri *et al.* in skin fibroblasts.¹³

We also compared two different approaches to evaluate the inhibition of uptake of the two endocytic probes. We qualitatively estimated the inhibitory effects on confocal laser scanning microscopy (CLSM) images and we quantitatively assessed internalization of biomarker by flow cytometry. The latter method has the advantage of being a relatively easy to use method to quantitatively describe the inhibitory effect by analyzing a large population of cells (50,000 in our study). Although flow cytometry integrates the total fluorescence per cell, CLSM gives a visual presentation of what the intracellular fluorescence pattern looks like at the single cell level. CLSM images, therefore, give complementary information to the flow cytometry data. When we compared the effects of potassium depletion on hTf uptake in ARPE-19 and D407 cells by CLSM, we observed a similar intracellular distribution (*i.e.*, an enrichment in the perinuclear region) of the labeled hTf in the absence of potassium depletion. However, very different profiles were observed in the potassium depleted cells where in ARPE-19 cells the label was scattered throughout the cytosol and in D407 cells it was constrained to the plasma membrane. This confirmed the flow cytometry data where the plasma membrane fraction of transferrin in D407 cells is lost by acid washing and thus, in these cells, the amount of internalized label was very low in K⁺-depleted cells. Despite the fact that potassium depletion did not affect the uptake of transferrin in three of the five cell lines it has a significant effect downstream of the plasma membrane, possibly by inhibiting its traffic to recycling compartments. Thus valuable endocytic information can be gained by combining microscopy and flow cytometry analysis. Nonetheless, care should be taken when drawing conclusions on inhibitory effects of inhibitors, solely based on confocal images; first of all it is important that laser intensities and detector sensitivities should be identical when comparing images. Second, since only a limited number of cells are usually observed in microscopy images, interpretation might be biased if the inhibitor effect is heterogeneous (see for example [Figure 6b](#)).

Although it is clear that endocytosis inhibitors should be used with care, there is scope for their continued use for unraveling the uptake mechanisms of endocytic probes for cell biology and drug or gene-delivery systems, especially when different methods are combined. New and more specific endocytosis inhibitors are urgently required and there is an equal need for new specific biomarkers, which associate specifically with certain endocytic pathways or vesicles. But as is often the case, a particular protein may be involved in

a number of uptake mechanisms (e.g., dynamin) or be required for traffic at several cell locations (e.g., clathrin). Undoubtedly the combination of inhibitors and biological strategies to specifically inhibit endocytosis (e.g., RNA interference or transfection of dominant-negative proteins) will provide more information. This, however, is reliant on being able to silence a protein without affecting cell viability and other endocytic pathways and identifying a suitable probe whose access is constrained to one pathway.

In this work, we have given additional experimental evidence to highlight the care needed in the interpretation of data obtained from using chemical inhibitors of endocytosis. We have found that the inhibitory effect is extremely cell type dependent, as is the concentration for minimizing cellular toxicity and maximizing inhibitory effect. Moreover, chemical compounds can produce pronounced side effects on cellular morphology and the inhibition is not always as specific for a particular endocytic pathway as is sometimes stated in literature. We conclude that treatment with endocytosis inhibitors, which have been applied successfully on one cell line, should not be blindly transferred to other cell lines without performing the necessary control experiments. Additionally, combining the use of chemical inhibitors with inhibition of specific pathways through small interfering RNA silencing or the expression of dominant-negative proteins is likely to give more meaningful data. This in turn can be complemented by fluorescence microscopy experiments using fluorescently labeled gene complexes and fluorescent tracers that are specific for certain endocytic pathways. The combined results may ultimately provide important information on the mechanisms by which nonviral gene vectors enter cells.

