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Limits and challenges in using transport inhibitors to characterize how nano-sized drug carriers enter cells

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Aim: In this work we illustrate limits and challenges associated with the use of pharmacological inhibitors to study how nanomedicines enter cells and show how such limits can be overcome. Materials & methods: We selected a panel of six common pharmacological inhibitors and a model nanoparticle–cell system. We tested eventual toxicity by measuring cell viability. We confirmed drug efficacy by measuring the uptake of control markers for the pathways involved by flow cytometry and fluorescence microscopy. Results & conclusion: We show how to optimize the use of pharmacological inhibitors and interpret the results generated. Furthermore, we demonstrate that some inhibitors cannot be used for nanomedicine studies because they lose their efficacy when serum is added, as required for nanoparticle exposure to cells.

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Keywords: drug carriers • drug delivery • endocytosis • flow cytometry • nanomedicine • nanoparticle uptake • silica nanoparticles • transport inhibitors • uptake pathways

Nano-sized drug carriers are used to improve the targeted delivery of drugs to their site of action [1–3]. Several products are now on the market and extensive research is still ongoing on how to further improve carrier design and nanomedicine efficacy. Recent debates within the field have highlighted that a better understanding of the mechanisms by which these materials are processed at cellular level is one of the factors that could enable such improvements [4–6].

Within this context, a very first and central question is how nanomedicines are internalized by cells. Cells use different processes to internalize extracellular materials. Several mechanisms of endocytosis have been identified and extensive reviews are available to summarize the key aspects of each of these different pathways. The field is very active and constantly updating [7–10].

It is generally recognized that nanomedicines typically enter cells by active processes, thus some form of endocytosis. These objects are, in fact, too large to simply diffuse inside cells [11,12] unless specifically engineered to do so [13]. However, in many cases the details of the molecular machinery involved are still unclear, as well as very challenging to characterize.

Several studies have tried to determine how the mechanisms of uptake change with nanoparticle (NP) type as a function of their size, charge, shape and other NP parameters [11,12,14,15]. For instance, charge density and the type of charge (positive, negative) can affect the mechanisms NPs use to enter cells, and surface hydrophobicity is also an important parameter affecting uptake [16–22]. More recently, it has emerged that also the medium in which the nanomedicines are dispersed and the resulting biomolecule corona on their surface can affect the entry mechanisms [23,24].

Overall, several methods are available to study transport in cells. Most classic approaches are based on methods to block a certain portal of entry and – in this way – determine its involvement in the uptake of the material of interest. These include, for instance, the use of pharmacological inhibitors and RNA interference or other approaches to knock down or transiently block the expression of key proteins involved in endocytosis. However, when attempting to block transport, cells can adapt by overactivating alternative mechanisms normally less relevant,
Table 1. Overview of the selected transport inhibitors.

<table>
<thead>
<tr>
<th>Inhibitor name</th>
<th>Mechanism of action</th>
<th>Pathways involved</th>
<th>Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIPA</td>
<td>Na⁺ /H⁺ exchanger pump inhibitor; blocks Rac1 and Cdc42 signaling [30,31]</td>
<td>Macropinocytosis</td>
<td>TRITC–Dextran [29]</td>
</tr>
<tr>
<td>CP</td>
<td>AP2 inhibitor; blocks endosome recycling [32]</td>
<td>CME</td>
<td>Dil–LDL [33,34]</td>
</tr>
<tr>
<td>MBCD</td>
<td>Cholesterol depletion</td>
<td>Multiple pathways (lipid-rafts and caveolae-mediated endocytosis) [26]</td>
<td>BODIPY FL CS-Lactosylceramide/BSA complex (LacCer) [28, 35]</td>
</tr>
<tr>
<td>Dyn</td>
<td>Dynamin inhibitor [36,37]</td>
<td>Multiple pathways [38]</td>
<td>Dil–LDL [33,34]</td>
</tr>
<tr>
<td>NZ</td>
<td>Binds to tubulin, blocking microtubule polymerization [39]</td>
<td>CME [40], macropinocytosis and other mechanisms [41,42]</td>
<td>Tubulin staining (α-Tubulin)</td>
</tr>
<tr>
<td>CytD</td>
<td>Mycotoxin that binds actin and blocks its polymerization [43]</td>
<td>Macropinocytosis [44], CME [45], CIE [38]</td>
<td>Actin staining (phalloidin)</td>
</tr>
</tbody>
</table>

The table shows the transport inhibitors selected for this study, their mechanism of action and pathways involved. The control markers used to test their efficacy are also indicated. CIE: Clathrin independent endocytosis; CME: Clathrin-mediated endocytosis; CP: Chlorpromazine hydrochloride; CytD: Cytochalasin; Dil: 3,3’-dioctadecylindocarbocyanine; Dyn: Dynasore; EIPA: 5-(N-ethyl-N-isopropylamiloride); MBCD: Methyl-β-cyclodextrin; NZ: Nocodazole; TRITC: Tetramethylrhodamine.

