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Distinct transduction modes of arginine-rich cell-penetrating peptides for cargo delivery into tumor cells

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ABSTRACT

The application of cell-penetrating peptides (CPPs) for delivering various cargo molecules with biological functions into cells has gained much attention in recent years. However, the internalization mechanisms and delivery properties of CPP–cargo remains controversial. In this study, low- and high-molecular-weight cargoes attached to arginine-rich CPPs were employed: the former was the fluorescein isothiocyanate-labeled nona-arginine (CPP–FITC), and the latter was the fluorescently labeled nona-arginine–avidin complex (CPP–avidin). We measured the intracellular trafficking of CPP–FITC and CPP–avidin in four cancer cell lines in a series of microenvironments altered by the presence or absence of serum, different temperatures and different incubation times. The results revealed that CPP–cargo delivery exhibited no specificity toward any cell line, but the levels were found to be related to cell type and cargo. Furthermore, their endocytic mechanisms were investigated via incubation with related endocytic inhibitors. Two different types of CPP–cargo were required to cross the plasma membrane to bind to cell surface-associated heparan sulfate proteoglycans in a time-dependent manner. CPPs and small cargoes attached to CPP may enter cells rapidly via direct translocation in addition to the endocytic route. Translocation of large components linked to CPP tended to be mediated by macropinocytosis in an energy-dependent manner with slower rates for larger compounds. In contrast, the clathrin-dependent pathway is not essential to the translocation of either type of CPP–cargo.

1. Introduction

Delivery of biologically active molecules across cell membranes to interact with intracellular targets is a persistent challenge. Unfortunately, most candidates are unable to cross the plasma membrane alone (Maiolo et al., 2005). However, numerous organisms have developed proteins, many of which are transcription factors that breach membranes through a variety of mechanisms (Joliot and Prochiantz, 2004). In the past decade, several peptides called cell-penetrating peptides (CPPs), which can translocate across cell membranes have been identified (Stephens and Pepperkok, 2001).

CPPs, also known as protein transduction domains (PTDs), are a collection of different families of short peptides believed to enter cells by penetrating cell membranes (Zhang et al., 2009; Torchilin, 2005). CPPs have become one of the most popular and efficient techniques for achieving intracellular access. CPPs may be derived from natural sources or be synthetically designed constructs. HIV Tat, the HIV transactivator of a transcription protein, was the first sequence found to be capable of translocating across cell membranes and gaining intracellular entry (Frankel and Pabo, 1988; Green and Loewenstein, 1988). Some of the most commonly used peptides include HIV-Tat, polyarginine, penetratin and transportan (Fonseca et al., 2009). These CPPs have successfully delivered proteins (Myrberg et al., 2007), nucleic acids (Järver and Langel, 2004), therapeutics (Aroui et al., 2009), quantum dots (Santra et al., 2005), MRI contrast agents (Kersemans et al., 2008) and nanocarriers (Torchilin et al., 2001; Sawant et al., 2008; Torchilin, 2008) into different cells *in vitro* and *in vivo*.

Previous studies have confirmed that the guanidinium groups in the arginine of HIV Tat played a greater role in facilitating cellular uptake than either charge or backbone structure. Nonaarginine (R9) was 20-fold more efficient than Tat 49–57 at cellular uptake as determined by Michaelis–Menton kinetic analysis (Wender et al., 2000). R9 was therefore chosen as an excellent representative for understanding intracellular delivery at a fundamental molecular level in this research.

The mechanisms underlying CPP cellular uptake is a matter of great controversy, even though it is indisputable that CPPs are promising drug delivery vectors. The detailed mechanisms for how CPP molecules enter cells remain unclear (Drin et al., 2003), although many recent reports have indicated different forms of endocytosis. The pathway implicates the involvement of

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clathrin-mediated endocytosis (Richard et al., 2005), caveolae/lipid raft-mediated endocytosis (Fittipaldi et al., 2003), or macropinocytosis (Kaplan et al., 2005). Most likely, CPPs utilize a combination of multiple internalization mechanisms (Wadia et al., 2004).

Understanding the modes of CPP transduction is fundamental to their use as delivery vectors for biologicals (e.g., peptides, proteins, and nucleic acids) and probes, especially in view of the increasing interest in the delivery of anticancer drugs (Lee et al., 2008). It is especially noteworthy that the cell-penetrating mechanism is dependent upon the properties of the CPPs, the selected cell lines, the concentrations of CPPs and the assay conditions (temperature or serum). Few researchers have attempted to compare the influence of the size or weight of the attached cargo on modes of uptake, although cargo as the unitary part of delivery may indeed affect the transportation or permeability of CPPs.

This study focused on endocytic mechanisms of CPP–cargo in four cancer cell lines including human breast cancer cells (MCF-7 and MDA-MB-231), rat glioma cells (C6), and mouse melanoma cells (B16F10). The scope of the present discussion was limited to R9-mediated cargo trafficking. Low- and high-molecular-weight cargoes attached to R9, CPP–FITC and CPP–avidin were employed to explore endocytic mechanisms with the help of well-known endocytic inhibitors that act by selectively blocking specific endocytic pathways. On the other hand, we also attempted to further clarify the delivery properties of the CPP–cargo by changing a series of factors, including incubation time, serum and temperature. Eventually, two distinct modes were discovered when different types of cargoes were delivered into tumor cells by arginine-rich CPPs. The detailed endocytic mechanisms and transduction properties provide better guidelines for the use of arginine-rich CPPs in the clinical applications for anticancer drug delivery.

2. Materials and methods

2.1. Materials

GLPK(FITC)RRRRRRRRR (CPP–FITC) and biotin-GLPRRRRRRRRR were synthesized by Shanghai GL Biochem Co. Ltd (Shanghai, China). The CPP–FITC conjugate was considered as an example of a low-molecular-weight CPP–cargo. Fluorescein isothiocyanate (FITC)-labeled avidin and 4% paraformaldehyde were purchased from Saichi Biotech Co. Ltd (Beijing, China). Heparin was obtained from Shandong Wanbang Biochem Co. Ltd (Shandong, China). Chlorpromazine and chloroquine diphosphate salt were provided by HongJing Chem Co. Ltd (Hubei, China). 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) was the product of Alexis Biochem Co. Ltd (USA). Trypsin was supplied by Sigma–Aldrich (USA). All reagents were of analytical grade.