MATERIALS AND METHODS

Materials. Chlorpromazine, M β CD, genistein, and lovastatin (mevinolin) were purchased from Sigma-Aldrich (Bornem, Belgium). Propidium iodide solution was from Invitrogen (Merelbeke, Belgium). Dulbecco's modified Eagle's medium (DMEM), OptiMEM, L-glutamine, fetal bovine serum, penicillin-streptomycin (5,000 IU/ml penicillin and 5,000 μ g/ml streptomycin), and phosphate-buffered saline were supplied by GibcoBRL (Merelbeke, Belgium). hTF-AlexaFluor633 and bovine serum albumin (BSA)-complexed BODIPY FL C₅-LacCer were purchased from Molecular Probes (Merelbeke, Belgium). BODIPY-labeled LacCer was applied to the cells as a complex with BSA, which makes the lipid marker water soluble and therefore presentable to cells.¹³

Cell culture. COS-7 cells (African green monkey kidney fibroblast cell line; ATCC number CRL-1651) and Vero cells (African green monkey kidney epithelial cell line; ATCC number CCL-81) were cultured in DMEM containing 10% fetal bovine serum, 2 mmol/l L-glutamine, and 2% penicillin-streptomycin. HuH-7 cells (human hepatocellular carcinoma cell line; HSRB number JCRB 0403) and ARPE-19 cells (retinal pigment epithelial cell line; ATCC number CRL-2302) were cultured in DMEM:F12 supplemented with 10% fetal bovine serum, 2 mmol/l L-glutamine, and 2% penicillin-streptomycin. D407 (retinal pigment epithelial cell line) cells⁴⁸ were a kind gift from Richard Hunt (University of South Carolina, Medical School, Columbia, SC) and were cultured in DMEM with high-glucose concentration (4,500 mg/l), 5% fetal bovine serum, 2 mmol/l L-glutamine, and 2% penicillin-streptomycin. All cells were grown at 37°C in a humidified atmosphere containing 5% CO₂.

Cytotoxicity analysis. The cytotoxicity of the different inhibition protocols was evaluated using the EZ4U cell proliferation and cytotoxicity assay kit (Biomedica, Vienna, Austria). This assay is based on the principle of

the classical MTT test where tetrazolium salts are reduced into intensely coloured formazan derivatives by mitochondrial enzymes which are rapidly inactivated within a few minutes after cell death. The assay was performed according to the manufacturer's instructions. Briefly, cells were seeded at 10⁵ cells/well in a 24-well plate for 24 hours. They were then incubated in OptiMEM with various concentrations of the described inhibitors for 2 hours. For potassium depletion, cells were washed twice with potassium depletion buffer (140 mmol/l NaCl, 20 mmol/l HEPES, 1 mmol/l CaCl₂, 1 mmol/l MgCl₂, 1 mg/ml D-glucose, pH 7.4), then incubated for 15 minutes with hypotonic buffer (1:1 potassium depletion buffer and H₂O) at 37°C and finally washed again three times with potassium-free buffer. Subsequently, the cells were immediately incubated with the EZ4U substrate (40 μ l substrate with 260- μ l culture medium per well) containing the tetrazolium salts. After 3 hours at 37°C, the absorbance of the coloured formazan was measured on a Wallac Victor Plate reader (Perkin Elmer-Cetus Life Sciences, Boston, MA) at 460 nm and corrected for the absorbance measured at 630 nm which represents nonspecific absorbance.

Inhibition and specificity studies. For inhibition and specificity studies, enough cells were seeded on sterile MatTek coverslip (1.5)-bottom dishes (MatTek, MA) (for confocal analysis) or six-well plates (for flow cytometry analysis) to reach 70% confluency 24 hours later. The cells were subsequently incubated with the indicated concentration of inhibitor in OptiMEM for the appropriate time. For potassium depletion, cells were processed as described above in potassium-free and hypotonic buffer. The cells were then incubated with 16.7 μ g/ml hTF or 0.81 μ mol/l LacCer for 15 minutes at 37°C in the presence of the relevant inhibitor or buffer. hTF and LacCer uptake under different conditions were then qualitatively and quantitatively evaluated using CLSM and flow cytometry.

CLSM. The internalization of the fluorescently tagged biomarkers hTF and LacCer was visualized and evaluated with a Nikon C1 confocal laser scanning microscope (Nikon Belux, Brussels, Belgium) equipped with a Plan Apo VC 60 \times 1.4 NA oil immersion objective lens and suitable optical elements to obtain fluorescent and differential interference contrast transmission images. BODIPY FL-labeled LacCer was excited with the argon ion 488-nm laser line and emission light was collected using a 500–530 nm band pass filter. AlexaFluor 633 labeled hTF was excited with the diode 639-nm laser line and emission light was collected using a 655-nm long pass filter. After removing the medium with the marker and inhibitor, cells were imaged in HMEM-G+I [DMEM without glucose (GibcoBRL)] with 5 mmol/l NaN₃, buffered with 10 mmol/l HEPES to a pH of 7.4 to deplete the cells of energy by inhibiting mitochondrial respiration.⁴⁹ Representative 2D confocal images were acquired approximately through the middle plane of the cell.