Among all of these methods, most studies on the uptake of nanomedicines make use of pharmacological inhibitors [11,14]. They are often preferred because they have a very fast action, thus limiting the possibility for cells to adapt, and they are seemingly easy to use. However, it is well known that pharmacological inhibitors possess limits associated with both their poor specificity and toxicity, and that their efficacy can strongly vary with the cell line in which they are used [28,29]. Within this context, here we have selected a panel of six representative pharmacological inhibitors among those most commonly applied in nanomedicine uptake studies [11,14,26–29]. The selected compounds are active on different elements of the uptake machinery and all major uptake mechanisms (as summarized in Table 1).

More in detail, the following compounds were chosen:

- Chlorpromazine and EIPA (5-(N-ethyl-N-isopropylamiloride), typically used as inhibitors of (respectively) clathrin-mediated endocytosis and macropinocytosis;
- Methyl-β-cyclodextrin (MBCD) to determine the effect of cholesterol depletion;
- Nocodazole to determine the role of microtubules;
- Cytochalasin D to determine the role of actin;
- The dynamin inhibitor dynasore.

Chlorpromazine is a compound used to block clathrin-mediated endocytosis [28,32]. Chlorpromazine is a cationic amphiphilic drug which inhibits the function of AP2, one of the key adaptor proteins in clathrin-mediated endocytosis. Chlorpromazine is also known to trap receptors inside the endosomes, thus blocking their recycling [32]. Therefore, this compound interferes with clathrin-mediated endocytosis at multiple levels.

Similarly, amiloride and its derivative EIPA are used as inhibitors of macropinocytosis: they block the Na⁺ /H⁺ exchanger pump in the plasma membrane, thus inhibiting macropinocytosis as a consequence of their interference with the pH of the cytosol close to the cell membrane where macropinosomes form [30,31]. The resulting acidification also blocks Rac1 and Cdc42 signaling, essential for macropinocytosis.

The other selected pharmacological inhibitors have a more direct mechanism of action in blocking a key component of endocytosis. More in detail, cytochalasin D is a mycotoxin that blocks actin polymerization by binding to the barbed end of F-actin [43]. Actin is known to be involved in several pathways. This means that caution should be taken into interpreting cytochalasin D as an inhibitor of, for instance, macropinocytosis [44], since also clathrin-mediated endocytosis [45] and several clathrin-independent endocytosis mechanisms [38] depend on actin.

Similarly, nocodazole is a compound that binds to tubulin, blocking microtubule polymerization [39]. Microtubules are essential components for clathrin-mediated endocytosis [40], macropinocytosis and possibly other mechanisms [41,42] and they also control vesicle intracellular trafficking [46].
Dynasore is commonly used to block dynamin, a key protein for vesicle fission [36,37], although its specificity is sometimes questioned [47]. Multiple pathways depend on dynamin activity and the involvement of dynamin is a common way to classify endocytosis [38].

Finally, MBCD is a common agent for cholesterol depletion. Cholesterol depletion can be a very disruptive treatment for cells, since cholesterol is essential for maintaining proper membrane permeability and fluidity. Moreover, exposing cells to MBCD affects not only caveolae-mediated endocytosis, as often implied, but almost every endocytic mechanism, strongly depending on the concentration used and cells tested [26].

Overall, none of these latter four compounds (cytochalasin D, nocodazole, dynasore, MBCD), alone, allow the identification of a specific pathway, since they all affect components of the transport machinery which are involved in multiple mechanisms.

The selected panel allows us to illustrate the challenges associated with the use of pharmacological inhibitors while trying to characterize the uptake of one model NP–cell system.

We use this example to discuss limits related to the mechanism of action of the selected compounds; we also show how the interpretation of the results generated can be complicated by inhibitor toxicity, and how this – if overlooked – can lead to wrong conclusions. Next, we illustrate the need for control markers to verify and demonstrate efficient inhibition in the cells and conditions tested, and discuss challenges associated with the selection and availability of such controls. Finally, we also show specific limits when these compounds are used for nanomedicine uptake studies. Overall, the approaches and experiments presented can be used to set up and optimize the use of these and other pharmacological inhibitors for nanomedicine uptake studies, and to avoid misinterpretation of the results that can be obtained without similar optimization.

Materials & methods

Cell culture

HeLa cells (ATCC CCL-2™) were grown in a complete cell culture medium (cMEM) composed by MEM (Gibco™) supplemented with 10% v/v fetal bovine serum (FBS, Gibco™) under standard conditions (37°C, 5% CO2). Cells at passages between four and maximum 20 were used.

