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Small molecule ligands for enhanced intracellular delivery of lipid nanoparticle formulations of siRNA

Yuen Yi C. Tam, PhD[a,*], Sam Chen, BSc[a], Josh Zaifman, PhD[b], Ying K. Tam, PhD[c], Paulo J.C. Lin, PhD[a], Steven Ansell, PhD[c], Michel Roberge, PhD[a], Marco A. Ciufolini, PhD[b], Pieter R. Cullis, PhD[a]

[a]*Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia, Canada*
[b]*Department of Chemistry, University of British Columbia, Vancouver, British Columbia, Canada*
[c]*AlCana Technologies, Vancouver, British Columbia, Canada*

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Abstract

Gene silencing activity of lipid nanoparticle (LNP) formulations of siRNA requires LNP surface factors promoting cellular uptake. This study aimed to identify small molecules that enhance cellular uptake of LNP siRNA systems, then use them as LNP-associated ligands to improve gene silencing potency. Screening the Canadian Chemical Biology Network molecules for effects on LNP uptake into HeLa cells found that cardiac glycosides like ouabain and strophanthidin caused the highest uptake. Cardiac glycosides stimulate endocytosis on binding to plasma membrane Na^+/K^+ ATPase found in all mammalian cells, offering the potential to stimulate LNP uptake into various cell types. A PEG-lipid containing strophanthidin at the end of PEG (STR-PEG-lipid) was synthesized and incorporated into LNP. Compared to non-liganded systems, STR-PEG-lipid enhanced LNP uptake in various cell types. Furthermore, this enhanced uptake improved marker gene silencing *in vitro*. Addition of STR-PEG-lipid to LNP siRNA may have general utility for enhancing gene silencing potency.

Key words: Lipid nanoparticle; Small molecule ligand; Cardiac glycosides; High-throughput screening; Gene silencing; siRNA; Targeting; Targeted delivery

Background

The use of small interfering RNA (siRNA) to silence disease-associated genes has considerable therapeutic promise.[1–4] However, realizing the potential of siRNA therapeutics requires sophisticated systems to deliver siRNA into target cells *in vivo*. This is because "naked" siRNA molecules are rapidly broken down in biological fluids, do not accumulate in target tissues and cannot penetrate target cell membranes to reach intracellular sites of activity even if they get there. Lipid nanoparticle (LNP) formulations of siRNA (LNP siRNA) have demonstrated significant potential for overcoming these problems for delivery of siRNA to hepatocytes following intravenous (i.v.) injection.[5–8] Recent improvements in the cationic lipid component of LNP siRNA systems have resulted in effective gene silencing in hepatocytes at dose levels as low as 0.02 μg siRNA/kg body weight in mouse models.[8]

The potency of LNP siRNA systems for gene silencing of hepatocyte genes is dependent, at least in part, on association with apolipoprotein E (ApoE) following i.v. administration.[9,10] The presence of the ApoE on the LNP facilitates uptake into hepatocytes via the LDL and scavenger receptors on hepatocytes and little gene silencing activity is observed in ApoE knock-out mice. The requirement for Apo E emphasizes the vital role of factors on the LNP surface that bind to and trigger uptake into target cells and indicates that unless an LNP is able to bind a serum protein that facilitates uptake into the cell of interest, or has ligands on the LNP surface that promote uptake, little gene silencing activity can be expected. This results in an interesting opportunity. Because a given siRNA is so specific for silencing a particular gene once it arrives in the cell cytoplasm, more generalized uptake into non-target tissue, which may not express that gene, could be of little consequence. As a result, LNP-associated ligands that encourage promiscuous uptake into a

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*Corresponding author:
E-mail address: tam7@mail.ubc.ca (Y.Y.C. Tam).

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wide variety of cells could well be of considerable utility in target validation and possibly therapeutic applications of siRNA.

Targeting ligands such as antibodies, antibody fragments and peptides against specific cell surface receptors have been used to target liposomes to specific cells.[11–14] However, there are many issues associated with use of such ligands, including induction of immune responses to the targeting ligand, cost, as well as formulation and characterization issues, among others. Small molecule targeting ligands conjugated to lipid anchors in LNP offer important potential advantages, notably reduced immunogenicity as well as much improved ease of ligand manufacture and formulation into LNP. For example, anisamide, which possesses high affinity for sigma receptors can be coupled to a PEG-lipid which is easily formulated into LNP and has been shown to increase delivery of LNP to prostate and lung cancer cells that over-express sigma receptors.[15–18]

In this work we conducted a screen of 800 molecules from the Canadian Chemical Biology Network library to identify small molecules that can enhance cellular uptake of LNP in a potentially general manner. This resulted in the identification of cardiac glycosides as a class of compounds that enhance LNP uptake into human cervical cancer HeLa cells by binding to an extracellular region of the Na^+/K^+ ATPase in the cell plasma membrane. Because Na^+/K^+ ATPase is expressed in all mammalian cells, it offers a potentially general target to stimulate uptake into a wide variety of cells. We synthesized a poly(ethylene)glycol (PEG) lipid containing a cardiac glycoside (strophanthidin, STR) at the distal end of the PEG moiety and show that LNP siRNA systems containing the STR-PEG-lipid exhibit enhanced uptake in a variety of cell types derived from different tissues and improved gene silencing properties *in vitro*.

Materials and methods

Materials

1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids (Alabaster, AL), whereas cholesterol was obtained from Sigma (St Louis, MO). 1,2-dilinoleyloxy-keto-N,N-dimethyl-3-aminopropane (DLinK-DMA), 2,2-Dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLinK-C2-DMA)and polyethylene glycol-dimyristol glycerol (PEG-s-DMG) were provided by Tekmira Pharmaceuticals Corporation (Burnaby, BC). The fluorescently-labeled lipid 3,3′-dioctadecyl-5,5′-di(4-sulfophenyl)-oxacarbocyanine, sodium salt (SPDiO) was purchased from Invitrogen (Burlington, ON). The small molecule library used in this study was obtained from the Canadian Chemical Biology Network. Ouabain and strophanthidin were purchased from Sigma.

