Pharm Res (2014) 31:1438–1449
DOI 10.1007/s11095-013-1046-z

RESEARCH PAPER

Imaging the Cytosolic Drug Delivery Mechanism of HDL-Like Nanoparticles

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Received: 28 November 2012 / Accepted: 1 April 2013 / Published online: 27 April 2013
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ABSTRACT

Purpose Molecular therapeutics often require an effective nanoparticle-based delivery strategy to transport them to cytosolic organelles to be functional. Recently, a cytosolic delivery strategy based on the scavenger receptor class B type I (SR-BI) mediated pathway has shown great potential for the effective delivery of theranostics agents into the cytoplasm of cells without detrimental endosomal entrapment. This study elucidates this unique delivery mechanism for improving cytosolic drug delivery.

Methods Multifluorophore-labeled HDL-mimicking peptide phospholipid scaffold (HPPS) nanoparticles were developed. Fluorescence imaging was utilized to examine HPPS transporting payloads into cells step by step through sequential inhibition studies.

Results HPPS specifically recognizes and binds to SR-BI, then interacts with SR-BI, which results in direct transport of payload molecules into the cell cytoplasm without entire particles internalization. The cytosolic transport of payloads occurred through a temperature- and energy-independent pathway, and was also different from actin- and clathrin-mediated endocytosis. Furthermore, this transport was significantly inhibited by disruption of lipid rafts using filipin or methyl-β-cyclodextrin.

Conclusions The cytosolic delivery of payloads by HPPS *via* SR-BI targeting is predominately mediated through a lipid rafts/caveolae-like pathway. This cytosolic delivery strategy can be utilized for transporting molecular therapeutics that require their action sites to be within cytosolic organelles to enhance therapeutic effect.

KEY WORDS cytosolic · drug delivery · imaging · lipid rafts · nanoparticle · SR-BI · targeting

INTRODUCTION

For anticancer drugs to be effective, an efficient delivery strategy is usually required to transport them through various *in vivo* barriers and such that it reaches its site of action in an active state. Many drugs, such as small molecular siRNAs, recombinant protein, and certain anti-oxidants interact with targets within the cytoplasm (1,2). Therefore, efficient transport of those drugs to the cytoplasm of cells is critical. Commonly studied nanoparticle-based drug

Electronic supplementary material The online version of this article (doi:10.1007/s11095-013-1046-z) contains supplementary material, which is available to authorized users.

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delivery systems, such as liposomes, polymeric nanoparticles and inorganic nanoparticles (quantum dots and gold nanoparticles) (3–6) typically localize within the endo-lysosomal compartment in cells following internalization, followed by detrimental drug inactivation by lysosomal degradation. To enhance therapeutic efficacy, a number of endosomal-escape strategies, such as light-triggered endosomal disruption (2,7), pH-responsive release mechanisms (2,8) and swelling induced physical disruption (4) have been investigated. However, all of these methods require entrapment of the molecules within the endosome, prior to release. An ideal alternative is to directly deliver drugs into cytosolic components of targeted cells without endosomal entrapment.

One reported method of cytosolic delivery is direct cell penetration or diffusion, but this method either requires high drug concentration or is only suitable for small molecules (< 1KDa) (9,10). Cell penetrating peptides (CPPs)–mediated delivery was initially thought to occur by direct cytosolic delivery *via* cell penetration. However, subsequent studies suggested that the observed cytosolic delivery could be attributed to experimental artifacts (11), and the endocytosis and endosomal escaping were indeed involved in the intracellular transport of most CPPs (10). In addition to CPPs, fusogenic liposomes formed by fusing sendai virus on the surface of liposome was reportedly able to deliver encapsulated nanoparticles for cytosolic controlled gene delivery (12). In 2008, a cytosolic drug delivery strategy based on lipid rafts transport was introduced (13). The lipophilic drugs on the nanoparticle surface would stream from the nanoparticle lipid monolayer directly onto the cell membrane *via* a contact-mediated transfer process. This method has only been applied in lipid-coated liquid perfluorocarbon emulsions. Recently, a direct cytosolic drug delivery strategy based on scavenger receptor class B type I (SR-BI) has been discovered (14), taking advantage of the intrinsic targeting property of HDL-like nanoparticles. The SR-BI pathway has already been explored by a number of research groups for delivering a variety of imaging (14–16) and therapeutic cargos (17–21) for the diagnostic and treatment of cancers and other diseases. Therefore, this strategy could have broad implications in delivery drugs direct into cell cytoplasm, thereby bypassing the detrimental endosomal trapping and evading the further step for endosomal release of drugs.

SR-BI was first identified as the receptor for HDL in 1996 (22). Its delivery pathway is currently well known on potential treatment of atherosclerosis since HDL-like particle is capable of transport of cholesterol from tissues back to the liver for catabolism through a process named as reverse cholesterol transport. As SR-BI enables delivery nanoparticles across the blood–brain barrier (23), SR-BI mediated delivery strategy holds a great potential for brain disease treatment. Moreover, SR-BI is upregulated in many cancers, including ovary, pancreas, lung, liver, prostate, and breast (17,19,21,24). Therefore, it is hypothesized that the targeted delivery of drugs, in particular small molecules and siRNA, *via* SR-BI will improve anti-tumor efficacy and reduce systemic toxicity.

In nature, the selective uptake of lipids, mainly cholesteryl esters (CE), mediated by HDL *via* SR-BI is considered a two-step process. The first binding step involves interaction with the SR-BI protein binding domain, and the second step involves lipid transport from the particle to cells, the mechanism of which has not been completely elucidated. Rodriqueza *et al.* proposed that SR-BI formed a non-aqueous channel and used this model to explain how CE is transported from HDL to the cell *via* SR-BI. This channel was thought to exclude water and serve as a conduit for hydrophobic CE molecules diffusing from bound HDL down their concentration gradient to the cell plasma membrane (25). However, this channel hypothesis is still debated and the mechanism of SR-BI mediated direct transport of lipid molecule into cell cytoplasm remains unclear.

To facilitate the utility of SR-BI pathway for delivery of a wide range of drugs for clinical applications, it is imperative to understand the SR-BI mediated delivery mechanism. In this study, utilizing fluorescence imaging techniques, we investigated the SR-BI mediated delivery mechanism using synthetic HDL-mimicking peptide phospholipid scaffold (HPPS) nanoparticles. By labeling each component (surface phospholipid, peptide, and core cargo)of the HPPS nanoparticle with a different fluorescent dye, we examined the intracellular uptake of HPPS using confocal microscopy and investigated the cytosolic delivery mechanism by a series of sequential inhibition studies. The data provides evidence that HPPS initially recognized and bound to SR-BI, and then interacted with the special protein domain on SR-BI. The interaction resulted in immediate particle dissociation, followed by internalization of the hydrophobic cargo into the cytosol. The phospholipids and peptides were mostly retained on the cell surface. The cytosolic delivery of hydrophobic cargos was mainly inhibited by disruption of cholesterol-rich lipid rafts with filipin or methyl-β-cyclodextrin(MβCD), suggesting that the cytosolic drug delivery by HPPS *via* SR-BI targeting is mainly mediated through a lipid rafts/caveolae-like pathway (Fig. 1).