2.2. Cells and culture conditions

MCF-7, MDA-MB-231, C6, and B16F10 cells were obtained from the Cell Culture Center of Union Medical University (Beijing, China). MCF-7 and MDA-MB-231 cells were maintained in DMEM (Macgene, Beijing, China) supplemented with 10% fetal bovine serum (FBS, Sanli, Wuhan, China), 1% L-glutamine, 1% nonessential amino acids, and 1% penicillin/streptomycin (Macgene, Beijing, China). C6 was cultured in HAMS F10 medium which included 35% horse serum (Sanli, Wuhan, China), 15% FBS, 1% L-glutamine, 1% nonessential amino acids, and 1% penicillin/streptomycin. B16F10 was maintained in RPMI 1640 medium containing 10% FBS, 1% L-glutamine, 1% nonessential amino acids, and 1% penicillin/streptomycin. Unless otherwise indicated, all incubations were performed at 37 °C in a humidified atmosphere containing 5% CO_2. All experiments were performed on cells in the exponential growth phase.

2.3. Preparation of CPP–avidin complex

The CPP–avidin complex was prepared via the strong biotin–avidin interaction (Al-Taei et al., 2006). Briefly, 250 μg of avidin–FITC was mixed by vortexing with biotin-GLPRRRRRRRRR solution in a blank culture medium at different molar ratios (1:1, 1:2, 1:4, 1:6 and 1:8) and incubated for 15 min at room temperature. A series of complexes were achieved and the optimal formation was established on the basis of maximum cellular uptake by flow cytometry. As a result, the CPP–avidin complex was achieved with an optimal composition of 1:4 (74.7 kDa) and adopted as the high-molecular-weight CPP–cargo model in the following studies.

2.4. Cellular uptake by flow cytometry

A total of 1×10^6 MCF-7, MDA-MB-231, C6, or B16F10 cells were seeded in 6-well plates for 24 h. Supernatants were removed and discarded. Then, the cells were incubated with CPP–FITC or CPP–avidin in corresponding blank culture (no serum) for different lengths of time. After treatment with different CPP–cargoes, the cells were washed with 0.1 M of phosphate-buffered saline (PBS, pH 7.4) and detached from cell culture through trypsin treatment and incubation at 37 °C for 3 min. Subsequently, the cells were centrifuged for 3 min and resuspended in 0.5 mL of PBS. A single cell suspension was prepared by filtration through a 300-mesh filter. The cells suspended in PBS were measured immediately by flow cytometry (Becton Dickinson, San Jose, CA, USA). In each case, the fluorescence of 10,000 vital cells was acquired. Flow cytometry was performed with a 488 nm argon laser. To avoid artificial results during fluorescence measurements and to differentiate between CPP–cargo that are within the cells and those that were stuck on the cell surface, some precautions had to be considered (Mueller et al., 2008; Nakase et al., 2008). For flow cytometry measurements, the electrostatic interaction between the positively charged CPPs and the negatively charged plasma membrane was neutralized by trypsin treatment as described above.

2.4.1. The effect of incubation time, serum and temperature on cellular uptake

The CPP–FITC or CPP–avidin was incubated with seeded cells for 0.5 h, 1 h, 2 h, 4 h, 8 h, and 24 h. Then, the same treatments listed above were performed for determining cellular uptake by flow cytometry. A standard cellular uptake method was established for cells that were incubated with these CPP–cargoes without serum. To assess the effect of serum on the internalization of CPP–cargo, we performed a revised procedure in which seeded cells were incubated with CPP–cargoes in complete cultures (with serum) from 0.5 h to 24 h. Two incubation temperatures (4 °C and 37 °C) were considered to estimate whether the CPP–cargo access to cells was energy dependent in each of the four cancer cell lines.

2.4.2. The effect of different endocytic inhibitors on cellular uptake

Four commonly used endocytic inhibitors were exploited to address the trafficking pathways of CPP–FITC and CPP–avidin across cells. Different attached cells were treated for 30 min with heparin (10 μM), chlorpromazine (30 μM), chloroquine (100 μM), or EIPA (50 μM) solutions which were dissolved in sterile PBS or DMSO. Then, incubation with these CPP–cargoes was performed for 1 h or 4 h, respectively, and analyzed by flow cytometry. The uptake experiments for CPP–FITC were performed in duplicate until similar consecutive results were obtained.

2.5. Laser confocal scanning microscopy

After culturing of 1×10^4 MCF-7 or B16F10 cells for 24 h in a glass-bottom dish, CPP–FITC (10 μM) or CPP–avidin (20 μM) solutions were added to each dish and incubated for 0.5 h, 1 h, 2 h, 4 h, 8 h, or 24 h at 37 °C. The medium was then removed, and the cells were washed with PBS (0.1 M, pH 7.4) followed by fixation with 4% paraformaldehyde for 10 min. The fluorescent images of the cells were analyzed using a laser scanning confocal microscope (Leica, TCS SP5, Germany).

3. Results

3.1. Effects of incubation time and serum on the internalization of CPP–cargo

Four cancer cell lines were treated with CPP–avidin for different times in corresponding serum-free or essential serum-containing culture media. The amount of internalized peptides was then quantified by flow cytometry. The results (Fig. 1) revealed that the delivery of CPP–avidin exhibited no specificity toward any cell line, but the amounts and patterns were related to cell type and incubation conditions. In addition, the intracellular trafficking of CPP–avidin was time dependent in both serum-containing and serum-free media. After a 24 h incubation in serum-containing medium, the uptake of CPP–avidin complexes was 1.6-fold, 2.0-fold, and 6.0-fold higher in C6, MDA-MB-231, and MCF-7 cells, respectively ($p < 0.05$), compared to serum-free medium. C6 displayed more pronounced uptake than the other cell lines at 4 h and 8 h and maintained this level after incubation for 24 h both in the absence and in the presence of serum. Similar fluorescent values of CPP–avidin were observed in B16F10, both in serum-containing and in serum-free groups, which demonstrated that the internalization of CPP–avidin was independent of the presence serum for this cell type.