Cell culture

All cells were incubated at 37 °C with 5% CO_2 unless indicated otherwise. The human cervix carcinoma cells (HeLa), carcinomic alveolar basal epithelial cells (A549), hepatocellular carcinoma cells (Hep3B), exocrine pancreatic carcinoma cells (PANC1), prostate adenocarcinoma cells (PC3) and mouse pancreatic beta cells (MIN6) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 0.1 mM non-essential amino acids (NEAA). The human ovarian clear cell carcinoma cells (TOV21G, JHOC-5 and JHOC-9), androgen-sensitive prostate adenocarcinoma (LNCaP), breast ductal carcinoma (MCF7), and breast adenocarcinoma (MDA-MB-231) were cultured in RPMI 1640 Medium supplemented with 10% FBS, 2 mM L-glutamine and 0.1 mM NEAA. All cell culture reagents were obtained from Invitrogen.

Cell lines stably expressing shRNA targeted to ATP1A1 (shATP1A1) or shRNA directed to a negative control sequence (shScramble) were constructed by transfection of shATP1A1 plasmid or shScramble plasmid (SABiosciences, MD) according to the manufacturer's instructions.

Preparation of siRNA-LNP

siRNA-Cy3 targeting mouse factor VII mRNA was obtained from Alnylam Pharmaceuticals (Cambridge, MA). siRNA (5′-TGGCCAAGGTCATCCATGA-3′) directed to glyceraldehyde 3-phosphate dehydrogenase (siGAPDH) was purchased from Dharmacon (Thermo Scientific, Pittsburg, PA). siRNA with a random sequence of low GC content (siScramble) was purchased from Invitrogen. siRNA was encapsulated in LNP using the preformed vesicle (PFV) procedure as previously described.[8] Briefly, lipids were mixed together in 30% ethanol and the mixture was slowly added to 50 mM citrate or acetate buffer, pH 4.0 under rapid vortexing followed by extrusion through two stacks of 80 nm polycarbonate filters at ~300 psi. The siRNA solution was then slowly added to the liposome dispersion equivalent of ten times siRNA amount under vortexing. The mixture was subsequently incubated at 31 °C for 30 min and dialyzed twice in 1× PBS for 18 h to remove ethanol. siRNA-Cy3 was encapsulated in LNP consisting of DLinK-DMA/DSPC/cholesterol/PEG-s-DMG/SPDiO at a molar ratio of 40/10/39.8/10/0.2 whereas siGAPDH and siScramble were encapsulated at a molar ratio of 40/18.8/40/1/0.2 for *in vitro* experiments using ouabain. The LNP composition used for subsequent *in vitro* uptake and knockdown experiments was DLinK-C2-DMA/DSPC/Cholesterol/PEG-s-DMG/STR-PEG or DSPE-PEG/SPDiO at a molar ratio of 40/14.8/40/4/1/0.2 and 40/17.3/40/1.5/1/0.2, respectively. All LNPs were ~80 nm in diameter measured by dynamic light scattering with intensity mode (Nicomp, Port Richey, FL). LNPs containing DSPE-PEG and STR-PEG have zeta-potential of 1.91 and 0.08 mV at pH 7.4, respectively as measured by a Zetasizer NanoZS (Malvern, Worcestershire, UK).

Small molecules and LNP treatment on 96-well plate

Cells were seeded at 8000 cells/well in 96-well ViewPlates (PerkinElmer, Shelton, CT) in 100 μl of medium and were allowed to grow overnight. Fresh medium containing LNP was added the next day. Small molecules were either added manually or pinned from 1000-fold stocks in DMSO using a pinning robot equipped with 0.4 mm pins (BioRobotics, Cambridge, UK). Cells were incubated for 24 h. Cells were then washed once in PBSCM (1× PBS containing 1 μM $MgCl_2$ and 0.1 μM $CaCl_2$), fixed in 3% paraformaldehyde containing 500 ng/ml Hoechst

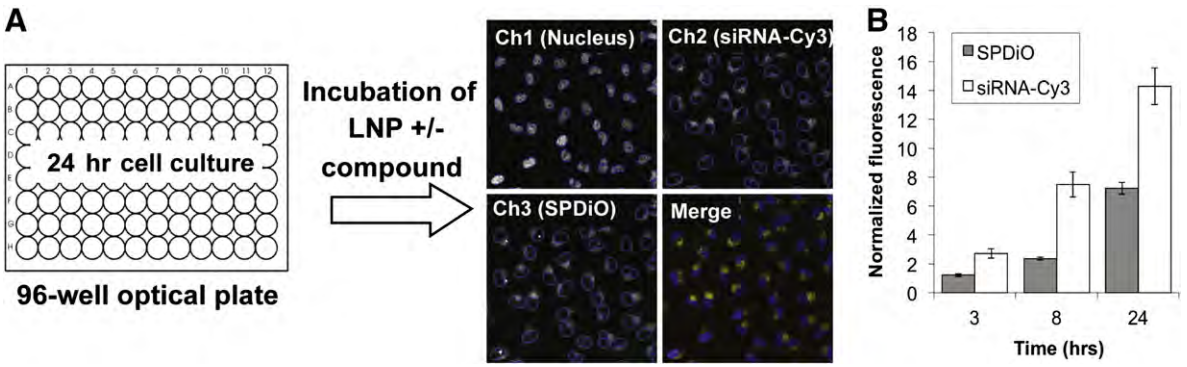


Figure 1. **(A)** Quantification of LNP uptake using a 96-well format. Cells were grown in 96-well optical plate for 24 h. Chemical compounds and LNP were added and incubated at 37 °C. Automated fluorescence microscopy was performed using a Cellomics Arrayscan. Representative images of HeLa cells are shown. Individual object segmentation based on the nuclear stain (Hoechst's stain), mask encompassing the cytoplasm and quantification of SPDiO and siRNA-Cy3 uptake were performed using the Cellomics Compartmental Analysis algorithms. **(B)** The Cellomics Arrayscan can be used to monitor progressive uptake of LNP over time. HeLa cells were grown in 96-well optical plates for 24 h before LNP treatment (5 μg/ml of siRNA-Cy3) for 3, 8 and 24 h. SPDiO and siRNA-Cy3 uptake was quantified using the Cellomics Compartmental Analysis algorithms. All values are means ± SD of 4 experiments.