MATERIALS AND METHODS

Nanoparticles Preparation

Fluo-BOA (Dioleyl Fluorescein) and DiR-BOA (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide

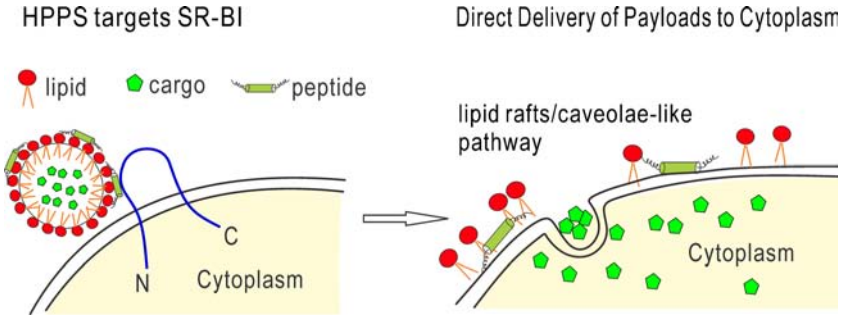

Fig. 1 The scheme illustrated a two-step intracellular delivery mechanism of HPPS nanoparticles: 1) HPPS recognize SR-BI and interact with SR-BI; 2) HPPS dissociate during interaction and directly transport hydrophobic cargos into the cytosol of cells mainly *via* a lipid-raft/caveolae-like mediated endocytosis pathway.

bis-oleate) were synthesized using previously reported methods (14). Cholesteryl oleate (CO) was obtained from Sigma-Aldrich Co. (MO, USA). 1, 2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhodamine B-labeled phospholipids) was purchased from Avanti Polar Lipids Inc. (AL, USA). The ApoA-1 mimetic, amphipathic α-helical peptide, Ac-FAEKFKEAVKDYFAKFWD-NH2, was purchased from GL Biochem Ltd. (Shanghai, China). Fluorescein isothiocyanate (FITC) labeled peptide was synthesized following the previously reported method (14). The multi dye labeled HPPS nanoparticles were defined as follows: 1) HPPS(Fluo-BOA): HPPS with its core loaded with Fluo-BOA; 2) Rhodamine B-lipid-HPPS: HPPS with the phospholipids labeled with Rhodamine B; 3) FITC-peptide-HPPS: HPPS with the peptide labeled with FITC; 4) Rhodamine B-lipid-HPPS(Fluo-BOA): HPPS with Rhodamine B-labeled phospholipids and Fluo-BOA loaded in core; 5) FITC-peptide-HPPS(DiR-BOA): HPPS with the peptide labeled with FITC and the core loaded with DiR-BOA. All of the HPPS particles were prepared according to a previously reported method (14). No significance size difference was observed between various differently labeled types of nanoparticles. The sizes of all the nanoparticles are between 18 and 23 nm (Fig. S4).

Cell Culture

ldl(mSR-BI) and LDL receptor-deficient Chinese hamster ovary(CHO) cells (ldlA-7) (26) were gifts from Dr. Monty Krieger (Massachusetts Institute of Technology, Cambridge, MA). ldlA-7 cells were cultured in Hams F-12 media with 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, and 5% fetal bovine serum (FBS). ldl(mSR-BI) cells were cultured under similar conditions with 300 μg/ml G418. All cells were grown at 37°C in an atmosphere of 5% CO_2. L-glutamine and penicillin-streptomycin were purchased from the ATCC (American Type Culture Collection). Fetal bovine serum (FBS), Hams F-12 media and trypsin-ethylenediaminetetraacetic acid (EDTA) solution were purchased from Gibco-Invitrogen Co. (CA, USA).

Confocal Microscopy

ldl(mSR-BI) cells and ldlA-7 cells (1.5×10^4 per well) were seeded into eight-well cover-glass-bottom chambers (Nunc Lab-Tek, Sigma-Aldrich) and incubated for 48 h at 37°C in an atmosphere of 5% CO_2 in a humidified incubator. The confocal image of the cells was taken by Olympus FV1000 Confocal Laser Scanning Microscope (Olympus, Tokyo, Japan) with excitation wavelengths at 488 nm for Fluo-BOA/FITC, 543 nm for Rhodamine B, and 633 nm for DiR-BOA.

Time-Dependent Uptake of Fluorescence Labeled HPPS

ldl(mSR-BI) cells seeded in chamber wells were incubated with HPPS(Fluo-BOA), Rhodamine B-lipid-HPPS and FITC-peptide-HPPS, respectively, at a particle concentration of 0.05 μM for 30 min, 1.5 h, 3 h, 6 h. After incubation, the cells were washed twice with PBS and replaced with fresh cell culture medium. The fluorescent signal of ldlA(mSR-BI) cells was imaged at different time points using confocal microscopy.

Uptake of Rhodamine B-Lipid-HPPS(Fluo-BOA) and FITC-peptide-HPPS(DIR-BOA) *via* SR-BI

ldl(mSR-BI) cells or ldlA-7 cells seeded in chamber wells were incubated with Rhodamine B-lipid-HPPS(Fluo-BOA) and FITC-peptide-HPPS(DiR-BOA), respectively, at a HPPS concentration of 0.05 μM . For inhibition studies, HDL or the blocker of lipid transport 1 (BLT-1) (Chembridge Corporation, CA, USA) was used in a 50 fold mole-excess and 10 μM, respectively. Each inhibitor was added into chamber wells 15 min before incubation with HPPS particles. After 1.5 h incubation, the cells were washed twice with PBS, replaced with fresh cell culture medium, and imaged by confocal microscopy.

Mechanism of Cytosolic Delivery by HPPS

2-Deoxy-D-glucose, sodium azide, Chlopromazine hydrochloride (CPZ), filipin, MβCD, and 5-(N-Ethyl-N-isopropyl)

amiloride (EIPA) were all purchased from Sigma-Aldrich Co. (MO, USA). Transferrin-AlexaFluor633 and Tetramethylrhodamine(TMR)-70 kDa-dextran were obtained from Molecular Probes, Inc. (OR, USA). Cytochalasin D (CytD) was purchased from Gibco-Invitrogen Co. (CA, USA). EEA1 (C45B10) Rabbit mAb was purchased from cell signaling Technology (MA, USA).

To determine the temperature influence on cytosolic delivery of hydrophobic cargos by HPPS, ldlA(mSR-BI) cells were incubated with 0.05 μM HPPS(Fluo-BOA) at different temperature (37°C, 18°C and 4°C) for 1.5 or 3 h. Confocal microscopy was used to examine the temperature effect on the cytosolic delivery of these nanoparticles. To determine if the cytosolic delivery depended on energy, experiments were performed under ATP depleted conditions (50 mM 2-Deoxy-D-glucose and 20 mM sodium azide). Transferrin-AlexaFluor633 (50 μg/ml) was added together with HPPS(Fluo-BOA) in cells as an ATP-dependent control. To determine the role of endocytic cellular processes on HPPS cytosolic delivery, experiments were performed under different inhibition condition: CPZ was used as a clathrin-mediated endocytosis specific inhibitor (10 μg/ml); transferrin-AlexaFluor633 (50 μg/ml) was added as a common marker for clathrin-mediated endocytosis and co-incubated with HPPS(Fluo-BOA); CytD (25 μM) was used as an actin-disrupted agent; TMR-70 kDa-dextran (0.5 mg/ml) was added as a control for actin filaments-involved process; filipin(10 μg/ml) was used for disruption of cholesterol-rich lipid rafts and MβCD (8 mM) was used to deplete membrane cholesterol (27); Amiloride hydrochloride (5 mM) and (EIPA) (100 μM) were used to inhibit macropinocytosis. TMR-70 kDa-dextran (0.5 mg/ml) was served as a common marker of macropinocytosis and incubated together with HPPS(Fluo-BOA). For all inhibition studies, cells were incubated with inhibitors for 15 min before addition of HPPS(Fluo-BOA) nanoparticles. After 1.5 h incubation, cells were rinsed twice with PBS, and then imaged immediately using confocal microscopy. For filipin inhibition studies, to determine if the cytosolic delivery could be recovered after the removal of filipin, cells were washed several times with PBS, and imaged at different time points post washing until 24 h.

Quantification and Statistical Analysis

Image J software (Wayne Rasband, NIH) was used to quantify average intensity in cytoplasm as previously reported (13). The Pearson coefficient was calculated using the existing Olympus FV1000 laser confocal scanning microscopy software (Olympus, Tokyo, Japan). Comparisons were made using the Student's *t*-test (two tailed) with p-values less than 0.05 were considered statistically significant.