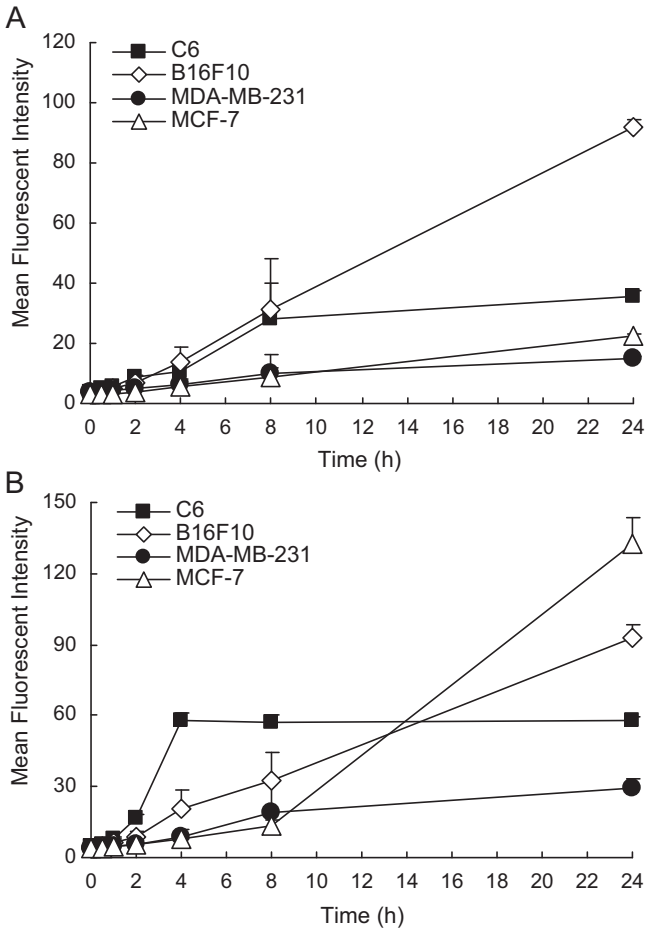

Fig. 1. Effect of incubation time and serum on the internalization of CPP–avidin complexes. C6, B16F10, MDA-MB-231, and MCF-7 cells were treated with 5 μM CPP–avidin complexes in serum-free (A) and serum-containing (B) media for 0.5 h, 1 h, 2 h, 4 h, 8 h, and 24 h. The cells were washed and analyzed by flow cytometry. Error bars indicate the standard deviation ($n = 3$).

3.2. Effects of temperature on the internalization of CPP–cargo

MCF-7 was selected as the representative cell system to assess transduction of CPP–FITC at different temperatures (Fig. 2A). Flow cytometry analysis revealed that CPP–FITC was internalized abundantly after incubation for 0.5 h, 1 h, and 2 h both at 4 °C and 37 °C. In contrast, free FITC displayed negligible uptake at 2 h without the aid of CPPs. This phenomenon suggested that CPP–FITC penetrated plasma membranes rapidly. A similar degree of cellular trafficking of CPP–FITC was obtained after 2 h at 37 °C and 4 °C (i.e., the entry of CPP–FITC was only slightly less at the lower temperature), which implied that small cargoes attached to CPP likely crossed the plasma membrane probably in a temperature-independent manner.

The fluorescence intensities strengthened with increased incubation time at 37 °C for CPP–avidin (Fig. 2B). However, these levels

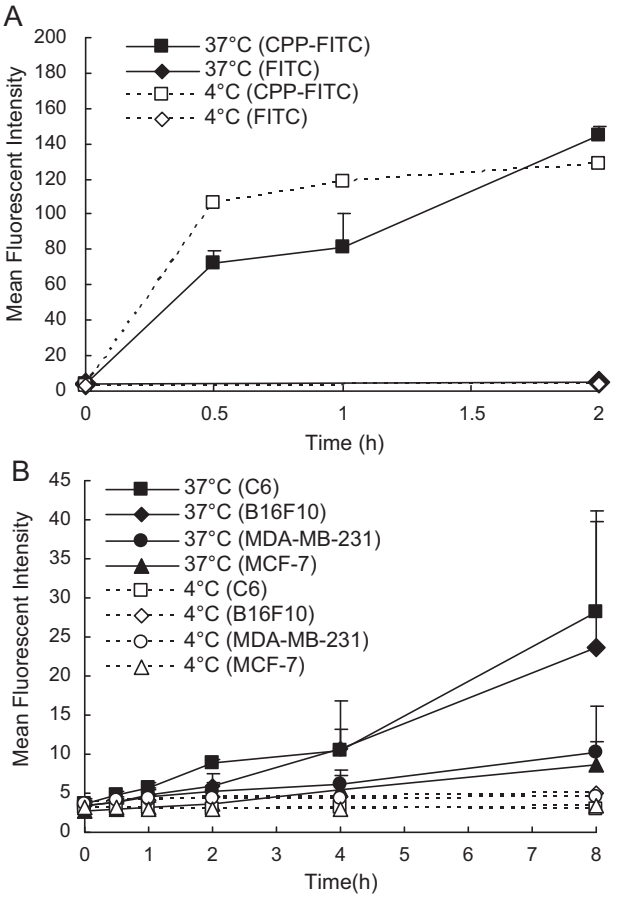

Fig. 2. Effect of temperature on the internalization of CPP–FITC and CPP–avidin. (A) MCF-7 cells were treated with 5 μM CPP–FITC at 37 °C ($n = 3$) and 4 °C ($n = 1$) in serum-free media for 0.5 h, 1 h, and 2 h. Meanwhile, MCF-7 cells were also treated with 5 μM FITC at two temperatures (37 °C and 4 °C, $n = 1$) in the same way. (B) C6, B16F10, MDA-MB-231, and MCF-7 cells were treated with 5 μM CPP–avidin complex at 37 °C ($n = 4$) and 4 °C ($n = 1$) in serum-free media for 0.5 h, 1 h, 2 h, 4 h, and 8 h. The cells were washed and analyzed by flow cytometry. Error bars indicate the standard deviation.

were relatively marginal when the complexes were tested at 4 °C. There were 9.2-fold, 4.8-fold, 2.6-fold, and 2.3-fold fluorescence intensity in C6, B16F10, MCF-7, and MDA-MB-231, respectively, at 37 °C compared with the values at 4 °C after an 8 h incubation. Lowering the incubation temperature from 37 °C to 4 °C led to a remarkable drop in fluorescence intensity, which demonstrated decreased access of CPP–avidin complexes to cells. The results revealed that the transport of macromolecules or nanoparticles fused to CPP was partly inhibited at a low temperature. These phenomena differed from that of small molecular cargoes attached to CPP (CPP–FITC). From Fig. 2, intracellular access of low-molecular-weight cargoes was faster than that of high-molecular-weight components bound to CPP.