33342 (Invitrogen) for 15 min, washed once in PBSCM and stored in 100 μl of PBSCM.

Imaging and image analysis

Plates were imaged using a Cellomics Arrayscan VTI HCS Reader (Thermo Scientific, Pittsburg, PA). Images were acquired using a 20× PlanFluor objective and an XF93 filter set. Object identification and image analysis were performed using the Cellomics Compartmental Analysis algorithm. Cellular SPDiO and siRNA-Cy3 fluorescence intensities were measured for a minimum of 400 cells and the average pixel intensity was examined. For confocal microscopy, cells grown on glass coverslips were washed once in 1× PBS, fixed in 3% paraformaldehyde containing 500 ng/ml Hoechst 33342 for 15 min, washed again and mounted on slides. Images were captured on an Olympus FV1000 (Olympus, Center Valley, PA) laser scanning microscope and cellular SPDiO fluorescence intensity was analyzed using ImageJ (NIH, http://rsb.info.nih.gov/ij/).

Immunoblotting

HeLa cells were plated in twelve-well plates for indicated times. They were then washed in PBS and extracted in RIPA buffer (1% NP-40 and 0.5% Deoxycholic in 1× PBS) supplemented with protease inhibitors (Roche Applied Science, Laval, PQ). Total protein quantified by the Bradford Assay was analyzed by immunoblotting using antibodies to GAPDH, β-actin (Abcam, Cambridge, MA) or ATP1A1 (Millipore, Billerica, MA). Antigen–antibody complexes in immunoblots were detected using Millipore Immobilon Western Chemiluminescent HRP Substrate (Millipore). Band intensities were quantified using ImageJ.

Synthesis of STR-PEG-lipid

Strophanthidin was obtained from MP Biomedicals, DSPE-PEG-NH_2 from Avanti Polar Lipids, and 2,4,6-trichlorobenzoyl chloride from TCI America (Portland, OR). Reagent grade triethylamine (Et_3N) was stored over potassium hydroxide pellets. All other reagents were obtained from Sigma or Fisher Scientific (Ottawa, ON) and used as received. Dry solvents were distilled under an atmosphere of nitrogen from standard drying agents: tetrahydrofuran (THF) from sodium benzophenone ketyl; dichloromethane (CH_2Cl_2) and pyridine from calcium hydride. The scheme for synthesizing STR-PEG-lipid is outlined in Figure 4. The complete synthesis method is given in Supplemental Materials.

Inhibition assay of Na^+/K^+ ATPase activity

Inhibition potencies of cardiac glycosides and lipids were determined by assaying the ATPase activity of the purified Na^+/K^+ ATPase from porcine cerebral cortex (Sigma) at 14 different inhibitor concentrations according to the manufacturer's instructions. Relative ATPase activities as a function of inhibitor concentration were fitted to a three-parameter logistic equation and inhibitor concentrations for half-maximal inhibition (IC_{50}) were calculated using GraphPad Prism (La Jolla, CA).

Results

LNP uptake into cells can be assayed in a high-throughput manner using the Cellomics Arrayscan apparatus

As a first step in this study, a quantitative uptake assay to measure levels of LNP accumulated in cells was developed using fluorescent probes for nuclei (Ch1), siRNA (Ch2) and lipid (Ch3), and the automated fluorescence microscope Cellomics Arrayscan (Figure 1, *A*).The LNP formulation used in this study (DLinK-DMA/DSPC/cholesterol/PEG-s-DMG/SPDiO at a molar ratio of 40/10/39.8/10/0.2) is a potent siRNA delivery system for silencing genes in hepatocytes *in vivo*.[8] HeLa cells were incubated overnight in 96-well optical plates. Fluorescently-labeled LNPs were added to cells the next day and incubated for 3 h, 8 h and 24 h (Figure 1, *B*). Cells were then fixed and washed before scanning. Hoechst's stain, which stains the cell