RESULTS

Direct Cytosolic Delivery of Hydrophobic Cargos by HPPS Without Nanoparticle Internalization

HPPS mimics HDL in both lipid particle structure and function. The particle itself has a small size (less than 25 nm), spherical shape when loaded with hydrophobic cargo (14), and the ability to offload hydrophobic cargos to cells *via* SR-BI targeting. HPPS is comprised of three components: a phospholipid shell, peptide and hydrophobic core. In our previous study, we have validated that after 1 h incubation, HPPS directly transported the core loaded hydrophobic cargo into cytoplasm of cells *via* SR-BI targeting while most of the peptides and phospholipids were retained on surface of the cell (14). To further investigate the SR-BI mediated cytosolic delivery mechanism of HPPS, three individual labeled fluorescent HPPSs: HPPS(Fluo-BOA), Rhodamine B-lipid-HPPS and FITC-peptide-HPPS, were prepared with their core, lipid and peptide labeled with Fluo-BOA, Rhodamine B and FITC, respectively. Time-dependent intracellular uptake of these fluorescent HPPSs were examined using confocal microscopy. As shown in Fig. 2, within 30 min of incubation, core molecules started to internalize into the cytoplasm of SR-BI positive ldlA(mSR-BI) cells, while the lipid and peptide components were only observed on the surface of cell membrane. With increasing incubation time, greater amounts of core loaded molecules were transported homogeneously into the cytoplasm, whereas increasing amounts of lipids and peptides were retained on the cell membrane. These results suggest that unlike most of nanocarriers delivering drugs into cells with the entire particle being internalized, HPPS particles dissociated on the cell membrane and directly transported core payloads into cell cytoplasm while leaving lipid and peptide components on the cell membrane. After 1.5 h incubation, a small amount of lipids and peptides were internalized in cells, but their subcellular distribution was not homogeneous within the cytoplasm. We believe that this internalization of lipids and peptides occurs after HPPS dissociation, thus is likely independent of the HPPS delivery pathway. The time-dependent intracellular uptake fate of a dual dye labeled HPPS, Rhodamine B-lipid-HPPS(Fluo-BOA) (HPPS with phospholipids labeled by Rhodamine B and core loaded with Fluo-BOA), further demonstrated the cytosolic delivery of HPPS without entire nanoparticle internalization, evidenced by the complete separation of the signals of Fluo-BOA and Rhodamine B lipid upon internalization: Fluo-BOA was found to be localized in the cytoplasm, while Rhodamine B lipid stayed on the cell surface (Supplementary Material Fig. S1).

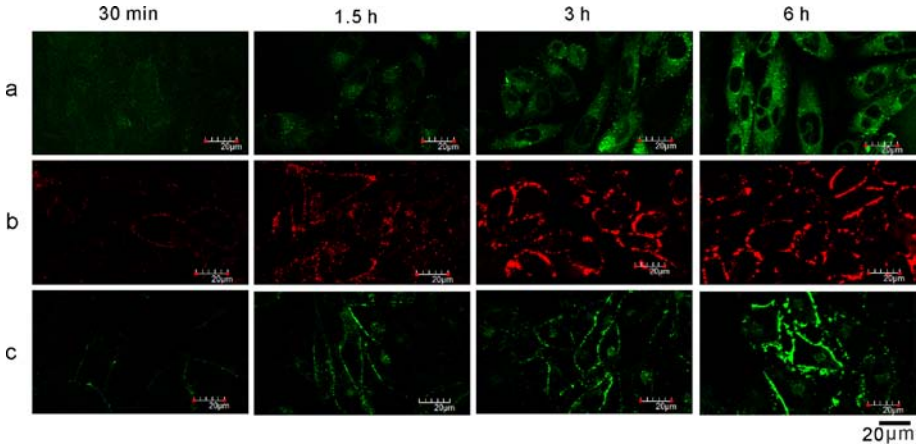

Fig. 2 Confocal imaging of time-dependent intracellular uptake of the following three fluorescent HPPS in SR-BI overexpressed ldlA(mSR-BI) cells: **a** HPPS(Fluo-BOA), the core of HPPS was loaded with Fluo-BOA, **b** Rhodamine B-lipid-HPPS, the phospholipids of HPPS were labeled with Rhodamine B, and **c** FITC-peptide-HPPS, the peptides of HPPS were labeled with FITC. All of images were taken under the same condition after incubation with 0.05 μM of HPPS.

SR-BI-Mediated Cytosolic Delivery of Hydrophobic Cargos by HPPS

After validating that HPPS directly delivered hydrophobic cargo into the cell cytoplasm without intact particle internalization, we next examined the role of SR-BI in the direct cytosolic delivery. A series of confocal experiments were performed to track the intracellular uptake of Rhodamine B-lipid-HPPS(Fluo-BOA), yielding the following results (Fig. 3a): (1) HPPS recognized SR-BI positive ldlA(mSR-BI) cells and directly delivered core loaded Fluo-BOA into cytoplasm while leaving Rhodamine B lipid on the cell membrane; (2) HPPS neither bound to nor entered in ldlA7 cells which are SR-BI negative.; (3) Excess native human HDLs efficiently inhibited the binding and uptake of HPPS in SR-BI positive cells, indicating that HDLs competed with HPPS in binding to SR-BI, which resulted in completely blocking of HPPS uptake; (4) The blocker of lipid transport 1 (BLT-1), a specific SR-BI inhibitor capable of efficiently inhibiting the lipid transfer by HDL (28), was used to inhibit the lipid interaction between HPPS and cells. With BLT-1 inhibition, both signals of Fluo-BOA core and Rhodamine B-lipid were retained on the surface of cells and colocalized well with a high Pearson coefficient value of 0.89 (Fig. 3a bottom), indicating that BLT-1 did not influence HPPS binding to SR-BI, but completely inhibited cytosolic delivery of hydrophobic payloads by HPPS. The high degree colocalization of phospholipids and core payloads demonstrated that HPPS nanoparticles remained intact when binding to SR-BI. In addition, similar inhibition phenomenon was observed on cell uptake of another dual dye-labeled HPPS, FITC-peptide-HPPS(DiR-BOA) (HPPS with core labeled by DiR-BOA and peptide labeled by FITC, Fig. 3b) and other two single dye-labeled HPPSs: HPPS(Fluo-BOA) and Rhodamine B-lipid-HPPS(Supplementary Material Fig. S2). These data suggest that SR-BI plays a key role in HPPS binding to cells and BLT-1 can block the interaction between HPPS and SR-BI cells which completely inhibited the cytosolic delivery of hydrophobic payloads by HPPS.

After clarifying the key role of SR-BI on HPPS binding and interaction, we next investigated the mechanism in which the HPPS core payloads were transported into the cytoplasm upon HPPS interaction with SR-BI. In general, most molecules are taken up into cells by endocytosis except some essential small molecules, such as sugars and ions, traverse the plasma membrane through the action of integral membrane protein pumps or channels. The endocytosis pathways could be divided into two broad categories, namely, phagocytosis and pinocytosis. Phagocytosis only occurs in specialized mammalian cells and is responsible for the uptake of particulate matter larger than around 0.5 μm in diameter, such as small-sized dust particles, cell debris, micro-organisms and even apoptotic cells . Pinocytosis occurs in almost all cells and can be subdivided into the following basic categories: clathrin-mediated endocytosis, macropinocytosis and caveolae/lipid rafts mediated endocytosis (29). Generally, endocytosis is affected by temperature, energy, cytoplasmic contractile elements, such as actin. To define the role of these factors in the cytosolic delivery of HPPS, experiments with broad inhibition of transport pathway were conducted by modulating the temperature of the cell environment (37°C,18°C,4°C), depleting ATP (sodium azide and 2-deoxyglucose) and disrupting actin (CytD).