3.3. Heparan sulfate receptors involved in CPP–cargo internalization

Membrane-associated heparan sulfate proteoglycans (HSPGs) is reported to play crucial roles in the endocytic uptake of arginine-rich peptides; however, internalization may be a function of a distinct endocytic process called macropinocytosis (Richard et al., 2005; Nakase et al., 2008). In this study, heparin was used as a competitor for cell membrane-associated HSPG, and the cellular uptake of CPP–FITC and CPP–avidin was investigated for tested cell lines in the presence of heparin.

As shown in Fig. 3A, after incubation for 1 h, CPP–FITC uptake weakened remarkably in all cell types in the presence of heparin compared with the heparin-free group. CPP–avidin entry was suppressed significantly in C6 ($p < 0.01$) and MDA-MB-231 ($p < 0.05$) cells, but not as significantly in B16F10 and MCF-7 cells in the presence of heparin compared to the control after 4 h (Fig. 3B). Collectively, the results revealed that CPP–FITC internalization was related to HSPG-dependent mechanisms for these cell lines. Regarding CPP–avidin complexes, HSPGs-dependent mechanisms were only relevant in C6 and MDA-MB-231 cell lines.

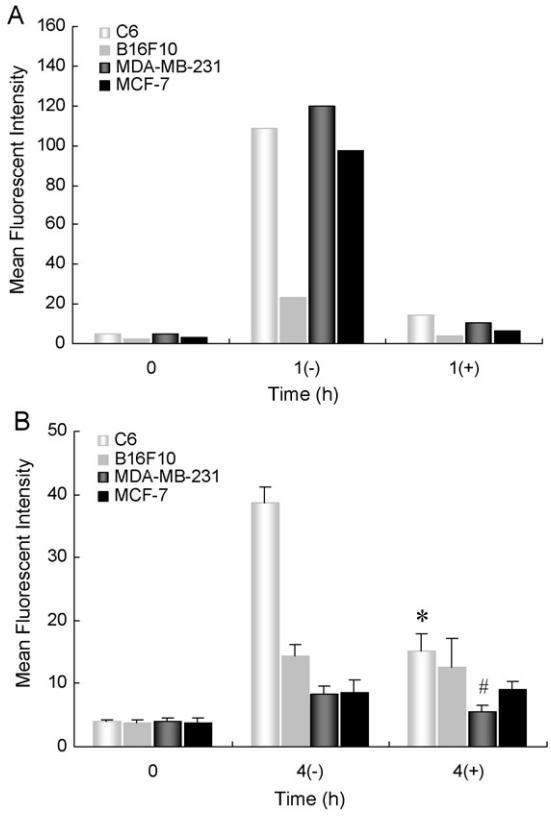

Fig. 3. Involvement of heparan sulfate receptors in the cellular uptake of CPP–cargoes. (A) CPP–FITC (5 μM) was incubated with C6, B16F10, MDA-MB-231, and MCF-7 cells for 1 h in the absence (−) and presence (+) of 10 μM heparin ($n = 2$). (B) CPP–avidin (5 μM) was incubated with four cancer cell lines for 4 h in the absence (−) and presence (+) of 10 μM heparin ($n = 3$–4). The cells were washed and analyzed by flow cytometry. Error bars indicate the standard deviation. #$p < 0.05$ and *$p < 0.01$ vs. 4 h (−).

3.4. CPP–cargo uptake involves clathrin-independent endocytic pathways

Studies on arginine-rich peptide uptake in cells with specific endocytotic pathways chemically inhibited have yielded mixed results. Clathrin-mediated endocytosis, known as the major and best-characterized endocytotic pathway, has been proposed as the primary mechanism for the uptake of arginine-rich transporters (Richard et al., 2005; Schmidt et al., 2010). To test whether CPP–cargo uptake involved clathrin-mediated endocytosis, CPP–FITC and CPP–avidin uptake was measured in the presence or absence of chlorpromazine, a known inhibitors of this endocytic route (Fig. 4). Chlorpromazine intensified the cellular fluorescence of CPP–FITC to different extents in the tested cell lines (Fig. 4A). C6 displayed the highest level in the presence of chlorpromazine, which was approximately 16-fold higher than the fluorescence level in the absence of chlorpromazine after incubation for 1 h. Unlike CPP–FITC, however, slight or negligible reinforcement of the CPP–avidin uptake was detected in all of the cell lines after 4 h of treatment (Fig. 4B). These data provided evidence that both types