Flow cytometry analysis. Before measuring the cell-associated fluorescence with flow cytometry, it is imperative to remove the fraction of fluorescently labeled markers, hTF and LacCer, that are associated with the plasma membrane but not internalized. For transferrin a well-established acid wash step was used for this purpose.⁵⁰ Following incubation with the marker for 15 minutes, the cells were washed twice with cold HMEM-G+I to inhibit metabolic activities followed by 1 minute incubation in ice-cold acid wash buffer (0.2 mol/l acetic acid and 0.2 mol/l NaCl). The cells were then quickly washed twice with ice-cold HMEM-G+I. For LacCer, surface-associated fluorescent lipid is extracted out of the plasma membrane (back exchange¹⁵) by adding defatted BSA. This will only have access to plasma membrane localized fluorescent lipid and not the fraction that is internalized. After incubation with LacCer, the cells were washed twice with cold HMEM-G+I and then washed six times for 10 minutes with cold HMEM-G+I supplemented with 5% defatted BSA. After the removal of noninternalized fluorescent markers, the cells were finally detached with trypsin and EDTA, centrifuged and resuspended in ice-cold flow buffer (1% BSA and 0.1% NaN₃ in phosphate-buffered saline). The remaining (internal)

fluorescence of the cells was analyzed with a 5-color FC500 flow cytometer (Beckman Coulter, Nyon, Switzerland) equipped with an argon laser (excitation at 488 nm). To stain dead cells, propidium iodide stock solution was diluted till 10 µg/ml in the cell suspension, 1 minute before analysis, to stain nuclei of (dead) cells with a permeated plasma membrane.

For quantification, the experiments were performed in triplicate and for each sample 50,000 events were collected by list-mode data that consisted of side scatter, forward scatter, and fluorescence emission centered at 530 nm (FL1, for AF488 and Bodipy FL) and 620 nm (FL3, for propidium iodide)). CXP software (Beckman Coulter) was used for the analyses. The data were generated from ungated cells. The internalization of fluorescent marker is calculated as the mean fluorescence value (average of three experiments) corrected for a 0% uptake and normalized to a 100% uptake. The 0% uptake was determined from cells at 4°C which have been incubated with the marker for 15 minutes and underwent the same acid wash or back exchange. The 100% uptake was determined from cells which have not been exposed to any inhibitor and have been incubated with the marker for 15 minutes followed by an acid wash or back exchange as described above.

SUPPLEMENTARY MATERIAL

Figure S1. Schematic presentation of a current classification of the endocytic mechanisms in mammalian cells.