The Effect of Temperature, Energy and Actin on the Cytosolic Delivery of Hydrophobic Cargos by HPPS

To investigate the effect of temperature on the cytosolic delivery, time dependent cellular uptake of HPPS(Fluo-BOA) were examined at 37°C,18°C and 4°C, respectively. The results showed (Fig. 4) that decreasing the incubation temperature resulted in the reduction of the intracellular uptake of Fluo-BOA. However, Fluo-BOA was still observed homogeneously in the cytoplasm of cells both 18°C

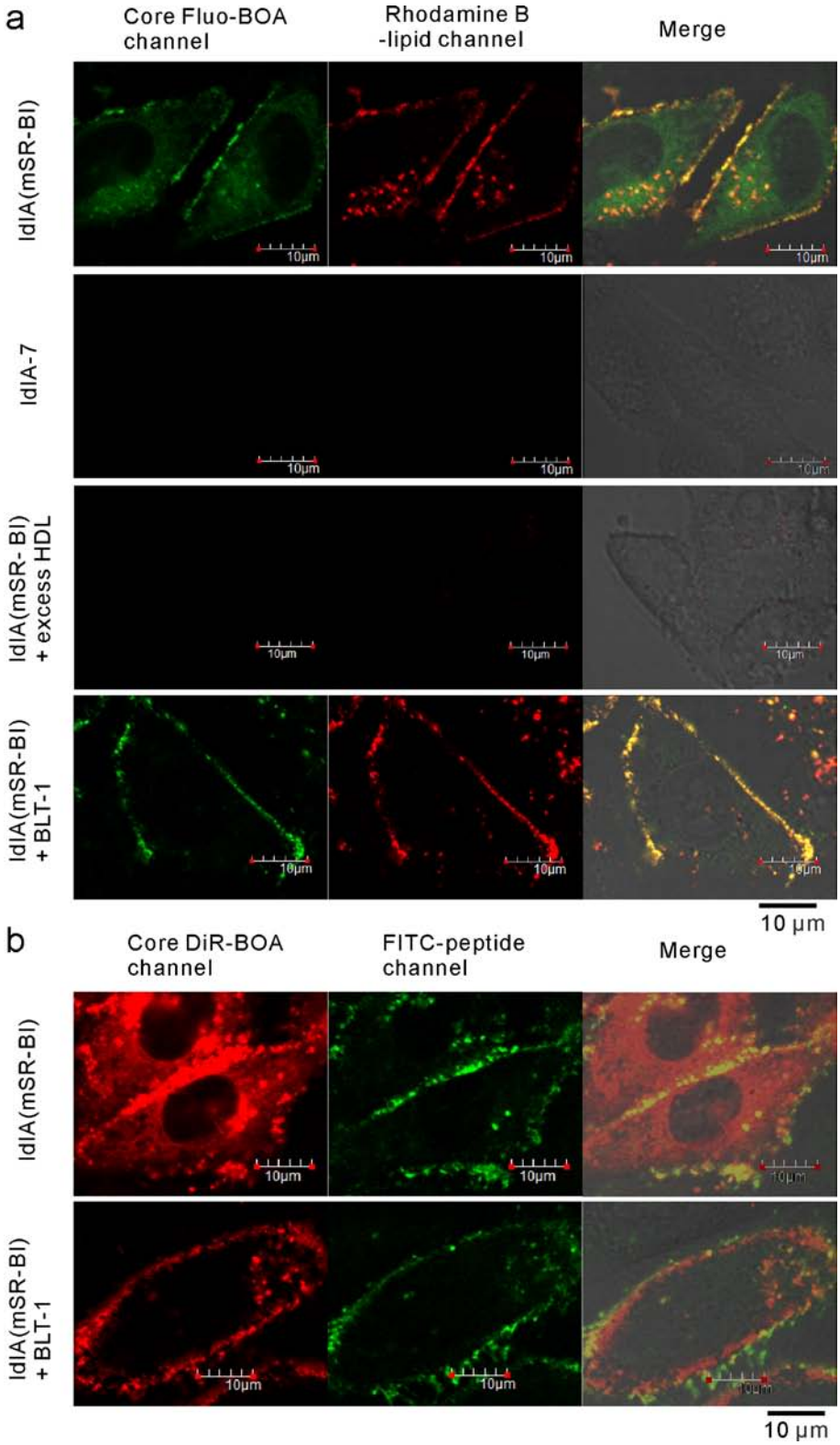

Fig. 3 The specific SR-BI mediated cytosolic delivery of hydrophobic cargos by HPPS. **a** The intracellular uptake of Rhodamine B-lipid-HPPS(Fluo-BOA) at different conditions: 1) in SR-BI positive ldlA(mSR-BI) cells (the *first row*), 2) in SR-BI negative ldlA-7 cells (the *second row*), 3) in ldlA(mSR-BI) cells with excess HDL inhibition(the *third row*), and 4) in ldlA(mSR-BI) cells with BLT-1 inhibition (the *fourth row*). **b** The intracellular uptake of FITC-peptide-HPPS(DiR-BOA) in ldlA(mSR-BI) cells (*upper row*) and with BLT-1 inhibition (*bottom row*). All of the images were acquired under the same condition after 1.5 h incubation with 0.05 μM of HPPS.

and 4°C (Fig. 4), indicating that the decrease of temperature can only decrease the rate of uptake but not inhibit the pattern of cytosolic delivery.

To further determine if the cytosolic delivery process is energy dependent, the uptake of HPPS(Fluo-BOA) under ATP depletion was studied. Transferrin-AlexaFluor633 was added into cells as a control and co-incubated with HPPS(Fluo-BOA), as transferrin recycling is known to be ATP dependent (30). Confocal microscopy was applied to monitor the uptake of transferrin and HPPS by using

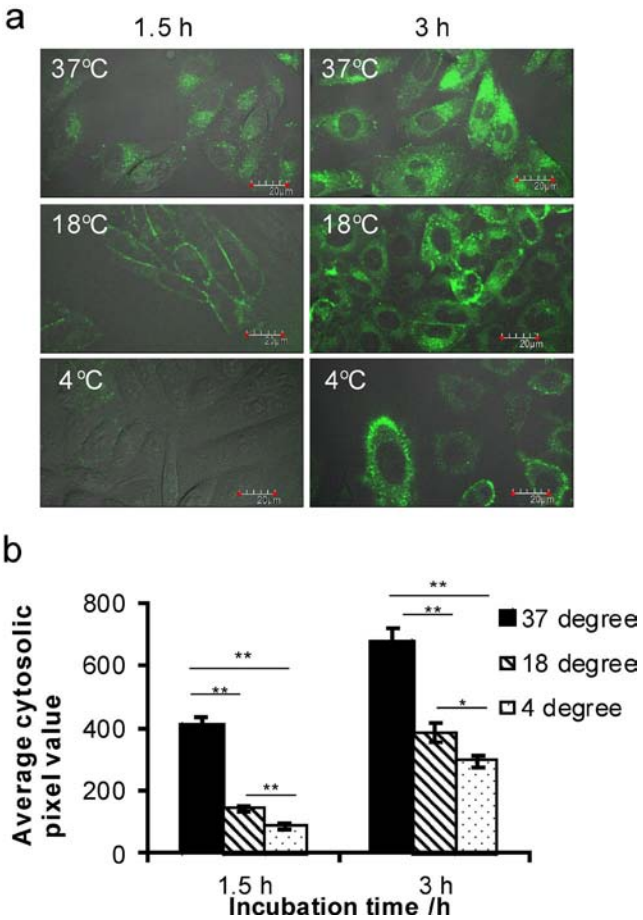

Fig. 4 Temperature influence on the cytosolic transport of Fluo-BOA by HPPS. **a** Time-dependent uptake of HPPS(Fluo-BOA) in ldlA(mSR-BI) cell after incubation with 0.05 μM of nanoparticles at 37°C, 18°C, and 4°C, respectively. All the images were taken under the same condition. **b** The corresponding signal intensity of Fluo-BOA in cell cytoplasm under the different conditions. The signal of Fluo-BOA in cytoplasm was quantified by the average fluorescence pixel value using Image J software and the error bars represent SEM ($N > 70$), and the Student's *t*-test (two tailed) was used to determine significant differences, ** *p*- values < 0.01,*p-values < 0.05.