NP characterization

Plain fluorescently labeled silica NPs (SiO2 NPs) of 50 nm diameter, with excitation and emission wavelengths of, respectively, 569 and 585 nm were purchased from Kisker Biotech. NP hydrodynamic diameter by dynamic light scattering and ζ-potential were measured using a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) using disposable capillary cells. In order to assess NP stability, 100 μg/ml NPs were dispersed in 1 ml of dH2O, PBS or MEM supplemented with 10% v/v fetal bovine serum (FBS, Gibco™; cMEM) and measured at 20°C immediately or after 1 and 24 h at 37°C, 5% CO2. Results are the average of five runs of at least three measurements.

Studies with pharmacological inhibitors of endocytosis

In order to characterize NP uptake mechanisms, HeLa cells were treated with inhibitors of endocytosis prior to and during exposure to the NPs. Briefly, 50,000 cells/well were seeded in a 24-well plate (Greiner Bio-One) 24 h prior to the experiments. Then, cells were preincubated for 10 or 20 min (in the case of nocodazole) in cMEM with the different inhibitors at different concentrations in order to determine best conditions for their use. The optimized conditions were as follows: 5-(N-ethyl-N-isopropyl)amiloride (EIPA) 100 μM, chlorpromazine hydrochloride 10 μg/ml, MBCD 2.5 mg/ml (all from Sigma-Aldrich, MO, USA), dynasore 25 μg/ml and nocodazole 5 μM (Biovision, CA, USA), cytochalasin D 2.5 μg/ml (ThermoFisher Scientific, MA, USA). After preincubation, 100 μg/ml SiO2 NPs in cMEM were added to cells with or without the different drugs. Drug efficacy was assessed by measuring uptake of fluorescently labeled control markers. Total 2 μg/ml of 3,3’-dioctadecyldiacarbocyanine-labeled (Dil)-LDL, ThermoFisher Scientific) in MEM with or without 10% FBS or 15 μg/ml of fluorescently labeled human transferrin (Alexa546-TF, ThermoFisher Scientific) were used as markers for clathrin-mediated endocytosis. Total 1 μg/ml of BODIPY FL C5-Lactosylceramide/BSA complex (LacCer, Thermo Fisher Scientific) in MEM with or without 10% FBS was used as a marker for caveolae-mediated and lipid raft dependent endocytosis. Total 250 μg/ml TRITC 10 kDa Dextran (ThermoFisher Scientific) in cMEM was used as a marker for macropinocytosis. Samples were collected and prepared for flow cytometry as described below.
Alternatively, the efficacy of cytochalasin D and nocodazole on, respectively, actin or microtubule disruption was assessed by immunohistochemistry as described in the Supplementary Information.

Flow cytometry analysis
Cell fluorescence intensity following uptake of NPs and control markers was measured by flow cytometry. After different exposure times, cells were washed once with cMEM and twice with phosphate-buffered saline (PBS) in order to remove the excess of fluorescent NPs and markers and limit the presence of eventual NPs adhering outside of the cell, which could affect the flow cytometry measurement \([48,49]\). Subsequently, cells were collected using 0.05% trypsin–EDTA, centrifuged and resuspended in PBS for measurement. Cell fluorescence was recorded using a Cytoflex Flow Cytometer (Beckman Coulter, CA, USA) with a 488 nm laser. Data were analyzed using Flowjo software (Flowjo, LLC). Double scatter forward and side-scattering plots were used to set gates in order to exclude cell debris and cell doublets. A total of at least 20,000 cells were acquired per sample and each sample was performed in triplicate. When, due to toxicity, fewer cells were measured, a symbol has been added on the data point and the number of cells acquired has been specified in the caption. The results in Figures 1–6 and Supplementary Figures 1, 3 and 4 are expressed as the median cell fluorescence intensity of the obtained distributions, averaged over the three technical replicates. Error bars are the corresponding standard deviation. In Supplementary Figure 5 and 7, instead, the uptake results – calculated as described above – in cells exposed to NPs in the presence of a drug inhibitor have been normalized by the uptake in control cells without drugs. This allows one to obtain a percentage of uptake in respect to control cells. Supplementary Figure 7 shows the normalized results obtained in at least three independent experiment replicates, while Figure 7 shows the corresponding averaged results and standard deviations.

MTT assay & immunohistochemistry
Cell viability after treatment with the different inhibitors was assessed via MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Efficacy of nocodazole and cytochalasin D was assessed by immunostaining. The detailed methods are included in the Supplementary Information.

Results
As a first step, a panel of pharmacological inhibitors was selected for this study, together with appropriate control markers to optimize their use. Table 1 summarizes the mechanism of action of the different inhibitors and the pathways involved.

In order to optimize the use of this kind of compound and avoid misinterpretations, two requirements are essential: first, testing their efficacy in blocking the pathway of interest and second, testing their toxicity on the cells chosen for the study. To this aim, here, HeLa epithelial ovarian cancer cells were selected as a model cell line commonly used both in the endocytosis and in the nanomedicine fields \([11,14,50,51]\).