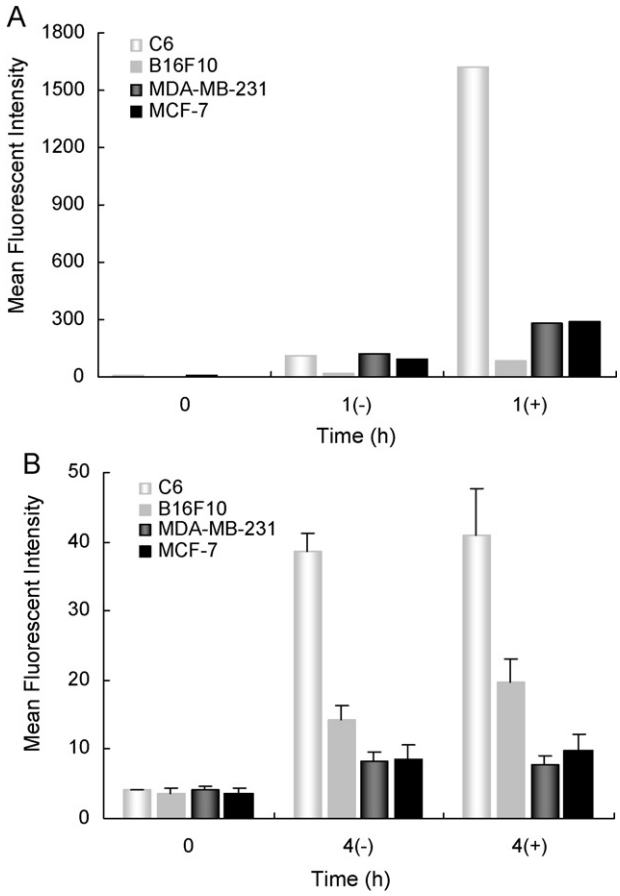

Fig. 4. Effect of chlorpromazine on the cellular uptake of CPP–cargoes. (A) C6, B16F10, MDA-MB-231, and MCF-7 cells were incubated with CPP–FITC (5 μM) for 1 h at 37 °C in the absence (−) and presence (+) of 30 μM chlorpromazine ($n = 2$). (B) Four cancer cell lines were incubated with CPP–avidin (5 μM) for 4 h at 37 °C in the absence (−) and presence (+) of 30 μM chlorpromazine ($n = 3$–4). The cells were washed and analyzed by flow cytometry. Error bars indicate the standard deviation.

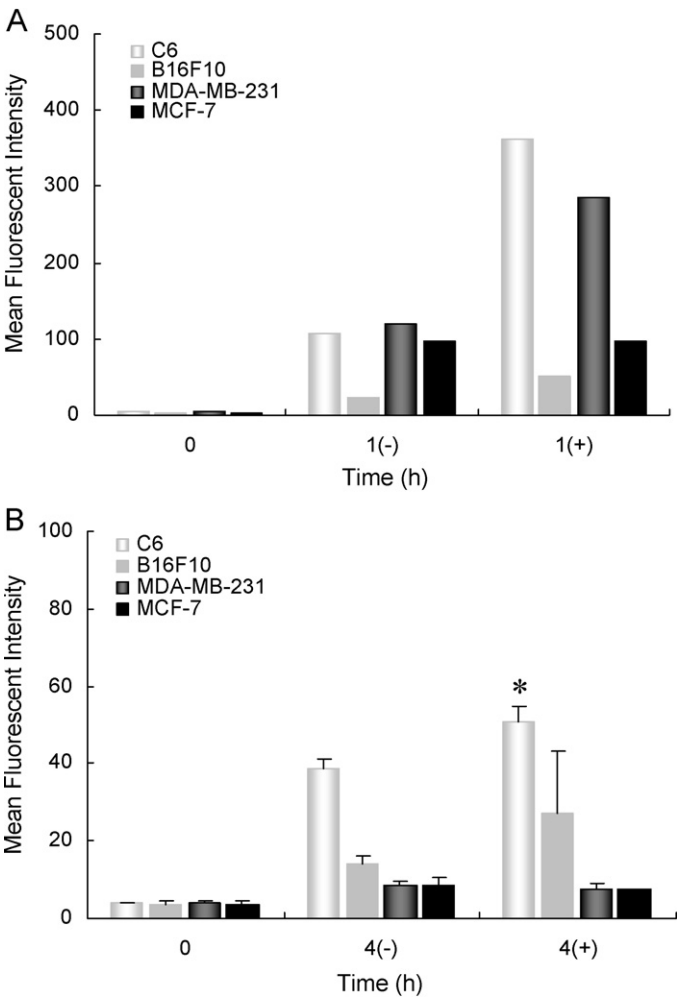

Fig. 5. Effect of chloroquine on the internalization of CPP-cargoes. (A) C6, B16F10, MDA-MB-231, and MCF-7 cells were incubated with CPP–FITC (5 μM) for 1 h at 37 °C in the absence (−) and presence (+) of 100 μM chloroquine ($n = 2$). (B) Four cancer cell lines were incubated with CPP–avidin (5 μM) for 4 h at 37 °C in the absence (−) and presence (+) of 100 μM chloroquine ($n = 3$–4). The cells were washed and analyzed by flow cytometry. Error bars indicate the standard deviation. *$p < 0.05$ vs. 4 h (−).

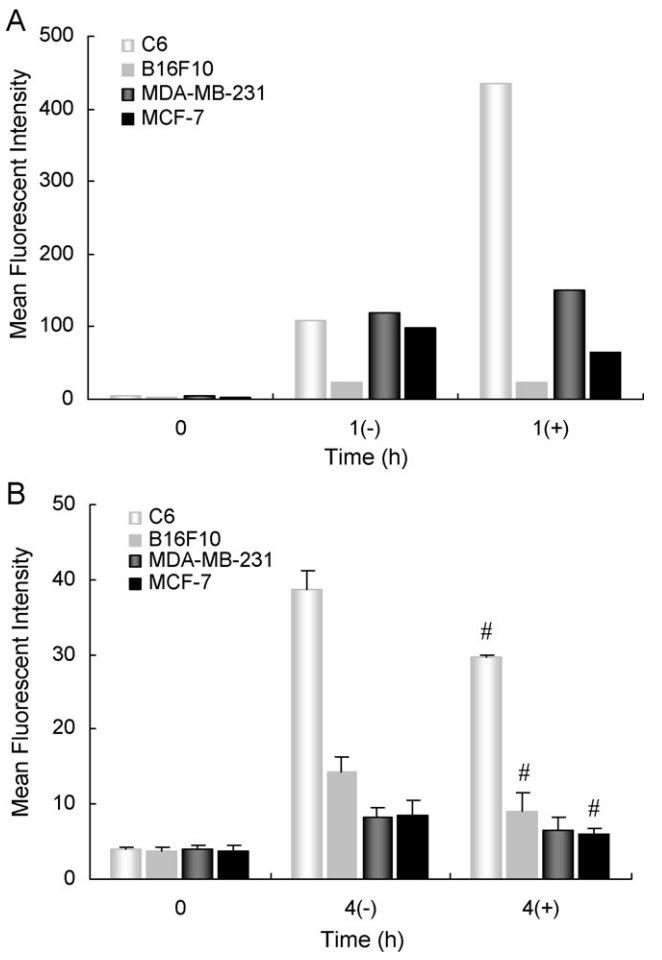

Fig. 6. Effect of EIPA on the cellular uptake of CPP–cargoes. (A) C6, B16F10, MDA-MB-231, and MCF-7 cells were incubated with CPP–FITC (5 μM) for 1 h at 37 °C in the absence (−) and presence (+) of 50 μM EIPA ($n = 2$). (B) Four cancer cell lines were incubated with CPP–avidin (5 μM) for 4 h at 37 °C in the absence (−) and presence (+) of 50 μM EIPA ($n = 3$–4). The cells were washed and analyzed by flow cytometry. Error bars indicate the standard deviation. #$p < 0.05$ vs. 4 h (−).

of CPP–cargoes did not require the clathrin-dependent endocytic pathway for intracellular access.