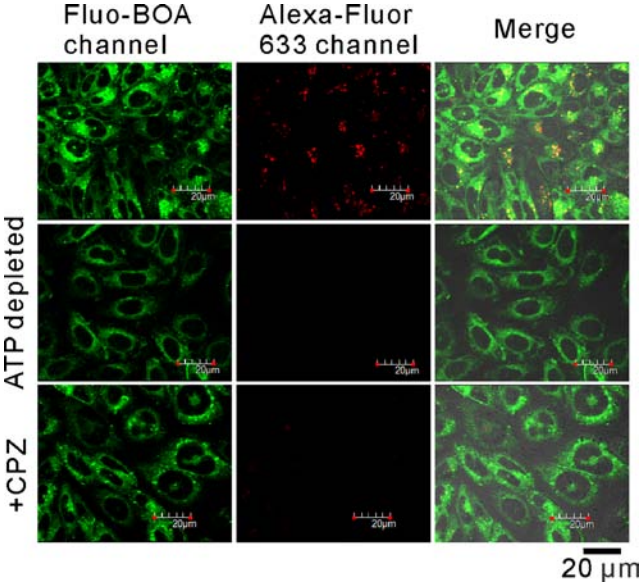

Fig. 5 Energy and chlorpromazine(CPZ) influence on the cytosolic delivery of hydrophobic cargos by HPPS. The ldlA(mSR-BI) cells were treated with ATP depleted agents or CPZ for 15 min and then incubated with HPPS(Fluo-BOA) together with Alexa-Fluor 633 transferrin for 1.5 h. All the images were taken at two fluorescence channels: Fluo-BOA signal (*green*): Ex. 488 nm, Em. 500–600 nm and Alexa-Fluor 633 signal (*red*): Ex. 633 nm and Em. 650 long pass.

different laser settings (for Alexa excited at 633 nm, emission at 650 nm long pass, for Fluo-BOA excited at 488 nm, emission between 500 and 600 nm). As shown in Fig. 5, under the ATP depleted condition, the uptake of transferrin-AlexaFluor633 was completely blocked, while the uptake of Fluo-BOA was not. These data indicate that the cytosolic delivery of hydrophobic cargos by HPPS is not ATP-dependent.

Next, to examine the role of actin in HPPS delivery, cytochalasin D (CytD), an actin-disrupted agent (13) was used to disrupt the transport pathway mediated by actin filaments. As shown in Fig. 6, the cytosolic delivery of Fluo-BOA by HPPS could not be inhibited by CytD, while the uptake of TMR-70 kDa-dextran, a control molecule, was significantly reduced under the same condition. Overall, the cytosolic delivery of Fluo-BOA by HPPS could not be inhibited by either low temperature (4°C, 18°C) (Fig. 4), ATP depletion (Fig. 5), or CytD (Fig. 6), suggesting that the cytosolic delivery of hydrophobic cargos by HPPS is neither governed by a general endocytic process nor an energy-dependent process.

Cytosolic Delivery of Hydrophobic Cargos by HPPS is not Dependent on Clathrin-Mediated Endocytosis Pathway

Clathrin-mediated endocytosis plays an important role in the cellular uptake of many macromolecules, such as LDL and transferrin (29). To determine if the transport of hydrophobic cargos by HPPS was mediated by this pathway, experiments under specific inhibition condition with chlorpromazine (CPZ) were performed. During the incubation, the transferrin uptake was significantly inhibited by CPZ while the cytosolic delivery of Fluo-BOA by HPPS was not (Fig. 5), indicating that the cytosolic delivery of hydrophobic cargos by HPPS is independent of the clathrin-mediated endocytosis pathway.

Cytosolic Delivery of Hydrophobic Cargos by HPPS is Correlated with Lipid Rafts/Caveolae Mediated Pathway

We next investigated if the cytosolic delivery of hydrophobic cargos by HPPS was mediated by lipid-raft/caveolae using

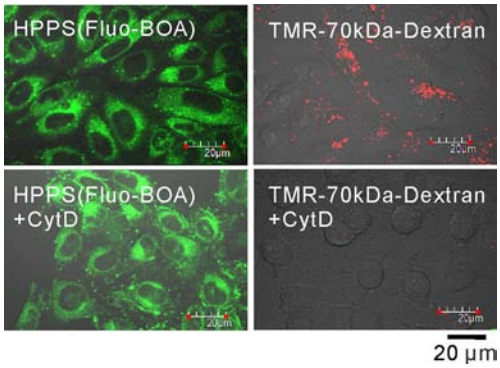

Fig. 6 Actin effect on the cytosolic delivery of hydrophobic cargos by HPPS. IdlA(mSR-BI) cells were co-incubated with HPPS(Fluo-BOA) and TMR-70 kDa-dextran (an indicator of actin-mediated transport) for 1.5 h. For inhibition study, cytochalasin D(CytD) was added to cells 15 min prior to HPPS(Fluo-BOA) and TMR-70 kDa-dextran to disrupt the actin-mediated transport pathway. All the images were taken at two fluorescence channels: Fluo-BOA signal (*green*): Ex. 488 nm, Em. 500–530 nm and TMR signal (*red*): Ex. 543 nm, Em. 560 nm long pass.

the filipin-cholesterol disruption method. Filipin is an inhibitor which can disrupt lipid rafts and caveolae structures by interacting with 3-H-hydroxysterols in the plasma membrane to form filipin–sterol complexes, a major component of glycolipid microdomains and caveolae (27,31). This process specifically inhibits the lipid rafts/caveolae-mediated pathways. Under filipin treatment, the Fluo-BOA fluorescence signal was only observed in the cell membrane, but not in the cytoplasm (Fig. 7a), indicating that the cytosolic delivery of hydrophobic cargos was efficiently inhibited by filipin. In addition, MβCD which disrupts lipid rafts by depleting cholesterol was also used for the inhibition study (27). Upon MβCD treatment, minimal Fluo-BOA fluorescence signal was observed in the cytoplasm (Fig. 7b), indicating that the cytosolic delivery of payloads was also inhibited by MβCD, which was consistent with filipin inhibition. The quantification of relative HPPS(Fluo-BOA) uptake in cytoplasm further supported this inhibition effect (Fig. 7c). Compared with the normal condition, the cytosolic delivery of Fluo-BOA by HPPS was minimally influenced by CPZ, CytD and ATP depletion with relative uptake ratios of 88.6%, 95.2% and 106.6%, respectively. However, HPPS delivery was significantly inhibited by BLT-1, filipin and MβCD treatment with relative uptake ratios of 10.9%, 11.7% and 14.6% respectively (Fig. 7c). All relative uptake ratios were normalized to the uptake determined under normal conditions. As the process of filipin-inhibition is known to be reversible (31), the cell activity after filipin treatment was next investigated to determine whether the inhibition effect was truly induced by cholesterol disruption rather than other confounding effects, such as toxicity. Briefly, after 1.5 h incubation with filipin, cells were washed three times by PBS, incubated with fresh medium for 24 h, and then imaged by confocal microscopy. As shown in Fig. 7a, after 24 h incubation with fresh medium, the Fluo-BOA fluorescence signal was observed in the cytoplasm rather than on the cell membrane, indicating that the Fluo-BOA molecules which were on the cell membrane under filipin inhibition further internalized in cytoplasm following removal of filipin. Taken together, the cytosolic delivery of hydrophobic delivery by HPPS is cholesterol