Thus, in order to test drug efficacy, control molecules entering cells via the mechanism affected by the transport inhibitors are required. Transferrin and LDL were selected to optimize the use of chlorpromazine in blocking clathrin-mediated endocytosis \([33,34]\). In order to test the efficacy of cholesterol depletion by MBCD, the uptake of LacCer, a glycosphingolipid that resides preferably in lipid rafts and whose uptake depends on cholesterol, was measured \([28,35]\). Similarly, fluorescently labeled dextran, a fluid phase marker, was used to test the efficacy of the amiloride derivative EIPA in blocking macropinocytosis \([29]\). Finally, for cytochalasin D and nocodazole imaging of actin and microtubules, respectively, allowed us to easily assess the efficacy of the protocol applied in altering these structures.

Different doses of the selected inhibitors were applied to HeLa cells and their effect on the uptake of control markers was tested, together with the effect on the uptake of 50 nm silica, here chosen as a model NP. MTT assay and light microscopy imaging were used to detect eventual toxicity.

Even when performing simple experiments with control markers, further complications may arise: when we tested the uptake of transferrin in HeLa cells in the presence of chlorpromazine (Supplementary Figure 1), for short incubation times, exposure to chlorpromazine led to a strong reduction of transferrin uptake, suggesting inhibition of clathrin-mediated endocytosis. However, after 1 h, this reduction was fully lost. This could be due to cells adapting quickly to chlorpromazine and compensating in some way. However, a 1 h pre-exposure to chlorpromazine led to a strong reduction of transferrin uptake, ruling out this interpretation. Still, also in this case, after 1 h exposure to transferrin in the presence of chlorpromazine, inhibition was lost. Interestingly, no such loss of inhibition was observed using LDL as control marker instead of transferrin (Supplementary Figure 1). A possible
Figure 1. Chlorpromazine to block clathrin-mediated endocytosis. Uptake by flow cytometry of (A&C) 2 μg/ml Dil-LDL in sfMEM and (B&D) 100 μg/ml red silica nanoparticles in cMEM in HeLa cells exposed to different concentrations of chlorpromazine; the results are the average and standard deviation over three replicates of median fluorescence intensities of 20,000 cells, except where indicated (X: average of 2500 cells; Y: average of 13,000 cells); (C&D): the data of (A&B) are normalized for uptake in control cells without chlorpromazine; (E) viability measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for test and (F) light microscopy images of HeLa cells exposed for 4 h to chlorpromazine in sfMEM or cMEM (scale bar: 200 μm). % ctrl and ctrl+: performed as described in the methods.

au: Arbitrary units; % ctrl: Normalization; cMEM: Complete cell culture medium; Ctrl+: Positive control; Dil-LDL: 3,3′-dioctadecylylindocarbocyanine-labeled low density lipoprotein; sfMEM: Serum free cell culture medium.
Figure 2. EIPA to inhibit macropinocytosis. Uptake by flow cytometry of (A & D) 250 μg/ml 10 kDa tetramethylrhodamine dextran in cMEM and (B & E) 100 μg/ml red silica nanoparticles in cMEM in HeLa cells exposed to different concentrations of EIPA; the results are the average and standard deviation over three replicates of median fluorescence intensities of 20,000 cells, except where indicated (X = not enough cells [less than 500]; Y = average of 700 cells; Z = average of 8000 cells); (D & E) the data of (A & B) are normalized for uptake in control cells without EIPA; (C) viability measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for test and (F) light microscopy images of cells exposed for 4 h to EIPA in cMEM (scale bar: 200 μm).

au: Arbitrary units; cMEM: Complete cell culture medium; Ctrl +: Positive control; EIPA: 5-(N-ethyl-N-isopropyl)amiloride.
Figure 3. Dynasore to inhibit dynamin. Uptake by flow cytometry of (A) 2 μg/ml Dil-LDL in sfMEM, and (B) 100 μg/ml red silica nanoparticles in cMEM, in control cells and cells exposed to 25 μg/ml dynasore; (C) viability measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for test of cells exposed for 4 h to different doses of dynasore in cMEM. The % ctrl and ctrl + are performed as described in the methods; (D & E) uptake by flow cytometry of (D) 2 μg/ml Dil-LDL in sfMEM and cMEM and (E) 100 μg/ml red silica nanoparticles in cMEM in cells exposed to different concentrations of dynasore normalized by the uptake in control cells without dynasore; (F) light microscopy images of cells exposed for 4 h to different doses of dynasore in cMEM (scale bar: 100 μm). Flow cytometry data are the average and standard deviation over three replicates of the median fluorescence intensity of 20,000 cells except where indicated (X = not enough cells [less than 500]; Y = average of 4000 cells; Z = average of 10,000 cells). au: Arbitrary units; cMEM: Complete cell culture medium; % ctrl: Normalization control; ctrl + : Positive control; DiL-LDL: 1,1'-Dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate-labeled low density lipoprotein; sfMEM: Serum free cell culture medium.
Figure 4. *Methyl-β-cyclodextrin to deplete cholesterol.* Uptake by flow cytometry of (A) 1 μg/ml LacCer in sfMEM and (B) 100 μg/ml red silica nanoparticles in cMEM in control cells and cells exposed to 2.5 mg/ml MBCD; (C & D) uptake by flow cytometry of (C) 1 μg/ml LacCer in sfMEM and cMEM and (D) 100 μg/ml red silica nanoparticles in cMEM in cells exposed to different concentrations of MBCD, normalized by the uptake in control cells without MBCD. (E) Viability measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test and (F) light microscopy images of cells exposed for 4 h to different doses of MBCD in cMEM (scale bar: 100 μm). % ctrl and ctrl+: performed as described in the methods. Flow cytometry data are the average and standard deviation over three replicates of the median fluorescence intensity of 20,000 cells except where indicated (X = not enough cells [less than 500], Y: average of 5000 cells). % ctrl: Normalization control; au: Arbitrary units; cMEM: Complete cell culture medium; Ctrl: Control cells; Ctrl+: Positive control; MBCD: Methyl-β-cyclodextrin; sfMEM: Serum-free cell culture medium.
transport inhibitors to study nano-carrier uptake