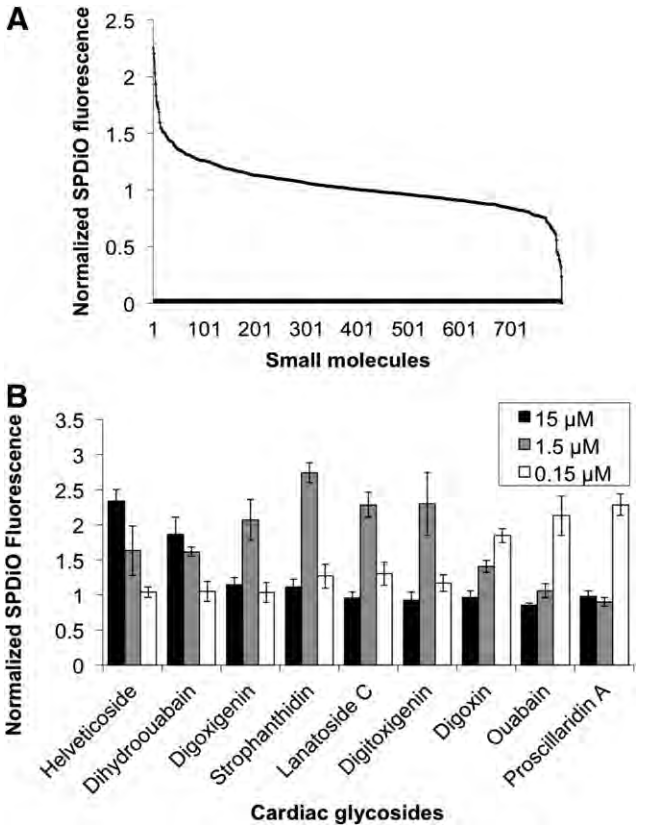


Figure 2. **(A)** Normalized uptake of fluorescently labelled LNP systems into HeLa cells following incubation with small molecules that are known drugs. HeLa cells were incubated with 800 small molecule drugs and 5 μg/ml of siRNA-Cy3-LNPfor 24 h as described under Methods. The fluorescence resulting from the accumulated SPDiO-labeled LNP following incubation with each individual compound was normalized to the LNP SPDiO fluorescence in cells untreated with any compound. The small molecules are sorted so that those giving rise to the highest LNP accumulation are on the left and the lowest on the right. **(B)** Effects of different cardiac glycosides on LNP uptake. HeLa cells were incubated with 50 μg/ml (lipid concentration) of empty LNP and each of 9 cardiac glycosides at 0.15 μM, 1.5 μM and 15 μM for 24 h. Cardiac glycosides on the x-axis are arranged by their affinity for the Na^+/K^+ ATPase, from the weakest (helveticoside) to the strongest (Proscillaridin A).[19] Cellular LNP SPDiO fluorescence in the presence of individual compounds was normalized to the LNP SPDiO fluorescence in cells untreated with any cardiac glycoside. All values are means±SD of 4 experiments.

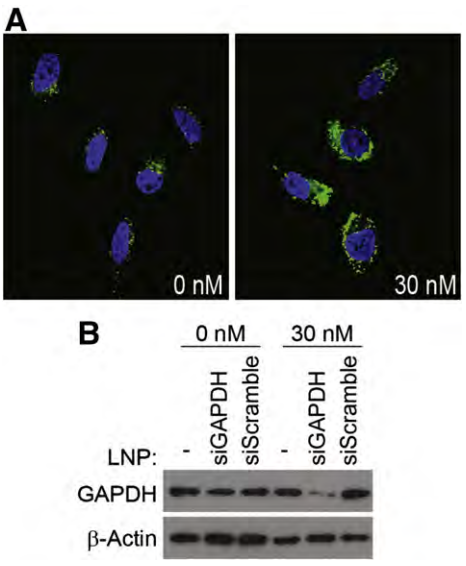


Figure 3. **(A)** Ouabain induces LNP uptake into HeLa cells. Confocal micrographs of HeLa cells treated with 10 μg/ml of siGAPDH-LNP and 0 nM or 30 nM of ouabain for 24 h. Cell nuclei were stained with Hoechst's dye in blue. SPDiO fluorescence is shown in green. **(B)** LNP siRNA-induced GAPDH knockdown is increased in the presence of 30 nM ouabain.Cells were treated with or without 10 μg/ml of siGAPDH-LNP or siScramble-LNP in the presence of 0 nM or 30 nM of ouabain for 24 h. LNP and ouabain were removed and cells were further incubated in fresh medium for 48 h. Equal portions of protein samples were analyzed by immunoblotting to GAPDH and β-actin which served as a loading control.

nuclei, was used to form the nuclear mask (blue line in Ch1, Figure 1, *A*) to identify valid objects or cells.

Cellular siRNA was monitored by the Cy3 fluorophore which was conjugated to the 3′ end of the siRNA sense strand (Ch2). The distribution of LNP lipid was reported by the fluorescent lipid, SPDiO (Ch3). A cellular mask (green line in Ch2 or Ch3, Figure 1, *A*), which was slightly larger than the nuclear mask but stayed within the cell boundary, was used to delineate the area from which Ch2 or Ch3 cytological features were measured. At least 400 cells were scored per well, and the average fluorescence intensity per pixel in each channel was measured for each cell region. Fluorescence values were normalized to untreated cells (Figure 1, *B*). A progressive time-dependent increase in intracellular SPDiO or siRNA-Cy3 fluorescence was observed following incubation of the cells with the fluorescently labeled LNP siRNA systems, indicating that LNP siRNA was taken up by cells in increasing amounts over time. This was taken as baseline behavior and the effect of added small molecules was assessed relative to this baseline.

Cardiac glycosides enhance uptake of LNP into HeLa cells

The influence of 800 compounds from the Canadian Chemical Biology Network collection of small molecules on LNP uptake into HeLa cells was assessed. Compounds were introduced into the 96-well optical plates containing cells and LNP using a pinning robot. Approximately 7.5 μM to 10 μM of each compound was transferred by each pin into each well. Approximately half of the molecules led to enhanced LNP uptake into HeLa cells as evidenced by increased cellular SPDiO fluorescence levels compared to control whereas the remainder decreased LNP uptake (Figure 2, *A*). Interestingly, among the seven molecules that led to the most uptake, three belonged to the cardiac glycoside family.

Cardiac glycosides are a diverse family of molecules that have been used to treat heart failure for many years.[20–22] They bind to the Na^+/K^+ ATPase on the plasma membrane thereby leading to an increase in intracellular Ca^{2+} concentrations and enhanced contractility for cardiac tissue. The binding site is on the extracellular side of the α-subunit of the enzyme. Binding of cardiac glycosides to the ATPase inhibits the enzyme and can also act as a signal transducer.[23–25] As important, for the purposes of this study, is the observation that binding of cardiac glycosides