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REFERENCES

- Wattiaux, R, Laurent, N, Wattiaux-De Coninck, S and Jadot, M (2000). Endosomes, lysosomes: their implication in gene transfer. *Adv Drug Deliv Rev* **41**: 201–208.
- Conner, SD and Schmid, SL (2003). Regulated portals of entry into the cell. *Nature* **422**: 37–44.
- Jones, AT (2007). Macropinocytosis: searching for an endocytic identity and role in the uptake of cell penetrating peptides. *J Cell Mol Med* **11**: 670–684.
- Grimmer, S, van Deurs, B and Sandvig, K (2002). Membrane ruffling and macropinocytosis in A431 cells require cholesterol. *J Cell Sci* **115**(Pt 14): 2953–2962.
- Rejman, J, Oberle, V, Zuhorn, IS and Hoekstra, D (2004). Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. *Biochem J* **377**(Pt 1): 159–169.
- Widera, A, Norouziyan, F and Shen, WC (2003). Mechanisms of TFR-mediated transcytosis and sorting in epithelial cells and applications toward drug delivery. *Adv Drug Deliv Rev* **55**: 1439–1466.
- Maxfield, FR and McGraw, TE (2004). Endocytic recycling. *Nat Rev Mol Cell Biol* **5**: 121–132.
- Mayor, S and Pagano, RE (2007). Pathways of clathrin-independent endocytosis. *Nat Rev Mol Cell Biol* **8**: 603–612.
- Simons, K and Toomre, D (2000). Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* **1**: 31–39.
- Parton, RG and Simons, K (2007). The multiple faces of caveolae. *Nat Rev Mol Cell Biol* **8**: 185–194.
- Pelkmans, L, Kartenbeck, J and Helenius, A (2001). Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. *Nat Cell Biol* **3**: 473–483.
- Torgersen, ML, Skretting, G, van Deurs, B and Sandvig, K (2001). Internalization of cholera toxin by different endocytic mechanisms. *J Cell Sci* **114**(Pt 20): 3737–3747.
- Puri, V, Watanabe, R, Singh, RD, Dominguez, M, Brown, JC, Wheatley, CL *et al.* (2001). Clathrin-dependent and -independent internalization of plasma membrane sphingolipids initiates two Golgi targeting pathways. *J Cell Biol* **154**: 535–547.
- Singh, RD, Puri, V, Valiyaveetil, JT, Marks, DL, Bittman, R and Pagano, RE (2003). Selective caveolin-1-dependent endocytosis of glycosphingolipids. *Mol Biol Cell* **14**: 3254–3265.
- Marks, DL, Singh, RD, Choudhury, A, Wheatley, CL and Pagano, RE (2005). Use of fluorescent sphingolipid analogs to study lipid transport along the endocytic pathway. *Methods* **36**: 186–195.
- Zuhorn, IS, Kalicharan, R and Hoekstra, D (2002). Lipoplex-mediated transfection of mammalian cells occurs through the cholesterol-dependent clathrin-mediated pathway of endocytosis. *J Biol Chem* **277**: 18021–18028.
- Glebov, OO, Bright, NA and Nichols, BJ (2006). Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. *Nat Cell Biol* **8**: 46–54.
- Medina-Kauwe, LK, Xie, J and Hamm-Alvarez, S (2005). Intracellular trafficking of nonviral vectors. *Gene Ther* **12**: 1734–1751.
- Khalil, IA, Kogure, K, Akita, H and Harashima, H (2006). Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. *Pharmacol Rev* **58**: 32–45.
- Elouahabi, A and Ruyschaert, JM (2005). Formation and intracellular trafficking of lipoplexes and polyplexes. *Mol Ther* **11**: 336–347.
- Midoux, P, Breuzard, G, Gomez, JP and Pichon, C (2008). Polymer-based gene delivery: a current review on the uptake and intracellular trafficking of polyplexes. *Curr Gene Ther* **8**: 335–352.
- von Gersdorff, K, Sanders, NN, Vandenbroucke, R, De Smedt, SC, Wagner, E and Ogris, M (2006). The internalization route resulting in successful gene expression depends on both cell line and polyethyleneimine polyplex type. *Mol Ther* **14**: 745–753.
- Douglas, KL, Piccirillo, CA and Tabrizian, M (2008). Cell line-dependent internalization pathways and intracellular trafficking determine transfection efficiency of nanoparticle vectors. *Eur J Pharm Biopharm* **68**: 676–687.
- Wong, AW, Scales, SJ and Reilly, DE (2007). DNA internalized via caveolae requires microtubule-dependent, Rab7-independent transport to the late endocytic pathway for delivery to the nucleus. *J Biol Chem* **282**: 22953–22963.
- Gabrielson, NP and Pack, DW (2009). Efficient polyethyleneimine-mediated gene delivery proceeds via a caveolar pathway in HeLa cells. *J Control Release* **136**: 54–61.
- Banks, GA, Roselli, RJ, Chen, R and Giorgio, TD (2003). A model for the analysis of nonviral gene therapy. *Gene Ther* **10**: 1766–1775.