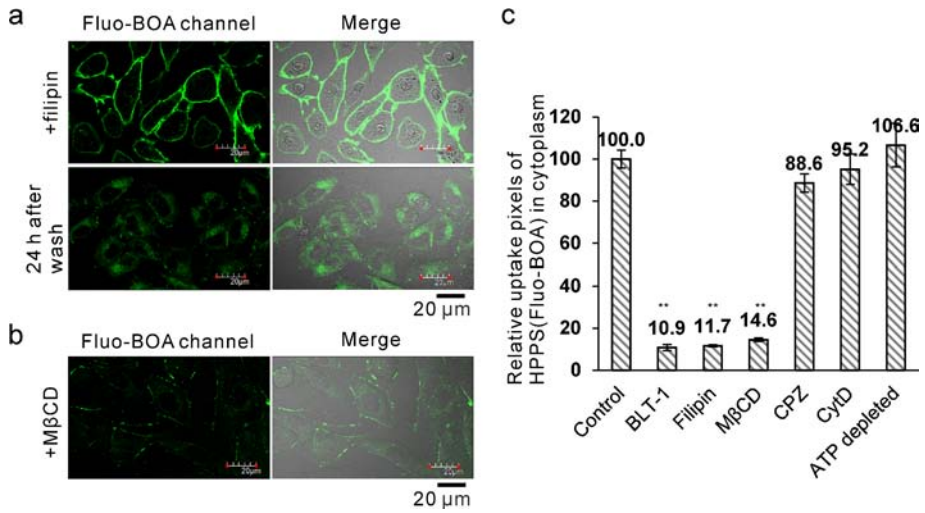

Fig. 7 **a** Filipin inhibition on cytosolic delivery of hydrophobic cargos by HPPS. Confocal imaging of IdlA(mSR-BI) cells: 1) after 1.5 h co-incubation with HPPS(Fluo-BOA) and Filipin (*upper row*) and 2) after 24 h re-growth in the fresh cell culture medium following 1.5 h co-incubation with HPPS(Fluo-BOA) and Filipin. **b** Methyl-β-cyclodextrin(MβCD) inhibition on cytosolic delivery of hydrophobic cargos by HPPS. Confocal imaging of IdlA(mSR-BI) cells after 1.5 h co-incubation with HPPS(Fluo-BOA) and MβCD. **c** The relative intracellular uptake of Fluo-BOA by HPPS delivery under different inhibition conditions. The uptake value of HPPS (Fluo-BOA) in cytoplasm was quantified by average fluorescence pixel value using Image J software. The relative intracellular uptake for each group were normalized to the control group which incubated only with HPPS(Fluo-BOA) without inhibitor. The error bar represents SEM ($N > 80$), and the Student's *t*-test (two tailed) was used to determine significant differences, ** *p*- values < 0.01.

dependent and correlate well with lipid rafts/caveolae mediated pathway.

Cytosolic Delivery of Hydrophobic Cargos by HPPS and Macropincytosis

In addition to clathrin-mediated endocytosis and lipid-raft/caveolae mediated endocytosis, macropinocytosis is another important endocytosis pathway for particle uptake. In general, if a drug is internalized by macropinocytosis, it should first enter into the endosome (29). However, the endosomal-liked (punctate spotted pattern) uptake by HPPS delivery even at a very early time points (30 min) was not observed. In addition, when cells were incubated with HPPS(Fluo-BOA) for 1.5 h, and then staining with an early endosomal marker- early endosome antigen 1(EEA1), minimal colocalization of Fluo-BOA and EEA1 was observed in cells as EEA1 signal was mostly observed in endosome while Fluo-BOA signal was uniformly distributed in the cytoplasm (Fig. S3B). The poor colocalization of Fluo-BOA and transferrin (another endosomal marker) in cells when they were simultaneously incubated (Fig. S3C) further differentiated HPPS delivery from endosome-related endocytosis. Furthermore, when cells were co-incubated with HPPS(Fluo-BOA) and a macropinocytosis marker, TMR-70kda dextran (13), the Fluo-BOA and TMR were taken up into the cells with poor colocalization (Fig. S3C). These data may suggest that macropinocytosis is not involved in the cytosolic delivery of HPPS. However, two typical macropinocytosis inhibitors, EIPA and amiloride (32), both reduced the cytosolic delivery of Fluo-BOA by HPPS. Their ability as macropinocytosis inhibitors were confirmed by their effective inhibition of cell uptake of TMR-70kDa-Dextran, a macropinocytosis marker at the same inhibition conditions (Fig. S3A).

DISCUSSION

Many physiological barriers exist in systemic delivery of molecular therapeutics for cancer treatment. Desirable drug delivery systems should be able to avoid filtration, phagocytosis and degradation in the bloodstream. It should also be capable of being transported, across the vascular endothelial barrier and diffuse through the extracellular matrix to reach its intended target cell. More importantly, once the drug vehicle reaches the cell, it should be able to effectively transport the drugs into subcellular sites where the drugs elicit their action. These sites are often found within cytosolic organelles, as such, illustrates the importance of being able to traverse the plasma membrane. In contrast to small molecules, nanoparticle delivery systems cannot easily diffuse across the cell membrane owing to their large molecular weight and their intracellular uptake is generally facilitated by endocytic processes. Some endocytic processes, like clathrin-mediated endocytosis and macropincytosis result in endo-lysosomal trafficking of particles, thus often resulting in inefficient delivery of drugs. Direct cytosolic delivery mechanism may provide a way to enhance intracellular delivery of drugs to the cytoplasm without endosome incorporation and to avoid the efflux transporters (2).

In nature, HDL plays key roles in both cholesterol influx and efflux. ABCA1 receptor together with ABCG1 receptor is involved in cholesterol efflux process, while SR-BI receptor mediates the selective transport of lipid, mainly CE from HDL into cells (33,34). SR-BI mediated transport pathway has received considerable attention for drug delivery. Many modified HDL, reconstituted HDL, as well as hybrid HDL-gold nanoparticles have been developed for delivery of theranostics agents (15–17,19,20,35). We recently developed HPPS nanoparticles capable of direct delivery of the payloads to cytoplasm *via* SR-BI targeting (14). These particles are spherical in shape, and are monodipered with a small size (10–30 nm), which enables the particles to escape from the RES and penetrate poorly permeable tumor to improve the drug accumulation in the tumor (36). Most importantly, the ability of HPPS to facilitate cytosolic delivery of drugs *via* SR-BI targeting without endosomal uptake overcomes a common intracellular delivery barrier of nanoparticle drug delivery systems. In our previous study, we employed HPPS for the targeted delivery of a chemotherapeutic drug, paclitaxel oleate (PTXOL) and achieved good therapeutic effect in the target tissue while shielded the drug toxicity in non-targets tissues (18). In addition, as HPPS mimics HDL, all components of HPPS are biocompatible, thus no toxicity were observed even after an intravenous administration of 2,000 mg/kg HPPS in mice (18). Therefore, HPPS serves as an ideal drug delivery system candidate for efficiently transporting the cargos safely to targets while reducing system drug toxicity, thus improving therapeutic window. The peptide component of HPPS, FAEKFKEAVKDYFAKFWD, plays essential role in HDL- mimicking, both structurally and functionally. The peptide assembles the particles to form an amphipathic α-helical structure to stabilize the particle and enable functional HDL-mimicking for SR-BI targeting. HPPS first recognizes and binds to SR-BI, and then may interact with SR-BI to drive cytosolic delivery of payloads which can be completely blocked by BLT-1 inhibition. Previously, the Krieger group demonstrated that the exoplasmic cysteine Cys384 on SR-BI of HDL is critical for BLT-1 inhibition and normal lipid transport (26). Therefore, we hypothesize that HPPS specifically recognizes and binds to SR-BI, and then interacts with its protein domain including the Cys384 residue. During this interaction, the particles rapidly dissociate and the hydrophobic cargos are directly internalized into the cytosol of cells while the phospholipids and peptides components are mainly retained on the cell surface (Fig. 2). This process without

entire whole nanoparticles internalization is comparable to the mechanism known to govern HDL cholesterol uptake (37).