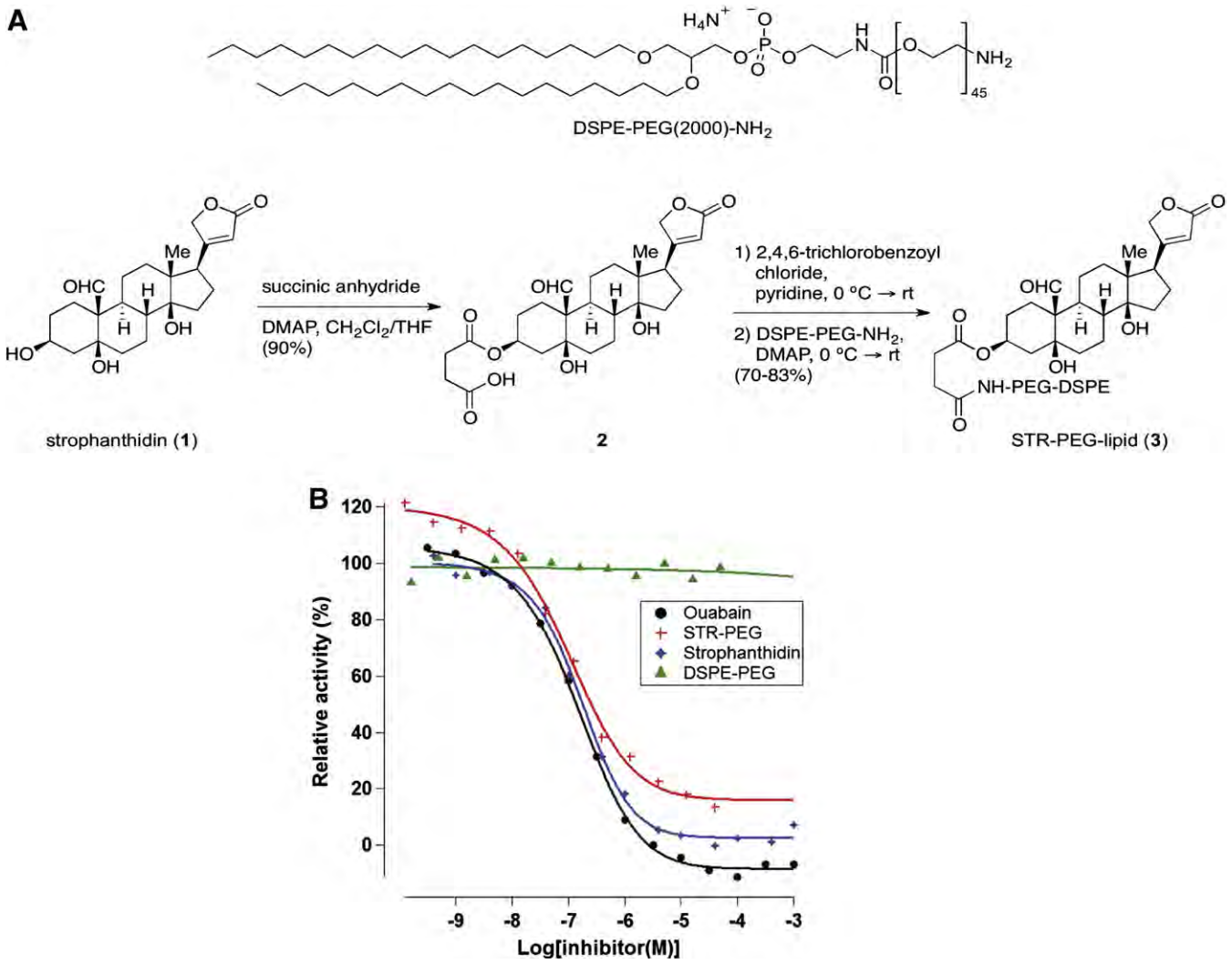


Figure 4. **(A)** Synthesis of STR-PEG-lipid.A handle for the conjugation of strophanthidin[1] to a PEG-functionalized phospholipid (DSPE-PEG-NH_2) was installed treating cardenolide **1** with succinic anhydride in the presence of 4-dimethylaminopyridine (DMAP) at room temperature to furnish carboxylic acid **2** in high yield. Exposure of succinate **2** to Yamaguchi's reagent in pyridine produced a mixed anhydride, which was directly treated with DSPE-PEG-NH_2 and DMAP, furnishing lipid conjugate **3** (STR-PEG-lipid) after chromatography on silica gel. **(B)** Na^+/K^+ ATPase activity is inhibited by STR-PEG-lipid. The ATPase inhibition assay (see Methods) was performed in the presence of ouabain, strophanthidin, STR-PEG-lipid and DSPE-PEG-lipid. Data represent the average of two independent experiments.

such as ouabain also induces endocytosis of the Na^+/K^+ ATPase via a caveolin- and clathrin-dependent mechanism.[26–28]

Members of the cardiac glycoside family possess different binding affinities for, and inhibitory effects on, theNa^+/K^+ ATPase.[19] We tested LNP uptake in HeLa cells in the presence of nine cardiac glycosides at three concentrations, 0.15 μM, 1.5 μM and 15 μM for 24 h (Figure 2, *B* and S1). The relative binding affinities of these cardiac glycosides for Na^+/K^+ATPase follow the series helveticoside < dihydroouabain < digoxigenin < strophanthidin < lanatoside C < digitoxigenin < digoxin < ouabain < proscillaridin A.[19] Helveticoside, being the weakest binder to the Na^+/K^+ ATPase, resulted in enhanced LNP uptake at the highest concentration whereas proscillaridin A which has the highest Na^+/K^+ ATPase affinity among the nine molecules tested, required the least amount to cause increased LNP uptake (Figure 2, *B*). Enhanced uptake was not observed at higher concentrations of strong binders, which is likely due to increased toxicity. Cell viability assay indeed shows that proscillaridin A and ouabain, but not strophanthidin, cause toxicity at concentration as low as 0.03 μM (Figure S2).

Ouabain induces LNP siRNA uptake and enhanced gene silencing effects

If cardiac glycosides enhance endocytosis of LNP siRNA systems, it would be expected that enhanced gene silencing effects would be observed as a result of increased amount of LNP siRNA systems in the cell. Previous studies have shown that incubation of LLC-PK1 cells with 50 nM ouabain for 12 h induces endocytosis of the Na^+/K^+ ATPase.[26–28] Increased uptake of LNP in HeLa cells incubated with 30 nM of ouabain for 24 h was observed by confocal microscopy (Figure 3, *A*).

Quantification of cellular SPDiO fluorescence showed that cells treated with 30 nM of ouabain contained 2.5 times higher levels of LNP than untreated cells. No significant cell death was observed when cells were treated at this concentration of ouabain.

The gene silencing potencies of LNP siRNA systems were determined in the presence or absence of ouabain using GAPDH as a target gene. GAPDH was chosen as a target gene since it is ubiquitously expressed at high levels in all cell types. LNP siRNA systems containing siGAPDH or the negative control siScramble were incubated with HeLa cells for 24 h in the presence or absence of ouabain. Because GAPDH has a relatively long half-life of ~38 h,[29] cells were further incubated in plain medium for 48 h before protein expression was analyzed. As shown in Figure 3, *B*, expression of GAPDH was substantially reduced only in cells treated with siGAPDH-LNP and ouabain. The gene silencing effect was strictly due to enhanced LNP uptake caused by ouabain as no changes in GAPDH expression were observed in cells not treated with ouabain or treated with LNP containing siScramble (Figure 3, *B*).