- Ivanov, AI (2008). Pharmacological inhibition of endocytic pathways: Is it specific enough to be useful? *Methods Mol Biol* **440**: 15–33.
- Wang, LH, Rothberg, KG and Anderson, RC (1993). Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. *J Cell Biol* **123**: 1107–1117.
- Larkin, JM, Donzell, WC and Anderson, RG (1986). Potassium-dependent assembly of coated pits: new coated pits form as planar clathrin lattices. *J Cell Biol* **103**(6 Pt 2): 2619–2627.
- Rodal, SK, Skretting, G, Garred, O, Vilhardt, F, van Deurs, B and Sandvig, K (1999). Extraction of cholesterol with methyl-β-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. *Mol Biol Cell* **10**: 961–974.
- Parton, RG, Joggerst, B and Simons, K (1994). Regulated internalization of caveolae. *J Cell Biol* **127**: 1199–1215.
- Nabi, IR and Le, PU (2003). Caveolae/raft-dependent endocytosis. *J Cell Biol* **161**: 673–677.
- Gonçalves, C, Mennesson, E, Fuchs, R, Gorvel, JP, Midoux, P and Pichon, C (2004). Macropinocytosis of polyplexes and recycling of plasmid via the clathrin-dependent pathway impair the transfection efficiency of human hepatocarcinoma cells [abstract]. *Mol Ther* **10**: 373–385.
- Nichols, BJ (2002). A distinct class of endosome mediates clathrin-independent endocytosis to the Golgi complex. *Nat Cell Biol* **4**: 374–378.
- Hinrichsen, L, Harborth, J, Andrees, L, Weber, K and Ungewickell, EJ (2003). Effect of clathrin heavy chain- and alpha-adaptin specific small interfering RNAs on endocytic accessory proteins and receptor trafficking in HeLa cells. *J Biol Chem* **278**: 45160–45170.
- Huang, F, Khvorova, A, Marshall, W and Sorkin, A (2004). Analysis of clathrin-mediated endocytosis of epidermal growth factor receptor by RNA interference. *J Biol Chem* **279**: 16657–16661.
- Vanden Broeck, D and De Wolf, MJ (2006). Selective blocking of clathrin-mediated endocytosis by RNA interference: epsin as target protein. *BioTechniques* **41**: 475–484.
- Benmerah, A, Bayrou, M, Cerf-Bensussan, N and Dautry-Varsat, A (1999). Inhibition of clathrin-coated pit assembly by an Eps15 mutant. *J Cell Sci* **112** (Pt 9): 1303–1311.
- Payne, CK, Jones, SA, Chen, C and Zhuang, X (2007). Internalization and trafficking of cell surface proteoglycans and proteoglycan-binding ligands. *Traffic* **8**: 389–401.
- Damke, H, Baba, T, Warnock, DE and Schmid, SL (1994). Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J Cell Biol* **127**: 915–934.
- Liu, SH, Marks, MS and Brodsky, FM (1998). A dominant-negative clathrin mutant differentially affects trafficking of molecules with distinct sorting motifs in the class II major histocompatibility complex (MHC) pathway. *J Cell Biol* **140**: 1023–1037.
- Lamaze, C and Schmid, SL (1995). The emergence of clathrin-independent pinocytic pathways. *Curr Opin Cell Biol* **7**: 573–580.
- Rejman, J, Bragonzi, A and Conese, M (2005). Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. *Mol Ther* **12**: 468–474.
- Mano, M, Teodósio, C, Paiva, A, Simões, S and Pedroso de Lima, MC (2005). On the mechanisms of the internalization of S4(13)-PV cell-penetrating peptide. *Biochem J* **390**(Pt 2): 603–612.
- Weyeremann, J, Lochmann, D and Zimmer, A (2005). A practical note on the use of cytotoxicity assays. *Int J Pharm* **288**: 369–376.
- Subtil, A, Gaidarov, I, Kobylarz, K, Lampson, MA, Keen, JH and McGraw, TE (1999). Acute cholesterol depletion inhibits clathrin-coated pit budding. *Proc Natl Acad Sci USA* **96**: 6775–6780.
- Huth, US, Schubert, R and Peschka-Süss, R (2006). Investigating the uptake and intracellular fate of pH-sensitive liposomes by flow cytometry and spectral bioimaging. *J Control Release* **110**: 490–504.
- Davis, AA, Bernstein, PS, Bok, D, Turner, J, Nachtigal, M and Hunt, RC (1995). A human retinal pigment epithelial cell line that retains epithelial characteristics after prolonged culture. *Invest Ophthalmol Vis Sci* **36**: 955–964.
- Yuwen, L, Yi, L, Cunxin, W, Songsheng, Q and Fengjiao, D (2000). Microcalorimetric studies of the inhibition of sodium azide on the mitochondrial metabolism of fish liver tissue. *Thermochimica Acta* **351**: 51–54.
- Sonnichsen, B, De Renzi, S, Nielsen, E, Rietdorf, J and Zerial, M (2000). Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. *J Cell Biol* **149**: 901–914.