3.5. Chloroquine increases the retention of CPP–cargoes in acidic compartments

Chloroquine is a potent lysosomotropic agent and inhibits endosomal acidification and consequently slows down endocytosis, thus leaving more time for endosomal escape. By preventing the acidification of endocytic compartments, the associated degradative activities are inhibited (Zaro et al., 2009; Duchardt et al., 2007). Different cancer cells were subsequently treated for 30 min with chloroquine, and then CPP–FITC and CPP–avidin were added and incubated for 1 h or 4 h, respectively. The data revealed that chloroquine caused a noticeable increase in uptake of CPP–FITC in C6, B16F10, and MDA-MB-231 cells, but not MCF-7 cells (Fig. 5A). The total internalization of CPP–avidin was augmented in C6 cells and demonstrated no significant difference in MDA-MB-231, B16F10, and MCF-7 cells in the presence of chloroquine (Fig. 5B). The assay presented further evidence for the endocytosis of CPP–avidin complexes. The findings allude to the possibility that a significant fraction of CPP–avidin is delivered into acidic cellular compartments and passes through intracellular endosomes and lysosomes. In any case, endocytosis is related to intracellular access for both types of CPP–cargoes, especially CPP–avidin complexes.

3.6. CPP–avidin complexes enter cells by macropinocytosis

Macropinocytosis is a rapid, lipid raft-dependent and receptor-independent form of endocytosis. It requires actin membrane protrusions that are enveloped into vesicles, termed macropinosomes (Wadia et al., 2004; Conner and Schmid, 2003). Amiloride is known to block the formation of macropinosomes in growth factor-stimulated cells without affecting coated pit-mediated endocytosis (Meier et al., 2002; West et al., 1989). To examine the involvement of macropinocytosis in CPP–cargo transduction, we determined whether EIPA, a potent analog of amiloride, affected CPP entry as an inhibitor of the Na^+/H^+ exchange.

In the presence of EIPA (Fig. 6), there was no decrease in CPP–FITC internalization in C6, MDA-MB-231, and B16F10 cells but a slight decrease in MCF-7 cells compared to the control (Fig. 6A). The assay suggested that the macropinocytosis route did not play a crucial role for CPP–FITC entry. However, for CPP–avidin (Fig. 6B), the levels were lower after treatment with EIPA in established cancer cells than in the EIPA-free group ($p < 0.05$). Thus,

macropinocytosis might be involved in intracellular transduction of CPP–avidin complexes.

3.7. Laser confocal microscopy study

The transduction of CPP–FITC and CPP–avidin was monitored visually using a laser confocal microscope to capture images of their distribution at different time points. MCF-7 and B16F10 were used as cell model systems for this observation. Confocal images of fixed cells following a series of incubation times revealed significant differences regarding the uptake characteristics of two types of CPP–cargoes (Fig. 7).

The fluorescence of CPP–FITC (Fig. 7A) was clearly observed in the cytoplasm of MCF-7 cells after incubation for only 0.5 h, longer incubation (1 h or 2 h) resulting in stronger fluorescence. It was remarkable that CPP–FITC was transported more into the cytoplasm than into the nucleus at 2 h. The uniform or continuous dispersion of CPP–FITC fluorescence in the cytoplasm suggested direct translocation or an alternative route for intracellular access of CPP–FITC.

CPP–avidin complexes revealed striking differences when compared with CPP–FITC. The intensity of these complexes was very low in MCF-7 cells after 2 h of treatment. The fluorescence clearly emerged at 8 h, however, gradually increased with increased incubation time (Fig. 7B). At equal concentrations of 5 μM, uptake of CPP–avidin at 24 h was only slightly weaker than CPP–FITC at 2 h. Similar uptake characteristics of CPP–avidin were observed in B16F10 cells (Fig. 7C). With increased incubation time, the fluorescence of CPP–avidin became fuzzy and patchy, probably because the CPP–avidin complex is relatively large for attachment to the cell surface. The punctate signals observed in the MCF-7 and B16F10 cells also substantiated the notion that endosomal uptake of CPP–avidin occurred in the studied cell lines.

4. Discussion

We estimated the ability of CPPs to deliver different cargoes into cells by following the cellular uptake of CPP–FITC and CPP–avidin by flow cytometry and fluorescence microscopy. Internalization mechanisms of these CPP–cargoes were investigated by changing a series of microenvironments and adding different endocytic inhibitors. R9 was taken in consideration as representative of various CPP families because of its excellent translocation efficiency. Instead of synthesizing a CPP–protein conjugate, we used the complex of a biotinylated CPP with fluorescein-labeled avidin to follow the translocation activity (Al-Taei et al., 2006).

The intracellular uptake studied on CPP–avidin revealed that CPP–avidin entry was time-dependent and increased with increased incubation time. In addition, no specificity was observed toward any cell line, but the amount and rate of internalization depended on the cell type and cargo (Figs. 1, 2 and 8). The results were consistent with our previous experiments on CPP–FITC (Ma and Qi, 2010) and other reports (Säälik et al., 2004; Iwasa et al., 2006). The contrasting CPP–cargo trafficking in C6 cells in all of the experiments was ascribed to the exocytosis of C6, which was previously observed in other experiments (Ma and Qi, 2010). In C6, the appearance of a plateau between 8 h and 24 h of incubation for CPP–avidin uptake also supported this hypothesis (Fig. 1).

Very few studies have investigated the influence of serum on the transduction of various CPP–cargoes. The presence and absence of serum is a factor that needs to be carefully assessed if these *in vitro* models are to be designed to be more closely representative of the *in vivo* studies (Kosuge et al., 2008). Serum proteins have a considerable effect on the internalization of oligoarginine peptides, especially in the case of longer peptides. The presence of serum because it quenches the effective peptide concentration, significantly increases the threshold concentration of efficient intracellular CPP access (Kosuge et al., 2008). However, the protein transduction efficiency of CPP–avidin in the study displayed cell type selective enhancement in the presence of serum (Fig. 1). Specifically, clear reinforcement was observed in MCF-7, as well as moderate increases in C6 and MDA-MB-231 and negligible changes in B16F10. The phenomenon may be accounted for by considering that a majority of protein cargoes are delivered into cells by CPPs, whereas other components access cells probably with the aid of nutritional component uptake pathways.

