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THE USE OF INHIBITORS TO STUDY ENDOCYTIC PATHWAYS OF GENE CARRIERS: OPTIMISATION AND PITFALLS

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Short title:

EVALUATING INHIBITORS TO STUDY ENDOCYTIC PATHWAYS

ABSTRACT

Non-viral 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, since 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 endocytic inhibitors (chlorpromazine, genistein, MßCD and potassium depletion) on cell viability and endocytosis in five well described cell lines. We found that chlorpromazine and to a lesser extent MßCD 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 that are reported to enter cells via clathrin dependent and independent 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 questions the significance of performing endocytosis studies with these agents in the absence of very stringent controls.

INTRODUCTION

Successful gene delivery relies on the development of non-toxic 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, non-viral 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 non-viral gene complexes may therefore help to design more effective gene carriers.

The majority of reports on cellular uptake mechanisms suggests that endocytosis is the preferred route of cell entry of non-viral gene carriers [1]. As shown in **Fig. 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 non-selective endocytic mechanism for internalizing suspended macromolecules and the internalized vesicles or macropinosomes can have sizes up to 5 μm [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 (LDL) receptor, the transferrin receptor and the

Epidermal Growth Factor (EGF) 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 sub-classification 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]. Certain pathogens, like SV40 virus and Cholera toxin subunit B (CTB) 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 CTB 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 clathrin independent 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 clathrin independent endocytosis. Flotillin dependent endocytosis was recently characterized as a dynamin-2, caveolin-1 and clathrin independent pathway [17]. Most cells also have the capacity to constitutively internalise fluid via less well characterised 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 non-viral 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]. While there are several tools available for elucidating the endocytic pathways involved in the uptake of gene complexes, the present paper focuses on the use of these inhibitors which can interfere with the endocytic processes.

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-ß-cyclodextrin (MßCD) and genistein are evaluated as inhibitors of CIE. MßCD is a cyclic oligomer of glucopyranoside that inhibits cholesterol dependent endocytic processes by reversibly extracting the steroid out of the plasma membrane [30]. MßCD 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 indispensible events in the caveolae mediated uptake mechanism [31,32]. Here, we evaluate the usefulness of these four well characterised inhibitors of endocytosis in 5 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 microscopy, we evaluate the capacity and specificity of the agents to interfere with the uptake of endocytic markers. As endocytic markers we utilised two well characterised probes: human transferrin (hTf) for CDE and lactosylceramide (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 MßCD. 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 h. In case of K^+-depletion, cytotoxicity was determined following treatment with the different buffers as described in Materials and Methods. As positive and negative controls, cells were exposed for 2 h 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 **Fig. 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 (**Fig. 1a**). All cell lines retained >85% viability upon K^+-depletion (**Fig. 1b**). MßCD exhibited only very slight cytotoxicity to ARPE-19, D407 and COS-7 cells at 10 mM, while Vero and especially HuH-7 cells were clearly more sensitive to the effects of this agent (**Fig. 1c**). Finally, none of the tested genistein concentrations had a noticeable effect on the viability of the five cell lines tested (**Fig. 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 internalised by CDE and CIE respectively, after incubation with different inhibitor concentrations. K^+-depletion and chlorpromazine are reported to specifically inhibit CDE, whilst MßCD 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 MßCD on LacCer uptake is shown in **Fig. 2**. **Fig. 2a** shows control cells after incubation with LacCer for 15 min and the probe is shown to label the plasma membrane and intracellular vesicles in the perinuclear region. Incubating the cells with MßCD prior to LacCer addition significantly reduced its uptake in a time and concentration dependent manner (**Fig. 2b-f**). However, longer incubation times (> 2 h) and higher concentrations (> 5 mM) had major morphological effects and the cells tended to detach from the growing surface during the experimental procedure. **Fig. 2f** already shows that MßCD can have pronounced effects on the cellular morphology. Like MßCD, 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 chlorpromazine has strong effects on the ratio of forward scatter and side scatter of Vero cells (**Fig. 3**). We see a shift in the scatter profile to cells which exhibit more side scatter (**Fig. 3b**). The shift becomes even larger after LacCer and Back exchange treatment (**Fig. 3c**). This indicates a marked effect of the inhibitor treatment on cell morphology. However, after an additional PI staining, it seemed that only 1,2% extra dead cells were measured after chlorpromazine treatment and even less after chlorpromazine and back exchange (**Fig. 3**). The dead cells are probably all lost during the washing steps prior to FC analysis. Slight morphological effects were also observed after K^+-depletion (not shown here). Based on the images in **Fig. 2** and the toxicity data (**Fig. 1**) we determined that 2 h incubation with 5 mM MßCD 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 h for Vero and COS-7 cells, 10 µg/ml chlorpromazine for 2 h for ARPE-19, D407 and HuH-7 cells, 400 µM genistein for 2 h, 5 mM MßCD for 2 h and 15 min incubation with hypotonic buffer between the washing steps with K^+-free buffer for K^+-depletion.