Research Article

Figure 5. Cytochalasin D for actin depolymerization. (A) Fluorescence and light microscopy images of HeLa cells exposed for different times to 2.5 μg/ml cytoD; green: actin staining by TRITC–Phalloidin; blue: DAPI stained nuclei. Scale bar: 50 and 150 μm, respectively. (B) Uptake by flow cytometry of 100 μg/ml red silica nanoparticles in complete cell culture medium in control cells and cells exposed to 2.5 μg/ml CytoD (average and standard deviation over three replicates of the median fluorescence intensity of 20,000 cells); (C) viability by MTT test of cells exposed for 4 h to different doses of CytoD in complete cell culture medium. Normalization and ctrl + performed as described in the methods. au: Arbitrary units; Ctrl: Control cells; Ctrl +: Positive control; CytoD: Cytochalasin D; TRITC: Tetramethylrhodamine.

interpretation is that, after longer exposure, transferrin (and not LDL) adheres out of the cell membrane, polluting the flow cytometry measurements. Alternatively, cells exposed to transferrin in the presence of chlorpromazine may adapt and use alternative routes for its uptake. Further studies are required to understand this phenomenon.

Supplementary Figure 1 also shows controls for cells exposed to dynasore. As previously mentioned, dynamin is involved in several pathways including clathrin-mediated endocytosis; thus, we expected dynasore to reduce the uptake of both LDL and transferrin. Instead, we found that transferrin uptake was only partially reduced, while LDL uptake was strongly inhibited over time. This is again an example to illustrate the complexity of outcomes, even when simply setting up similar control experiments.

As mentioned above, 50 nm silica NPs were chosen as a common model used in many cell–NP studies because of their size, comparable with many drug carriers, and because of their well-known stability, even in cell culture medium [52,53]. Prior to their application, their size distribution and ζ-potential in different media over time were determined (Supplementary Figure 2 & Table 1). The results confirmed good dispersion and stability over time, also in the conditions applied for exposure to cells.

Figure 1 shows the results obtained for cells exposed to chlorpromazine at different concentrations. For the lower chlorpromazine concentration tested, the inhibition of the control marker LDL was already extremely strong, suggesting that these conditions are efficient in blocking the pathway (Figure 1A). However, only a minor effect
could be observed on NP uptake. NP uptake inhibition was much stronger at higher chlorpromazine concentrations. However, in these conditions, strong toxicity could be detected (Figure 1B). Thus, the observed reduction in NP uptake is most likely a simple consequence of the very strong toxicity at higher chlorpromazine concentrations rather than a proof of uptake by clathrin-mediated endocytosis.

It is important also to note that while NPs were added to cells in the presence of proteins to allow corona formation, controls with labeled transferrin or LDL are usually performed in absence of serum to avoid competition with the unlabeled transferrin and LDL already present in the serum. However, it is well known that drug efficacy can be limited by protein adsorption. Thus, as an ulterior control, uptake of labeled LDL in the presence of chlorpromazine was also measured in medium containing serum. The results clearly showed that chlorpromazine efficacy was not affected by the presence of proteins (Supplementary Figure 3). This suggests that the absence of effect of chlorpromazine on NP uptake was not due to loss of drug efficacy in the presence of serum.