Endocytosis is an energy dependent pathway and is usually suppressed at low temperatures. A standard protocol was used that included a trypsin treatment before the analysis of the cell suspensions by flow cytometry because trypsin can remove surface-bound peptides (Mueller et al., 2008; Nakase et al., 2008). A rapid and highly efficient internalization of CPP–FITC, both at 37 °C and at 4 °C, was subsequently observed (Fig. 2A). This fact supports the notion that the translocation of CPPs, or small molecules attached to CPP, is achieved via direct and non-endocytic mechanisms (Takeuchi et al., 2006; Ter-Avetisyan et al., 2009). The general decrease in the uptake of CPP–avidin at 4 °C compared with 37 °C, which was very pronounced for all cancer cells (Fig. 2B), proved that the intracellular access of CPP–avidin was energy dependent. We thus presumed that the transduction of macromolecules or nanoparticles fused to CPP was unique from small molecular cargo attached to CPP or CPP itself.

Cell penetration of CPPs obeys three fundamental steps: (i) binding to some component of the plasma membrane, (ii) the cell entry process, and (iii) subsequent release into the cytoplasm or nucleus. However, none of these steps are well understood, and conflicting reports have emerged (Ram et al., 2008). Generally, large molecular cargo delivered by CPPs requires the services of endocytic pathways (Gupta et al., 2005). However, there is still a certain discrepancy in the analysis of endocytic routes for CPP–cargo (Schmidt et al., 2010; Gupta et al., 2005; Richard et al., 2003).

It is now known that for most CPPs, entry into cells is mainly achieved through an endocytic mechanism, as quantified by flow cytometry after internalization. Several reports demonstrated that the classical clathrin-mediated endocytosis via clathrin-coated pits is one of the routes actively utilized by CPP–cargo in the internalization process (Säälik et al., 2004; Rinne et al., 2007). However, some publications argue against the importance of clathrin in the internalization process and present evidence in favor of other types of endocytosis involved in the translocation of CPP–cargo (Fittipaldi et al., 2003; Ferrari et al., 2003). Even so, others have reported the involvement of macropinocytosis instead of clathrin-dependent or caveolin-dependent endocytosis as the main entry pathway for CPP–cargoes (Khalil et al., 2006), especially the arginine-rich peptides and their protein conjugates (Kaplan et al., 2005; Wadia et al., 2004; Nakase et al., 2004).

In this study, different endocytic inhibitors were used to investigate endocytic routes of CPP–FITC and CPP–avidin internalization. Our results indicated that HSPGs played an important role in both CPP–FITC and CPP–avidin access into cells. Adsorption of CPP–cargoes on the plasma membrane seemed to be the key for CPPs access, regardless of cargo volume. CPP–FITC more easily adhered to cells than CPP–avidin due to the low payload and high density, which was determined from the degree of heparin inhibition (Fig. 3). Suppression by chlorpromazine did not clearly indicate that CPP–avidin entry was dependent on clathrin-mediated endocytosis for these cells (Fig. 4). Chloroquine increased the retention of both types of CPP–cargoes in acidic compartments, which, however, suggested that intracellular access of these CPP–cargoes may occur via endocytosis by passing through intracellular endosomes