The rate of cytosolic transport of Fluo-BOA by HPPS delivery could be slowed down but not inhibited with lower temperature treatment (4°C, 18°C) (Fig. 4). This observation is consistent with findings reported for cholesteryl ester(CE) transport by HDL (25,38), where the uptake rate of CE by HDL delivery was reduced under cool condition, and lowering temperature not only reduced the rate of lipid transfer, but also decreased the SR-BI binding (38). In HPPS delivery, the Fluo-BOA transport process was not completely inhibited by lowing temperature, suggesting that low temperature may have only influence the binding of HPPS to SR-BI, which resulted in slower uptake compared to normal conditions (37°C). In addition, the cytosolic delivery of hydrophobic cargos by HPPS *via* SR-BI has been validated as an energy independent process (Fig. 5), which was also consistent with that was previously reported for HDL (38,39): the cholesterol selective uptake by HDL did not depend on ATP.

Although HPPS shares many similar characteristics as HDL in drug delivery, there are several important differences. Significant inhibition of HPPS delivery by filipin or MβCD indicate that the cytosolic transport of hydrophobic cargos by HPPS is related to lipid-raft/caveolae mediated pathway. However, filipin and MβCD has been shown to have negligible influence on the selective uptake of CE by HDL (24,39). This difference suggests that the structure of plasma-derived HDL may not be as simple as that of HPPS assembled by a simple synthetic 18 amino acid peptide. Therefore, the cytosolic delivery mechanism may involve multiple pathways and filipin or MβCD alone could not significantly inhibit the CE uptake by HDL.

Unlike most lipid rafts/caveolae mediated pathways, which are commonly known as ATP-dependent (13), HPPS transport process was energy independent. The cytosolic delivery of cargos by HPPS was significantly inhibited by filipin or MβCD, demonstrating that the delivery was cholesterol dependent and related to lipid rafts/caveolae. Therefore, we speculate that the cytosolic delivery of payloads by HPPS occurs exclusively *via* an energy independent, lipid rafts/caveolae-like pathway.

Furthermore, the exact location of SR-BI in the cell is still debated. In 1999, Graf GA and collegues demonstrated that SR-BI was located in caveolae (40). In contrast to this, Peng and colleagues found that the SR-BI to be clustered on microvillar extensions (41). However, in 2004, Brissette *et al.* showed in HepG2 cells that SR-BI was present in lipid rafts devoid of caveolin (24). In 2008, the SR-BI was also reported in the late endosome compartment (42). Our data showing the cytosolic delivery of hydrophobic cargos by HPPS *via* SR-BI was predominately mediated by lipid rafts/caveolae-like pathway and may provide some clue to support the location of SR-BI in caveolae/lipid rafts.

In our study, although the cytosolic delivery of HPPS was reduced by two typical macropinocytosis inhibitors, either EIPA or amiloride (Fig. S3A), the HPPS delivery pattern was found to be significantly different from the endosomal-related endocytosis shown in Fig. S3B and Fig. S3C, suggesting that the macropinocytosis might not involve in this cytosolic delivery process. EIPA and amiloride inhibit macropinocytosis by blocking Na+/H+ exchange, which could result in the changes in the cell microenvironment, such as the pH value. It has been reported that amiloride could affect several other pathways at the concentrations for inhibiting Na+/H+ exchange (32,43). Therefore, we speculate that the reduction of HPPS uptake by either EIPA or amiloride was likely due to their influence on cell activity, and the cytosolic delivery of hydrophobic cargos by HPPS might not be related to macropinocytosis. Further studies to illustrate the relationship between macropinocytosis and HPPS cytosolic delivery are required.

One limitation of this study is the possibility that conjugation of various fluorescent labels on HPPS may have an influence on the lipid packing and/or the surface chemistry of the nanoparticle. This in turn may affect its interactions with the functional components involved in the uptake pathway under study. However, as shown in Fig. 1d of our previous publication (14), the uptake of HPPS labeled with FITC-PEG2000-lipid was also mediated *via* SR-BI pathway, and most of the lipids were observed on the cell membrane during the uptake, which was similar to that of the Rhodamine B-lipid labelled HPPS in this study. The DLS data further supported that no significant size difference between FITC-lipid-HPPS (20.9±3.1 nm) and Rhodamine B-lipid HPPS (20.9±2.1 nm) (Fig. S4). As only 1% of fluorophore-lipid was mixed with 99% of normal DMPC to prepare the fluorophore-lipid-HPPS, we don't believe that different fluorophore-labeled lipid would influence on the property of HPPS. In addition, the other method may also be required on the investigation of mechanism in future, such as radiolabelling other than fluorescence imaging. In this study, using fluorescence microscopy techniques we investigated the SR-BI mediated cytosolic delivery mechanism of synthetic HPPS nanoparticles. The knowledge acquired from studying this delivery mechanism could better guide the design of other nanoparticles for efficient intracellular delivery of drugs. The SR-BI mediated delivery pathway not only allow for direct cytosolic delivery of core loaded cargo but also enable the delivery of surface loaded cholesterol modified siRNA to cytosol *via* SR-BI (21).

CONCLUSION

In summary, the cytosolic delivery of hydrophobic cargos by HPPS follows the SR-BI process without entire nanoparticle

internalization through a hypothesized two-step process (Fig. 1). Firstly, HPPS binds to SR-BI, which could be blocked by excess HDL competition. Secondly, HPPS likely interacts with SR-BI at Cys 384 domain, which could be efficiently inhibited by BLT-1. HPPS was found to be intact during the binding and become dissociated after the interaction. Finally, hydrophobic cargos are transported into cell cytoplasm predominately by a lipid-raft/caveolae-like pathway, which could be inhibited by either filipin or MβCD but is energy-independent. The non uptaken components of HPPS, such as lipid and peptide, remain cell membrane-bound. This exclusive cytosolic delivery mechanism might provide a useful strategy for efficient delivery of drugs to improve their therapeutic effect.

ACKNOWLEDGMENTS AND DISCLOSURES

This study was conducted with the support of the China-Canada Joint Health Research Initiative (NSFC-30911120489, CIHR CCI-102936), DLVR Therapeutics, Canadian Institutes of Health Research, Ontario Institute for Cancer Research, Natural Sciences and Engineering Research Council of Canada, Canada Foundation for Innovation, National Natural Science Foundation of China (Grant No. 81172153), 111 Project of China (B07038), and the Joey and Toby Tanenbaum/Brazilian Ball Chair in Prostate Cancer Research.