Figure 2 shows a similar study performed on cells exposed to the macropinocytosis inhibitor EIPA. At the lower concentrations tested only a partial dose-dependent reduction of the uptake of the fluid-phase marker dextran was obtained (Supplementary Figure 4). At 100 μM EIPA, instead, uptake of dextran was strongly reduced, and at higher concentrations the effect did not improve further (Figure 2A). In the case of the NPs, 100 μM EIPA gave only a minor uptake reduction, but the effect was stronger for higher EIPA concentrations (Figure 2B). Cell viability measurements and microscopy clarified that in these conditions, as observed for chlorpromazine, the reduction

**Figure 6. Nocodazole to disrupt microtubules.** (A) Fluorescence and light microscopy images of HeLa cells exposed for different times to 5 μM nocodazole; red: α-Tubulin staining; blue: 4′,6-diamidino-2-phenylindole-stained nuclei. Scale bar: 50 and 150 μm, respectively. (B) Uptake by flow cytometry of 100 μg/ml red silica nanoparticles in cMEM in control cells and cells exposed to 5 μM nocodazole (average and standard deviation over three replicates of the median fluorescence intensity of 20,000 cells); (C) viability by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for test of cells exposed for 4 h to different doses of nocodazole in complete cell culture medium. Normalization and ctrl + performed as described in the methods.

au: Arbitrary units; Ctrl: Control cells; Ctrl +: Positive control.
in NP uptake was most likely only a side effect of the strong toxicity on cells (Figure 2C). Another interesting observation was that when using nontoxic EIPA concentrations (50–100 μM), the effect seemed stronger at the longer exposure times, suggesting a later involvement of macropinocytosis [54]. Size measurements showed a good stability of the dispersions up to 24 h (Supplementary Figure 2), excluding that such a result may be connected to particle agglomeration over time and activation of mechanisms commonly associated with the uptake of larger objects. Further studies are required to fully clarify these observations.

Figure 3 shows results in cells exposed to dynasore to inhibit dynamin. With this compound, we could observe a very good inhibition of LDL uptake at concentrations not associated with a decrease in cell viability (Figure 3A & C). In these conditions, NP uptake was not affected (Figure 3B). However, additional controls with LDL showed that dynasore efficacy was partially lost when serum was added (Figure 3D). Increasing dynasore concentration only partially reduced LDL uptake in serum, but still with almost no effect on NP uptake (Figure 3E). Furthermore, at these higher concentrations, strong toxicity was observed (Figure 3C & F).

Figure 4 shows similar results for MBCD. Cholesterol depletion was extremely effective in blocking uptake of the control marker LacCer (Figure 4A), while only a minor effect was observed on the uptake of the silica NPs (Figure 4B). However, as for dynasore, in the presence of serum the efficacy of MBCD was lost (Figure 4D), and increasing drug concentration only led to strong cell death (Figure 4C & F) without any sign of improved efficacy on LDL and NP uptake (Figure 4D & E).

Thus, other compounds not sensitive to the presence of proteins should be selected when studying the role of dynamin and cholesterol in the mechanisms of nanocarrier uptake into cells.
Figure 5 shows the results obtained with cytochalasin D, with additional optimization in Supplementary Figure 5: immunostaining and light microscopy confirmed drug efficacy (Figure 5A & Supplementary Figure 5A) and no effect on cell viability (Figure 5C & Supplementary Figure 5B). Interestingly, actin disruption clearly reduced NP uptake, suggesting a role for actin in the internalization mechanism (Figure 5B).

Finally, Figure 6 shows the results obtained with nocodazole to depolymerize microtubules (see also Supplementary Figure 6 for further controls). Microtubule disruption led to a reduction of NP uptake (Figure 6B), suggesting that the cytoskeleton is involved in the mechanism of internalization, or that altering the intracellular trafficking machinery has an effect on the uptake levels.

Discussion

Several parameters can affect the efficacy and toxicity of pharmacological inhibitors: these include not only the dose and exposure time, but also the length of the preincubation with the inhibitor, the medium in which they are dispersed (with or without serum), cell density and cell passage number. For instance, higher cell numbers may require higher drug concentrations and are typically associated with lower toxicities. Similarly, cells at higher passage numbers can become less sensitive to these compounds (For this reason, as specified in the materials and methods, we have used cells up to a maximum of 20 passages to exclude similar effects).

In order to be able to test and optimize all of these parameters, appropriate control markers, known to enter cells via specific endocytic pathways, should be selected. As easy as this may sound, for several of the endocytosis mechanisms described in literature such specific markers may be missing or are constantly debated, since endocytosis is still a very active field of research. The selection of such control markers is relatively easy in the case of clathrin-mediated endocytosis, for which molecules such as LDL or transferrin can be used, as here. The selection of appropriate cargoes to test the efficacy of the inhibitors on pathways other than clathrin-mediated endocytosis is more complicated. Usually, cholesterol depletion is used to block uptake mediated by caveolae, and molecules such as cholera toxin B or SV40 as markers for this pathway. However, it has been shown that cholera toxin is more complicated. Usually, cholesterol depletion is used to block uptake mediated by caveolae, and molecules such as cholera toxin B or SV40 as markers for this pathway. However, it has been shown that cholera toxin seems to be able to enter different cells independently of caveolae and via different mechanisms, including both clathrin-mediated endocytosis and clathrin-independent endocytosis [55,56]. Even more importantly, the actual role of caveolae in endocytosis is strongly debated [57,58]. In fact, it has been shown that in many cell types caveolae do not even pinch off from the plasma membrane [59]. In these cases, just a fraction of caveolae is mobile and undergoes endocytosis, mainly if activated by a ligand [60]. Molecules such as LacCer can thus be used, as done here, to confirm the efficacy of cholesterol depletion. Nevertheless, as discussed in the introduction, it is also important to keep in mind that cholesterol depletion affects several pathways (as also summarized in Table 1); thus, the results obtained with this compound need to be interpreted with care.