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 respectively hTf and LacCer was assessed quantitatively in the different cell lines by flow cytometry. As can be seen in **Fig. 4a**, chlorpromazine treatment inhibited the uptake of hTf by approximately 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. While 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 **Fig. 4b**. In general, both genistein and MßCD reduced LacCer uptake. We note that, while MßCD effectively inhibited LacCer uptake (> 60% inhibition) in all cell lines, post incubation treatments including back exchange and trypsinisation often reduced the number of cells that were available for subsequent analysis because cells started to detach during MßCD treatment (see also **Fig. 2f**). In the study of Rodal et al and in other examples [30,33], MßCD was used in combination with an inhibitor of cholesterol biosynthesis lovastatin. We therefore performed the same experiments where the preincubation periods were performed in the presence of both MßCD and lovastatin. The same inhibitory effects on endocytosis were observed as we did in cells treated with MßCD alone (Data not shown).

The flow cytometry data was visually confirmed by confocal microscopy. Images in **Fig. 5** show the cell type dependency of genistein on the cellular uptake of LacCer. From left to right, the confocal images show an increase in inhibition of LacCer internalization (**Fig. 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 **Fig. 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, while others will not show any inhibition at all. Since 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 MßCD for inhibiting CIE was evaluated via studying the uptake of hTf. Chlorpromazine treatment had no inhibitory effect on LacCer uptake (**Fig. 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. MßCD at the other hand, was highly effective at inhibiting this probe in all cell lines, which points out the lack of specificity of MßCD treatment. Again, these results were supported by confocal microscopy data. **Fig. 8** shows that K^+-depletion has a contrasting effect in both RPE cell lines: while 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 **Fig. 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 (**Fig. 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 non-viral 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 since it will allow correlation of the effects of specific carrier characteristics with cellular uptake, intracellular processing and subsequent gene expression. The internalization and post-endocytic 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 (RNAi) 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 inhibitors [42] 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 non specific 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, while all other tested cell lines, especially the COS-7 cells, were very sensitive to this agent. Similarly, MßCD 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. MßCD while the 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 post-endocytic 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 help leading to reliable interpretations and conclusions drawn 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 internalised 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 MßCD 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 clathrin independent 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 we did 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 when applying higher chlorpromazine concentrations (data not shown). It may be the case that inhibition of clathrin coated uptake may upregulate 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 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). While 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. Secondly, 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 **Fig. 6b**).

While it is clear that endocytosis inhibitors should be used with care, there is scope for their continued use for unravelling 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. RNAi 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.

CONCLUSION

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 siRNA 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 non-viral gene vectors enter cells.

MATERIALS AND METHODS

Materials

Chlorpromazine, MßCD, genistein and lovastatin (mevinolin) were purchased from Sigma Aldrich (Bornem, Belgium). Propidium iodide (PI) solution was from Invitrogen (Merelbeke, Belgium). Dulbecco's modified Eagle's medium (DMEM), OptiMEM, L-glutamine (L-Gln), foetal bovine serum (FBS), penicillin-streptomycin (5000 IU/ml penicillin and 5000 µg/ml streptomycin) (P/S) and phosphate buffered saline (PBS) were supplied by GibcoBRL (Merelbeke, Belgium). Human transferrin-AlexaFluor633 (hTF) and bovine serum albumin (BSA)-complexed BODIPY FL C_5-lactosylceramide (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 % FBS, 2 mM L-Gln and 2 % P/S. HuH-7 cells (human hepatocellular carcinoma cell line; HSRRB number JCRB 0403) and ARPE-19 cells (retinal pigment epithelial cell line; ATCC number CRL-2302) were cultured in DMEM:F12 supplemented with 10 % FBS, 2 mM L-Gln and 2 % P/S. D407 (retinal pigment epithelial cell line) cells [48] were a kind gift from Dr Richard Hunt (University of South Carolina, Medical School, Columbia,

USA) and were cultured in DMEM with high glucose concentration (4500 mg/l), 5 % FBS, 2 mM L-Gln and 2 % P/S. All cells were grown at 37°C in a humidified atmosphere containing 5 % CO_2.