Incorporation of a targeting lipid containing a cardiac glycoside increases LNP uptake in cell lines of various origins

The LNP systems employed here have a small positive charge which encourages association with negatively charged cell surfaces. The increased LNP uptake caused by free ouabain is attributed to increased plasma membrane turnover accompanying the increased endocytosis of Na^+/K^+ ATPase. A more direct way of stimulating uptake would be to incorporate a targeting lipid containing a cardiac glycoside into the LNP itself. For ease of synthesis, strophanthidin (STR), a relatively simple cardiac glycoside was chosen to test this approach. STR was conjugated to the distal end of a 2000 MW polyethylene glycol (PEG) lipid with distearyl (C18) fatty acid chains (Figure 4, *A*) as indicated in Materials and Methods and Supplementary Materials.

It is important to show that the STR incorporated into the PEG-lipid (STR-PEG-lipid) maintains an ability to bind to and inhibit the Na^+/K^+ ATPase. The inhibitory activity of the STR-PEG-lipid on the Na^+/K^+ ATPase was directly measured using an ATPase inhibition assay[30,31] (Figure 4, *B*). Purified Na^+/K^+ ATPase was incubated with different concentrations of inhibitor (10^{-10} M to 10^{-3} M) at pH 7.8 at 37 °C. The inhibitor concentration for half-maximal inhibition, IC_{50}, was 0.13 μM for ouabain and 0.18 μM for strophanthidin. STR-PEG-lipid exhibited an IC_{50} of 0.33 μM which is fully consistent with a maintained ability of the PEG-associated STR ligand to bind to the Na^+/K^+ ATPase. The limited increase in the IC_{50} can be attributed to inhibitory steric effects due to the presence of the conjugated PEG-lipid. Unmodified PEG-lipid was not able to inhibit the enzyme at any concentration tested.

The resulting STR-PEG-lipid could be easily formulated into LNP siRNA systems by simply including it as one of the lipid components during the standard PFV LNP siRNA formulation protocol described in Methods. Since the Na^+/K^+ ATPase is expressed in all tissues at variable levels,[32] the STR-PEG-lipid is expected to increase uptake of LNP in most, if not all, cell types. Uptake of LNP containing STR-PEG-lipid was examined in a number of cell lines originated from different tissues including the cervix (HeLa), ovary (TOV21G, JHOC-5 and JHOC-9), lung (A549), pancreas (PANC1 and MIN6), liver (Hep3B), prostate (LNCaP and PC3) and breast (MCF7 and MDA-MB-231) (Figure 5). Cells were treated with fluorescently-labelled LNP containing either 1 mol% of STR-PEG-lipid (STR-PEG-LNP) or 1 mol% of negative control DSPE-PEG-lipid (DSPE-PEG-LNP) for 24 h followed by imaging and quantitation of intracellular fluorescence. As shown in Figure 5, *A*, fluorescence microscopy showed a dramatic improvement in intracellular LNP accumulation for LNP containing the STR-PEG-lipid indicating that the targeting lipid strongly enhanced endocytosis of LNP (Figure 5, *A*). Quantitation of the fluorescence intensity of these images revealed that the presence of the STR-PEG-lipid resulted in a 2 to 5-fold enhancement in LNP uptake as compared to LNP containing PEG-lipid alone (Figure 5, *B*). Cell viability assays show that STR-PEG-LNP at 50 μg/ml (lipid concentration) is non-toxic. Toxic effects were only observed at STR-PEG-LNP concentrations as high as 500 μg/ml (Figure S3).

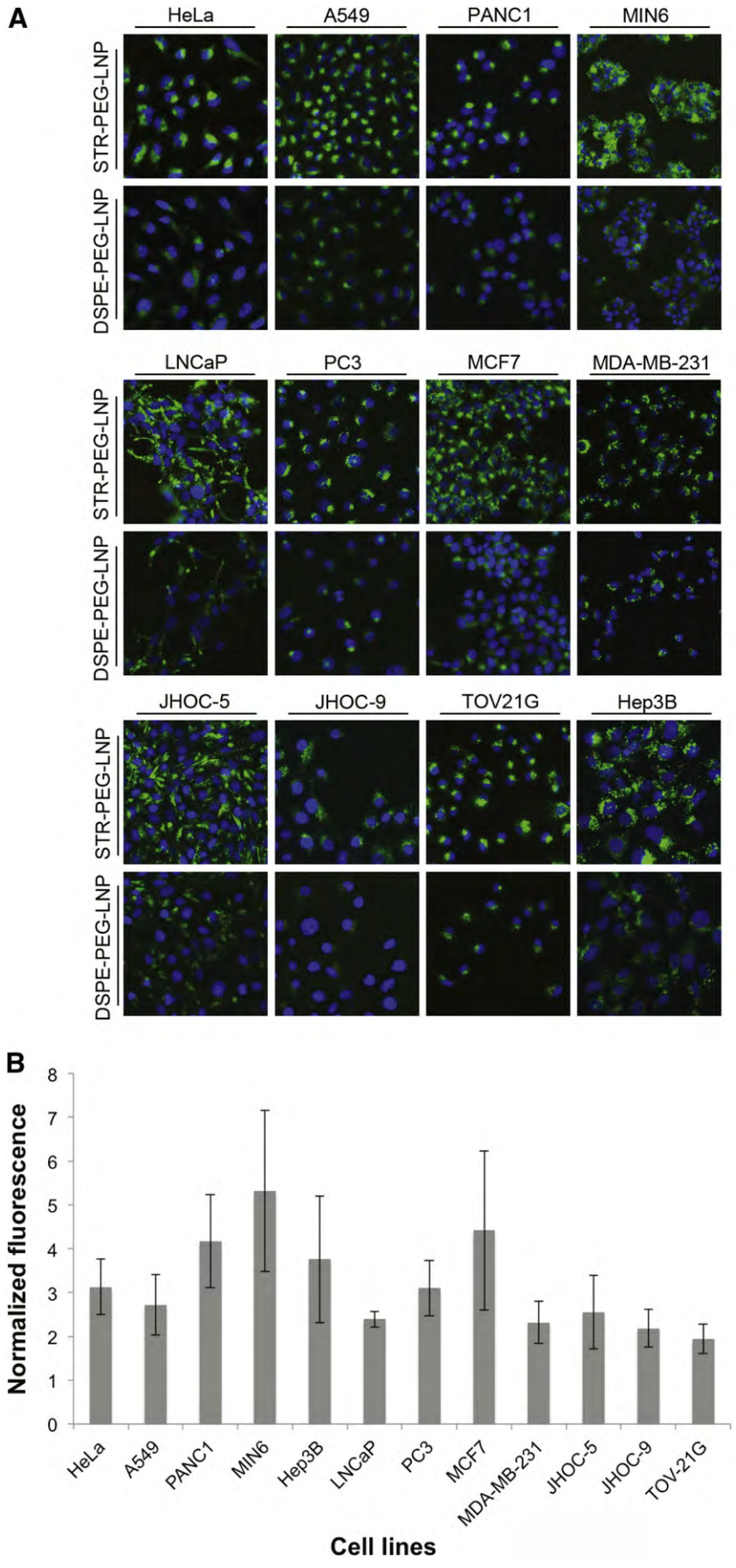
STR-targeted LNP systems exhibit increased gene silencing potency in HeLa cells