Next to the selected control markers, 50 nm silica NPs were chosen as a common model used in many cell–NP studies because of their well-known stability even in cell culture medium [52,53]. Furthermore, their size is comparable with many nanomedicines: drug carriers of 50–100 nm, commonly applied by parenteral administration, are in fact considered of optimal size to avoid renal clearance and limit macrophage recognition [61,62]. We stress that for studies on the mechanisms of uptake of NPs and drug carriers, it is crucial to include some information on NP dispersion and stability in the cell medium used for exposure to cells, as in Supplementary Figure 2 & Table 1 (unfortunately often missing): dispersion in cell culture medium can lead to agglomeration, and this could strongly affect the pathways involved in uptake.

Next to NP dispersion and stability, another key factor that needs to be considered for NP uptake studies is the choice of medium for NP exposure to cells. In fact, it is known that the biomolecular corona that forms on the surface of nano-sized objects once they are exposed to biological environments strongly affects their biological behaviour [63,64]. Studying uptake pathways in the complete absence of any form of corona, such as in serum-free media, may have totally different outcomes than in real applications [53]. Thus, here – as a first step – we have used a simple condition with NPs dispersed in standard cell culture medium, supplemented with 10% FBS. Further studies are required to determine uptake mechanisms in the presence of more realistic coronas.

Additionally, particular care was taken in performing uptake experiments as a function of time and to optimize conditions to allow exposing cells to the NPs in the presence of the inhibitors for relatively long time. Time-resolved experiments, in fact, provide more complete information on the kinetic of the process and are of particular importance when studying internalization of larger objects such as nanocarriers, typically occurring slower than the uptake of proteins or other macromolecules. Furthermore, shorter exposure times can be affected by particle adhesion to the outer cell membrane, which may confuse the results [49]: thanks to the optimization performed,
we have been able to expose cells to the inhibitors for up to 6 h, ensuring that the eventual contribution of NPs adhering outside cells was minimal in comparison with the amount of internalized NPs [48,49].

Overall, optimization of the protocols for the use of the selected panel of pharmacological inhibitors allowed us to define conditions in which controls showed a good efficacy of the drug tested, without strong effects on cell viability. Our results clearly show the importance of this aspect, for instance in the case of chlorpromazine and EIPA (Figures 1 & 2): as these examples illustrate, even though increasing drug concentration may result in lower uptake (thus, apparently a stronger inhibition), great care has to be taken in interpreting the results. Viability tests must be included to exclude that the lower uptake is not simply due to toxicity.

The results obtained with dynamin and MBCD in the presence of serum are an interesting example of a unique nano-specific challenge when using these pharmacological inhibitors for nanomedicine uptake studies: given the need to include some biological fluids to allow corona formation, some of these compounds – unfortunately – may become fully ineffective. When available, other compounds not sensitive to the presence of proteins or other biomolecules should be used, and overall it is essential that similar effects are tested to avoid misinterpretations.

A further aspect to consider is that even when protocols are optimized as we have shown here, reproducibility with these compounds can remain challenging. Supplementary Figure 7 shows results for independent experiments to illustrate this and Figure 7 the corresponding averaged results. The observed variability may be connected to small variations in cell density or drug preparation, which remain difficult to fully control in independent experiments. For drugs where only minor effects were observed on NP uptake, like chlorpromazine, these were easily lost when averaging over multiple replicates. Instead, where the inhibition was more substantial, as expected, it was easier to reproduce it in independent replicates. This overall suggests the need to set relatively high thresholds on inhibition efficacy.

Based on the results of Figure 7, and using high thresholds on the inhibitory effect (the red line in the figure is set at 40% inhibition), our results suggested that chlorpromazine effect on silica uptake is questionable, while a clear effect was observed with cytochalasin D and nocodazole, and upon longer exposure times also with EIPA.

The last challenge is the interpretation of these results to conclude on the pathway involved in the uptake of these NPs. While our aim in this work is mainly to illustrate the complexity of this question and challenges associated with the use of pharmacological inhibitors, rather than answering on how these selected model NPs enter cells, we include some considerations on the possible interpretation of the results.