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 10^5 cells/well in a 24-well plate for 24 h. They were then incubated in OptiMEM with various concentrations of the described inhibitors for 2 h. For potassium depletion, cells were washed twice with potassium depletion buffer (140 mM NaCl, 20 mM Hepes, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/ml D-glucose, pH 7.4), then incubated for 15 min with hypotonic buffer (1:1 potassium depletion buffer and H_2O) at 37°C and finally washed again 3 times with potassium-free buffer. Subsequently, the cells were immediately incubated with the EZ4U substrate (40 µl substrate with 260 µl culture medium per well) containing the tetrazolium salts. After 3 h at 37°C, the absorbance of the coloured formazan was measured on a Wallac Victor Plate reader (Perkin Elmer-Cetus Life Sciences, Boston, MA, USA) 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 corporation, MA, USA) (for confocal analysis) or 6-well plates (for flow cytometry analysis) to reach 70 % confluency 24 h 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 µg/ml hTf or 0.81 µM LacCer for 15 min 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 confocal laser scanning microscopy (CLSM) and flow cytometry.

Confocal laser scanning microscopy (CLSM)

The internalisation 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 60x 1.4 NA oil immersion objective lens and suitable optical elements to obtain fluorescent and differential interference contrast (DIC) 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 Merelbeke, Belgium) with 5 mM NaN_3, buffered with 10 mM 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

Prior to 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 min, the cells were washed twice with cold HMEM-G+I to inhibit metabolic activities followed by 1 min incubation in ice cold acid wash buffer (0,2 M acetic acid and 0,2 M 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 exchange [15]) 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 6 times for 10 min with cold HMEM-G+I supplemented with 5% defatted BSA. After the removal of non-internalized fluorescent markers, the cells were finally detached with trypsin and EDTA, centrifuged and resuspended in ice cold flow buffer (1 % BSA and 0,1 % NaN_3 in PBS). 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, PI stock solution was diluted till 10 µg/ml in the cell suspension, 1 min 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 PI)). CXP software (Beckman Coulter, Nyon, Switzerland) 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 min 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 min followed by an acid wash or back exchange as described above.

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SUPPLEMENTAL MATERIAL

Figure S1. Schematic presentation of a current classification of the endocytic mechanisms in mammalian cells. Proposed classification system for the endocytic mechanisms in mammalian cells. Endocytosis can be divided as phagocytosis and pinocytosis, but because phagocytosis is restricted to specialized cells, endocytosis and pinocytosis are often used as synonyms. Pinocytosis represents macropinocytosis, clathrin dependent endocytosis (CDE) and clathrin independent endocytosis (CIE).

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FIGURE LEGENDS

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

Figure 2. Optimization of the concentration of MßCD to inhibit CIE in D407 cells. Optimization of the concentration of MßCD to inhibit CIE in D407 cells. Cells were incubated with LacCer (green) following preincubation with increasing concentrations of MßCD for different incubation times. (**a**) positive control (**b**) 30 min 2.5 mM MßCD (**c**) 1 h 2.5 mM MßCD (**d**) 2 h 3 mM MßCD (**e**) 2 hr 5 mM MßCD and (**f**) 2 h 10 mM MßCD. 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 MßCD treatment, resulting in rounding up and lifting of the cells from the growth surface. All experiments were performed at least 3 times and representative images are shown. Scalebars = 20 µm.

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 h with 10 µg/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 prior to PI staining. The percentage of PI positive cells is shown in the right lower corner of the diagrams.

Figure 4. Efficacy of endocytosis inhibitors. Quantification of hTF (**a**) and LacCer (**b**) 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 MßCD. Values are given as means + SD of triplicates.

Figure 5. Intracellular distribution of LacCer after genistein treatment is dependent on cell type. Evaluation of LacCer uptake (green) in Vero (**a** and **f**), COS-7 (**b** and **g**), ARPE-19 (**c** and **h**), HuH-7 (**d** and **i**) and D407 (**e** and **j**) cells in the absence and presence of genistein. **a** - **e** control, **f** - **j** are the corresponding cell lines incubated with 400 µM genistein for 2 h. 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 3 times and representative images are shown. Scalebars = 20 µm.

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 µM genistein for 2 h prior to 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 µM genistein for 2 h prior to hTf addition (green; "+ Genistein").

Figure 7. Specificity of endocytosis inhibitors. Quantification of LacCer (**a**) and hTf (**b**) 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 MßCD. Values are given as means + SD of triplicates.

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 ARPE-19 (**a-d**) and D407 (**e** - **h**) cells. (**a**, **e**) control samples following incubation with hTf uptake, or (**c**, **g**) LacCer. (**b**, **f**, **d**, **h**) are the corresponding cell lines following K^+-depletion. All experiments were performed at least 3 times and representative images are shown. Scalebars = 20 µm.