In order to test whether LNP containing the STR-PEG-lipid could enhance gene knockdown as a result of increased LNP uptake, HeLa cells were treated with STR-PEG-LNP or DSPE-PEG-LNP containing siGAPDH for 72 h. GAPDH levels were analyzed by Western blotting (Figure 6, *A*) and quantified (Figure 6, *B*). These results show that STR-PEG-LNP induced more than ~50% GAPDH knockdown at 2.5 and 5 μg/ml of siRNA (Figure 6, *B*). However, cells treated with LNP containing unmodified PEG-lipid did not show silencing of GAPDH at any concentrations tested.

Uptake of STR-targeted LNP systems is dependent on expression levels of Na^+/K^+ ATPase

As a further confirmation that STR-targeted LNP systems are accumulated into cells following binding to Na^+/K^+ ATPase, we investigated the influence of Na^+/K^+ ATPase expression on STR-targeted LNP uptake. Stable cell lines expressing shRNA targeted to ATP1A1 (shATP1A1), the α_1-isoform of Na^+/K^+ ATPase, or a negative control sequence (shScramble) were constructed. The levels of ATP1A1 were significantly lower in shATP1A1 cells than shScramble cells or wild-type cells (Figure 6, *C*). STR-PEG-LNP uptake was reduced by ~65.6% and ~30.2% at 6 h and 8 h, respectively, in shATP1 cells

Figure 5. STR-PEG-lipid increases the uptake of LNP into a variety of cell lines. **(A)** 12 cell lines of various origins were treated with 50 μg/ml of lipid concentration of either STR-PEG-LNP or DSPE-PEG-LNP for 24 h. Represented fluorescence micrographs obtained by Cellomics Arrayscan are shown. **(B)** Fluorescence micrographs of cells treated with either STR-PEG-LNP or DSPE-PEG-LNP for 24 h were quantified as described in Materials and Methods. Cellular SPDiO fluorescence due to accumulated STR-PEG-LNP was normalized to SPDiO fluorescence in cells treated with DSE-PEG-LNP. All values are means ± SD of 6 experiments.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HeLa
A549
PANC1
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STR-PEG-LNP
DSPE-PEG-LNP
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JHOC-5
JHOC-9
TOV21G
Hep3B
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HeLa
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Cell lines

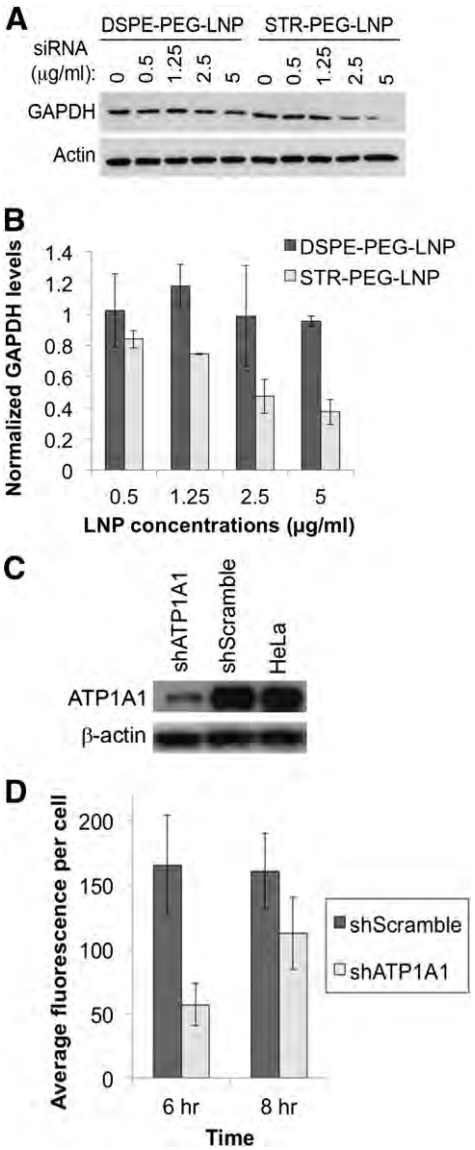

Figure 6. **(A)** LNP siRNA-induced GAPDH knockdown is increased when STR-PEG-lipid is incorporated into LNP siGAPDH systems. HeLa cells were treated with STR-PEG-LNP or DSPE-PEG-LNP encapsulating siGAPDH at various siRNA concentrations for 72 h. Equal portions of protein samples were analyzed by immunoblotting to GAPDH and β-actin which served as a loading control. **(B)** Quantitation of GAPDH levels. GAPDH and β-actin intensities in western blots were quantified. GAPDH levels were normalized to that of β-actin and reported relative to the untreated control group. **(C)** ATP1A1 expression is reduced in HeLa cell line stably transfected with shATP1A1 plasmid. Cells stably transfected with or without shATP1A1 or shScramble plasmid were lysed. Equal portions of protein samples were analyzed by immunoblotting to ATP1A1 and β-actin. **(D)** LNP uptake is dependent on expression of ATP1A1. Cells stably transfected with shATP1A1 or shScramble plasmid were treated with STR-PEG-LNP at 25 μg/ml of lipid concentration for 6 or 8 h. Confocal images were collected and SPDiO fluorescence was quantified using ImageJ. All values are means ± SD of 3 experiments.