The results obtained with chlorpromazine seem to exclude clathrin-mediated endocytosis, usually considered one of the main pathways for the uptake of NPs smaller than 100 nm [11], because of the size of clathrin coated pits. Other studies, however, have already contradicted similar findings [65,66]. The results obtained with cytochalasin D and nocodazole point toward an actin-driven mechanism with a clear role also for microtubules, and those with EIPA suggest that macropinocytosis may be involved at the longer exposure times. Unfortunately, with the selected panel, we could not conclude on the role of cholesterol and dynamin, which both help to classify uptake mechanisms.

Conclusion

Answering the question on the mechanisms that nano-sized drug carriers use to enter cells is an essential step for the drug-delivery community to improve the design of truly successful nanomedicines and targeted drugs. Another important aspect that this field needs to address is defining what the ‘appropriate corona’ is for a given nanomedicine application (for instance using bovine serum on human cells is likely not relevant, as is using serum for inhaled nanomedicines).

At the same time, the field of endocytosis is still highly active in defining and characterizing the different mechanisms cells use to internalize materials. This poses considerable challenges in highly interdisciplinary fields such as that of nanomedicine and points toward the need for a closer connection with the endocytosis community.

Within this context, pharmacological inhibitors are often applied to characterize nanomedicine uptake due to their apparent ease of use. However, stringent controls are required to demonstrate their effect using appropriate markers (where available). Moreover, toxicity should be monitored, since it could be easily misinterpreted for efficient inhibition.

Even when conditions are optimized, multiple independent replicate experiments are needed and results can be highly variable: time-resolved experiments with a high threshold on the inhibitory effect are needed to try to interpret the outcomes.
Overall, once applied with all the discussed precautions, pharmacological inhibitors are useful tools to allow simple screening on the uptake of nano-sized drug carriers. However, determining the pathways involved solely based on the results obtained with these compounds remains challenging because of the complications discussed here. Several other methods are available to characterize uptake pathways: these include for instance RNA interference, knock down or CRISPR/CAS9 to reduce or block the expression of key endocytic proteins, and imaging-based approaches to determine colocalization with proteins involved in uptake (for instance by immunolabeling and transmission electron microscopy, or by fluorescence labeling and imaging in live or fixed cells). The combination of multiple methods among those available (each presenting advantages and limits) is probably the best approach to try to fully characterize the pathways involved and answer this central question.

Future perspective
Despite its successes, the nanomedicine field has encountered several challenges in developing nano-sized drug delivery systems with efficient targeting capabilities and limited side effects. The improvement of targeted drugs requires a better knowledge of the interaction of these nano-sized carriers with live-cells and the body. Such knowledge will allow one to tune the nanomaterial design to achieve the desired biological behavior. However, both controlling nanomaterial design to achieve targeting and (as illustrated in this work) characterizing cellular pathways are extremely challenging tasks: a closer connection between the cell biology and drug-delivery communities is desirable to be able to tackle such challenges.

Summary points
- Transport inhibitors are very useful tools to characterize nanomedicine uptake pathways; however, they can have very strong effects on cells, confusing the outcomes.
- To properly use transport inhibitors, careful controls are needed to demonstrate their efficacy and exclude strong toxicity.
- Reproducibility of results with pharmacological inhibitors can be challenging; therefore, high thresholds on inhibition efficacy should be set.
- Time-resolved studies of nanoparticle uptake can help to better interpret the results.
- While serum must be used in order to study the uptake of nanomedicines in a realistic biological environment, some inhibitors such as methyl-β-cyclodextrin and dynasore fully lose their efficacy in the presence of serum. Different inhibitors which maintain their effect in the presence of serum are required for nanomedicine studies.
- Actin and microtubules seem to be involved in the uptake of 50 nm silica nanoparticles, as also macropinocytosis at later exposure times.

Supplementary data
To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/nnm-2018-0446

Author contributions
V Francia designed the experiments, performed most of the experiments, analyzed and interpreted the data, contributed to the drafting of the manuscript. C Reker-Smit and G Boel contributed to the optimization of the methods and to the experiments, the analysis and interpretation of the data and writing of the manuscript. A Salvati designed the experiments, interpreted the data and wrote the manuscript. All authors approved the final version of the manuscript.

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References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

• Review on the present knowledge and challenges in nanomedicine for cancer therapy.
•• This editorial underlines the need for ‘greater understanding of the mechanisms underlying macromolecule transport across cell membranes’.
• Review about the mechanisms of endocytosis used by cells.


**This is an excellent review discussing the challenges connected to the study of the uptake of nanoparticles, including challenges connected to the use of transport inhibitors.**


**Research article on the inhibitors of endocytosis and their limits.**


- This article highlights the importance of the presence of serum and the formation of a corona in the study of the uptake of nanoparticles.


- The authors review how the composition and characteristics of the nanoparticle corona influence the interactions of nanoparticles with cells.