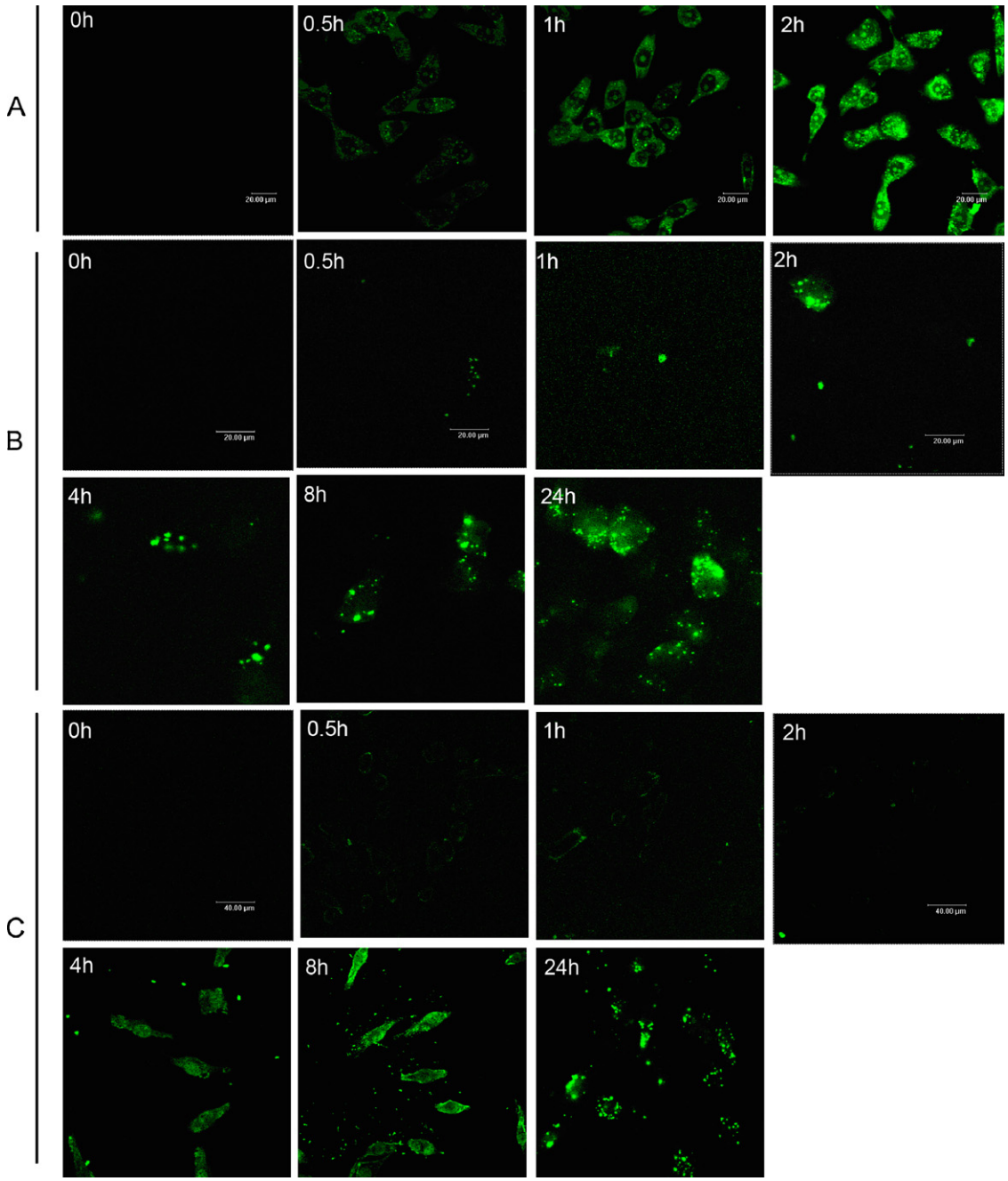

Fig. 7. Confocal microscopy images of MCF-7 and B16F10 cells incubated with CPP–cargo at 37 °C. (A) MCF-7 cells were treated with CPP–FITC (5 μM) for 0.5 h, 1 h, and 2 h at 37 °C. Scale bar: 20 μm. (B) MCF-7 cells were treated with CPP–avidin (5 μM) for 0.5 h, 1 h, 2 h, 4 h, 8 h, and 24 h at 37 °C. Scale bar: 20 μm. (C) B16F10 cells were treated with CPP–avidin (5 μM) for 0.5 h, 1 h, 2 h, 4 h, 8 h, and 24 h at 37 °C. Scale bar: 40 μm.

and lysosomes, this phenomenon was cell type specific and more pronounced for CPP–FITC in C6, B16F10 and MDA-MB-231 cells (Fig. 5). Furthermore, macropinocytosis was more likely to occur for high-molecular-weight cargoes that were fused to CPP from EIPA inhibition experiment (Fig. 6).

Intracellular distribution analysis of these CPP–cargoes was also visualized by confocal microscopy imaging to collect detailed information on the intracellular behaviors of CPPs and their cargoes (Fig. 7). The appearance of continuous fluorescence in MCF-7 cells was mainly attributed to the direct translocation of CPP–FITC, whereas weak signals for CPP–avidin complexes in MCF-7 and B16F10 cells showed that the endocytic mechanism was involved in the transduction of larger molecular cargoes linked to CPP.

Both translocation and endocytosis are internalization pathways utilized by arginine-rich CPPs (Fischer et al., 2004). The choice of one pathway versus the other depends on the peptide sequence (not the number of positive changes), the extracellular peptide concentration, and the membrane components (Jiao et al., 2009). Combining all of these observations, a possible model is described in Fig. 8 for the delivery of two types of CPP–cargo in cancer cells. Adsorption on the cell surface seems to be necessary for CPPs access, regardless of the cargo. CPP–FITC might enter cancer cells by rapid and direct translocation in combination with the endocytic pathway. The phenomenon was more marked in C6, MDA-MB-231, and MCF-7 cells than in B16F10. CPP–avidin complexes crossed the cell membranes mainly by macropinocytosis. The clathrin-mediated

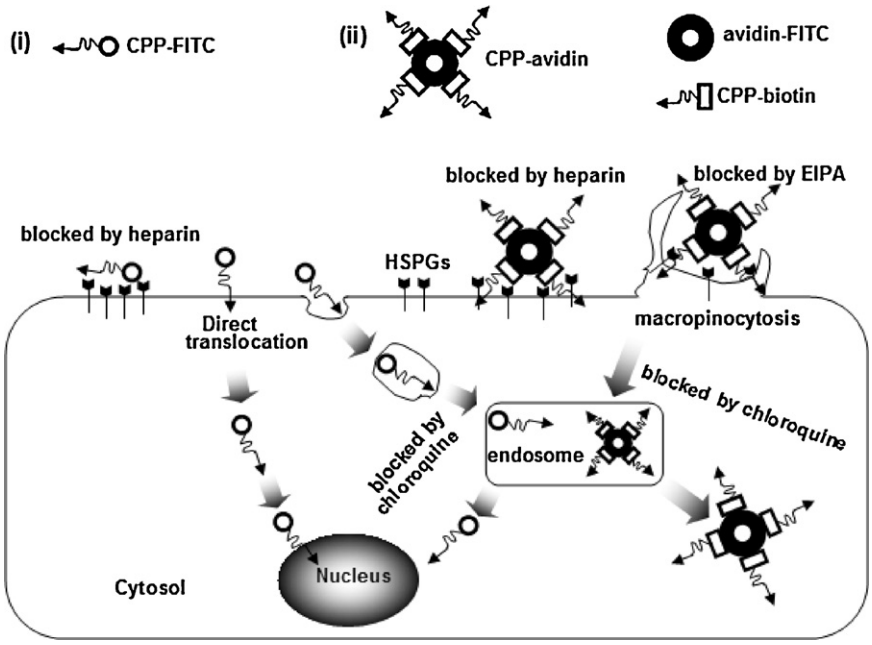

Fig. 8. Proposed model for CPP–cargo delivery into tumor cells. (i) CPP–FITC conjugates. Direct translocation in an energy independent manner is mediated by membrane potential or an endocytic mechanism. Binding with HSPGs occurs during this process. (ii) CPP–avidin complex. Interaction with HSPGs receptors on the cellular surface is important: macropinocytosis plays a dominant role in the intracellular trafficking of CPP–avidin.

pathway was not required for internalization of CPP–cargoes in any of the studied cell lines.

5. Conclusion

The uptake results for two types of CPP–cargo exhibited no specificity toward any cell line, but the levels were found to be related to cell type and cargo. Two distinct transduction modes for arginine-rich CPPs were observed for delivering different cargoes into tumor cells. Low-molecular-weight cargoes attached to CPP or CPP itself entered cells rapidly via direct translocation in combination with the endocytic route. High-molecular-weight cargoes fused to CPP tended to internalize through the macropinocytosis pathway in an energy-dependent manner.

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