compared to shScramble cells (Figure 6, *D*). These results are again consistent with an STR-PEG-LNP uptake process that occurs via binding tothe Na^+/K^+ ATPase.

Discussion

In this study we have identified a class of small molecules (cardiac glycosides) that enhance LNP uptake into a variety of cell types, have shown that incorporation of a representative cardiac glycoside (strophanthidin) into LNP siRNA systems via a PEG-lipid anchor results in enhanced uptake and gene silencing *in vitro* and have shown that uptake of this targeted LNP system is dependent on the expression of the ubiquitous cell-surface receptor, the Na^+/K^+ ATPase. Here we discuss the utility of the small molecule Cellomics screen for identifying targeting ligands and the potential of the strophanthidin-targeted LNP siRNA systems for target validation and therapeutic applications.

This study illustrates the considerable utility of developing screens for identifying small molecules that enhance LNP uptake into target cells. We focused our screening efforts on small molecules that are known drugs because the protein target, the binding affinities and the structure-activity relationships are often known. There is an obvious need for a secondary screen as many of the molecules increasing uptake may act on an intracellular receptor, which would not make them suitable as extracellular targeting ligands. In the case of the cardiac glycosides the secondary screen was straightforward, as it is well-known that they bind to an extracellular region of the Na^+/K^+ ATPase which is ubiquitously expressed on all mammalian cells.[21]

From a synthetic perspective, a targeting lipid incorporating a cardiac glycoside can be assembled by tethering an appropriately functionalized member of the family to commercially available activated PEG-lipids. It is known that cardiac glycosides bind to the active site of the ATPase with the unsaturated lactone deep in the binding pocket,[19,33] indicating that a logical place for conjugation would be through a functional group distant from the lactone, such as the C3-hydroxyl that is common to all members of this family; however, several members have a glycosidic group at this position complicating such functionalization. Of the tested cardiac glycosides, strophanthidin, digitoxigenin and digoxigenin possess free hydroxy groups at C3, with strophanthidin being the most affordable (Figure S1). Selective acylation of STR at C3 provided a handle for conjugation and provided a viable synthesis of the desired PEG-lipid conjugate. It is expected that further screening of small molecules libraries will lead to identification of other small molecule ligands that enhance cell uptake either through cell surface receptors specific to particular cells or to receptors, such as the Na^+/K^+ ATPase, that promote relatively non-specific uptake.

The results presented support accumulation of the non-targeted LNP siRNA systems through endocytosis as fluorescently-labelled LNPs appeared in punctate structures in HeLa cells (Figures 1, *A* and 3, *A*) and in other cells tested (Figure 5, *A*), in agreement with previous observations that (non-targeted) LNPs co-localize with the early endosomal marker in both Raw264.7 and primary antigen presenting cells.[34,35] The observation that more non-targeted LNPs were internalized upon ouabain treatment (Figure 3, *A*) is also consistent with an endocytotic mechanism. Ouabain has been shown to induce endocytosis of the Na^+/K^+ ATPase via a caveolin- and clathrin-

dependent mechanism.[26–28] Indeed, confocal microscopy suggested that both non-targeted and targeted LNPs reside in endosomal compartments containing transferrin, a marker for clathrin-mediated endocytosis (Figure S4). Furthermore, subcellular fractionation showed that a significant amount of the Na^+/K^+ ATPase was enriched in the endosomal fraction in cells treated with 30 nM of ouabain (data not shown).

The *in vivo* applications of LNP siRNA systems for silencing target genes are currently primarily limited to silencing liver (hepatocyte) target genes. The high potency of LNP siRNA for such applications is due in large part to the association of ApoE to the LNP following i.v. administration, which facilitates hepatocyte uptake.[9,10] In order to access other tissues alternative uptake ligands will need to be incorporated into the LNP. As noted previously, small molecule targeting ligands tethered to a lipid anchor in the LNP have considerable advantages compared to the use of peptide, protein or other larger ligands due to cost, ease of manufacturing and reduced immunogenicity amongst other factors. The incorporation of targeting ligands such as strophanthidin offers the possibility to stimulate relatively non-specific uptake and enhanced gene silencing in a variety of tissue types due to the ubiquitous expression of Na^+/K^+ ATPase. While the uptake of LNP into cells may be relatively non-specific, the highly specific nature of the siRNA cargo suggests considerable utility for target validation and possibly therapeutic applications in a broad range of diseases. For example, an LNP siRNA system designed to silence a tumour oncogene may be expected induce few "off target" effects if introduced into non-tumour tissue. Furthermore, the enhanced LNP uptake due to the targeting ligands can reduce the amount of drug used which translates into lower possible side effects and costs associated with the drug.

In summary, this study describes a screening approach to identify small molecule ligands to stimulate uptake of LNP siRNA systems into cells. We show that by screening small molecules that are known drugs additional benefits can be achieved in terms of knowledge of target proteins and structure–activity relationships that facilitate the design of ligand-PEG lipids that exhibit selective target binding properties and straightforward incorporation into LNP systems. In the particular case of the STR-PEG-lipid we demonstrate that the presence of this targeting agent in LNP siRNA systems results in enhanced uptake and gene silencing in a variety of cell types and may be of utility for targeted validation and possibly therapeutic applications when access to tissues other than hepatocytes is required.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2012.11.006.

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