REFERENCES

1. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature. 1998;391(6669):806–11.
2. Vasir JK, Labhasetwar V. Biodegradable nanoparticles for cytosolic delivery of therapeutics. Adv Drug Deliv Rev. 2007;59(8):718–28.
3. Mathew E, Hardee GE, Bennett CF, Lee KD. Cytosolic delivery of antisense oligonucleotides by listeriolysin O-containing liposomes. Gene Ther. 2003;10(13):1105–15.
4. Lee SH, Choi SH, Kim SH, Park TG. Thermally sensitive cationic polymer nanocapsules for specific cytosolic delivery and efficient gene silencing of siRNA: swelling induced physical disruption of endosome by cold shock. J Control Release. 2008;125(1):25–32.
5. Yezhelyev MV, Qi L, O'Regan RM, Nie S, Gao X. Proton-sponge coated quantum dots for siRNA delivery and intracellular imaging. J Am Chem Soc. 2008;130(28):9006–12.
6. Raoof M, Corr SJ, Kaluarachchi WD, Massey KL, Briggs K, Zhu C, *et al.* Stability of antibody-conjugated gold nanoparticles in the endolysosomal nanoenvironment: implications for noninvasive radiofrequency-based cancer therapy. Nanomedicine. 2012;8(7):1096–105.
7. Berg K, Selbo PK, Prasmickaite L, Tjelle TE, Sandvig K, Moan J, *et al.* Photochemical internalization: a novel technology for delivery of macromolecules into cytosol. Cancer Res. 1999;59(6):1180–3.
8. Hu Y, Litwin T, Nagaraja AR, Kwong B, Katz J, Watson N, *et al.* Cytosolic delivery of membrane-impermeable molecules in dendritic cells using pH-responsive core-shell nanoparticles. Nano Lett. 2007;7(10):3056–64.
9. Petros RA, DeSimone JM. Strategies in the design of nanoparticles for therapeutic applications. Nat Rev Drug Discov. 2010;9(8):615–27.
10. Madani F, Lindberg S, Langel U, Futaki S, Graslund A. Mechanisms of cellular uptake of cell-penetrating peptides. J Biophys. 2011;2011:414729.
11. Richard JP, Melikov K, Vives E, Ramos C, Verbeure B, Gait MJ, *et al.* Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. J Biol Chem. 2003;278(1):585–90.
12. Kunisawa J, Masuda T, Katayama K, Yoshikawa T, Tsutsumi Y, Akashi M, *et al.* Fusogenic liposome delivers encapsulated nanoparticles for cytosolic controlled gene release. J Control Release. 2005;105(3):344–53.
13. Partlow KC, Lanza GM, Wickline SA. Exploiting lipid raft transport with membrane targeted nanoparticles: a strategy for cytosolic drug delivery. Biomaterials. 2008;29(23):3367–75.
14. Zhang Z, Cao W, Jin H, Lovell JF, Yang M, Ding L, *et al.* Biomimetic nanocarrier for direct cytosolic drug delivery. Angew Chem Int Ed Engl. 2009;48(48):9171–5.
15. Frias JC, Williams KJ, Fisher EA, Fayad ZA. Recombinant HDL-like nanoparticles: a specific contrast agent for MRI of atherosclerotic plaques. J Am Chem Soc. 2004;126(50):16316–7.
16. Cormode DP, Skajaa T, van Schooneveld MM, Koole R, Jarzyna P, Lobatto ME, *et al.* Nanocrystal core high-density lipoproteins: a multimodality contrast agent platform. Nano Lett. 2008;8(11):3715–23.
17. Lacko AG, Nair M, Paranjape S, Johnso S, McConathy WJ. High density lipoprotein complexes as delivery vehicles for anticancer drugs. Anticancer Res. 2002;22(4):2045–9.
18. Yang M, Chen J, Cao W, Ding L, Ng KK, Jin H, *et al.* Attenuation of nontargeted cell-kill using a high-density lipoprotein-mimicking peptide–phospholipid nanoscaffold. Nanomedicine (Lond). 2011;6(4):631–41.
19. Shahzad MM, Mangala LS, Han HD, Lu C, Bottsford-Miller J, Nishimura M, *et al.* Targeted delivery of small interfering RNA using reconstituted high-density lipoprotein nanoparticles. Neoplasia. 2011;13(4):309–19.
20. McMahon KM, Mutharasan RK, Tripathy S, Veliceasa D, Bobeica M, Shumaker DK, *et al.* Biomimetic high density lipoprotein nanoparticles for nucleic acid delivery. Nano Lett. 2011;11(3):1208–14.
21. Lin Q, Chen J, Jin H, Ng KK, Yang M, Cao W, *et al.* Efficient systemic delivery of siRNA by using high-density lipoprotein-mimicking peptide lipid nanoparticles. Nanomedicine (Lond). 2012 Jul 26.
22. Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, Krieger M. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science. 1996;271(5248):518–20.
23. Kratzer I, Wernig K, Panzenboeck U, Bernhart E, Reicher H, Wronski R, *et al.* Apolipoprotein A-I coating of protamine-oligonucleotide nanoparticles increases particle uptake and transcytosis in an *in vitro* model of the blood–brain barrier. J Control Release. 2007;117(3):301–11.
24. Rhainds D, Bourgeois P, Bourret G, Huard K, Falstrault L, Brissette L. Localization and regulation of SR-BI in membrane rafts of HepG2 cells. J Cell Sci. 2004;117(Pt 15):3095–105.
25. Rodrigueza WV, Thuahnai ST, Temel RE, Lund-Katz S, Phillips MC, Williams DL. Mechanism of scavenger receptor class B type I-mediated selective uptake of cholesteryl esters from high density lipoprotein to adrenal cells. J Biol Chem. 1999;274(29):20344–50.
26. Yu M, Romer KA, Nieland TJ, Xu S, Saenz-Vash V, Penman M, *et al.* Exoplasmic cysteine Cys384 of the HDL receptor SR-BI is critical for its sensitivity to a small-molecule inhibitor and normal lipid transport activity. Proc Natl Acad Sci U S A. 2011;108(30):12243–8.

27. Simons K, Toomre D. Lipid rafts and signal transduction. Nat Rev Mol Cell Biol. 2000;1(1):31–9.
28. Nieland TJ, Penman M, Dori L, Krieger M, Kirchhausen T. Discovery of chemical inhibitors of the selective transfer of lipids mediated by the HDL receptor SR-BI. Proc Natl Acad Sci U S A. 2002;99(24):15422–7.
29. Conner SD, Schmid SL. Regulated portals of entry into the cell. Nature. 2003;422(6927):37–44.
30. Podbilewicz B, Mellman I. ATP and cytosol requirements for transferrin recycling in intact and disrupted MDCK cells. EMBO J. 1990;9(11):3477–87.
31. Schnitzer JE, Oh P, Pinney E, Allard J. Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. J Cell Biol. 1994;127(5):1217–32.
32. Masereel B, Pochet L, Laeckmann D. An overview of inhibitors of Na(+)/H(+) exchanger. Eur J Med Chem. 2003;38(6):547–54.
33. Krieger M. Charting the fate of the "good cholesterol": identification and characterization of the high-density lipoprotein receptor SR-BI. Annu Rev Biochem. 1999;68:523–58.
34. Wang N, Lan D, Chen W, Matsuura F, Tall AR. ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. Proc Natl Acad Sci U S A. 2004;101(26):9774–9.
35. Yang S, Damiano MG, Zhang H, Tripathy S, Luthi AJ, Rink JS, *et al.* Biomimetic, synthetic HDL nanostructures for lymphoma. Proc Natl Acad Sci U S A. 2013;110(7):2511–6.
36. Cabral H, Matsumoto Y, Mizuno K, Chen Q, Murakami M, Kimura M, *et al.* Accumulation of sub-100 nm polymeric micelles in poorly permeable tumours depends on size. Nat Nanotechnol. 2011;6(12):815–23.
37. Nieland TJ, Ehrlich M, Krieger M, Kirchhausen T. Endocytosis is not required for the selective lipid uptake mediated by murine SR-BI. Biochim Biophys Acta. 2005;1734(1):44–51.
38. Pagler TA, Rhode S, Neuhofer A, Laggner H, Strobl W, Hinterndorfer C, *et al.* SR-BI-mediated high density lipoprotein (HDL) endocytosis leads to HDL resecretion facilitating cholesterol efflux. J Biol Chem. 2006;281(16):11193–204.
39. Wustner D, Mondal M, Huang A, Maxfield FR. Different transport routes for high density lipoprotein and its associated free sterol in polarized hepatic cells. J Lipid Res. 2004;45(3):427–37.
40. Graf GA, Connell PM, van der Westhuyzen DR, Smart EJ. The class B, type I scavenger receptor promotes the selective uptake of high density lipoprotein cholesterol ethers into caveolae. J Biol Chem. 1999;274(17):12043–8.
41. Peng Y, Akmentin W, Connelly MA, Lund-Katz S, Phillips MC, Williams DL. Scavenger receptor BI (SR-BI) clustered on microvillar extensions suggests that this plasma membrane domain is a way station for cholesterol trafficking between cells and high-density lipoprotein. Mol Biol Cell. 2004;15(1):384–96.
42. Ahras M, Naing T, McPherson R. Scavenger receptor class B type I localizes to a late endosomal compartment. J Lipid Res. 2008;49(7):1569–76.
43. Koivusalo M, Welch C, Hayashi H, Scott CC, Kim M, Alexander T, *et al.* Amiloride inhibits macropinocytosis by lowering submembranous pH and preventing Rac1 and Cdc42 signaling. J Cell Biol. 2010;188(4):547–63.