IJC
International Journal of Cancer

Lipid nanoparticle siRNA systems for silencing the androgen receptor in human prostate cancer *in vivo*

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The androgen receptor (AR) plays a critical role in the progression of prostate cancer. Silencing this protein using short-hairpin RNA (shRNA) has been correlated with tumor growth inhibition and decreases in serum prostate specific antigen (PSA). In our study, we have investigated the ability of lipid nanoparticle (LNP) formulations of small-interfering RNA (siRNA) to silence AR in human prostate tumor cell lines *in vitro* and in LNCaP xenograft tumors following intravenous (i.v.) injection. *In vitro* screening studies using a panel of cationic lipids showed that LNPs containing the ionizable cationic lipid 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA) exhibited the most potent AR silencing effects in LNCaP cells. This is attributed to an optimized ability of DLin-KC2-DMA-containing LNP to be taken up into cells and to release the siRNA into the cell cytoplasm following endocytotic uptake. DLin-KC2-DMA LNPs were also effective in silencing the AR in a wild-type AR expressing cell line, LAPC-4, and a variant AR expressing cell line, CWR22Rv1. Importantly, it is demonstrated that LNP AR-siRNA systems containing DLin-KC2-DMA can silence AR gene expression in distal LNCaP xenograft tumors and decrease serum PSA levels following i.v. injection. To our knowledge, this is the first report demonstrating the feasibility of LNP delivery of siRNA for silencing AR gene expression *in vivo*.

Key words: androgen receptor, prostate cancer, lipid nanoparticles, liposomes, siRNA

Abbreviations: AR: androgen receptor; DLinDAP: 1,2-dilineoyl-3-dimethylammonium-propane; DLinDMA: 1,2-dilinoleyloxy-ketal-*N*,*N*-dimethyl-3-aminopropane; DLin-KC2-DMA: 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane; DLinKDMA: 1,2-dilinoleyloxy-ketal-*N*,*N*-dimethyl-3-aminopropane; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; LNP: lipid nanoparticle; PEG-C-DOMG: R-3-[(ω-methoxy-poly(ethyleneglycol)2000)carbamoyl)]-1,2-dimyristyloxypropyl-3-amine; PEG-S-DMG: 3-*N*-[(ω-methoxypoly (ethyleneglycol)2000)carbamoyl]-1,2-dimyristyloxy-propylamine; PSA: prostate specific antigen; SC: scrambled sequence; siRNA: small-interfering RNA

Additional Supporting Information may be found in the online version of this article

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Grant sponsors: Canadian Institutes of Health Research (MOP-86587) and the PCF-STAR Project, which is funded by Prostate Cancer Canada with the support of Safeway. Support in kind from Alnylam is also acknowledged.

DOI: 10.1002/ijc.27361

History: Received 29 Apr 2011; Accepted 25 Oct 2011; Online 16 Nov 2011

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Prostate cancer is the most frequently diagnosed nonskin cancer and the second-leading cause of cancer-related deaths in males.[1] It is projected that the number of new cases in the United States will exceed 200,000 in 2012. While often curable in its early stages by surgical or radiation ablation, the outcome for advanced or recurrent disease is less favorable, even if some form of androgen withdrawal therapy is employed. The cancer eventually progresses to a castration-resistant form that results in a median survival of 12–18 months.[2,3] Survival extensions of a few months can be achieved using docetaxel-based therapies[4]; however, more durable treatment options are clearly required.

A potentially new therapeutic strategy is to utilize RNA interference (RNAi) to silence critical genes involved in prostate cancer growth.[5–7] The androgen receptor (AR) represents one such target as the inappropriate activation/dysfunction of this protein is believed to be responsible for the development of castration-resistant prostate cancer.[6,8] Silencing of the AR using small-interfering RNA (siRNA) has been shown to increase cellular apoptosis due to disruption of the Bcl-xL-mediated survival signaling pathway.[9] Prior *in vivo* experiments using short-hairpin RNAs (shRNAs) against the AR have been shown to inhibit prostate tumor growth in mice and in castration-resistant disease and to cause tumor regression in 50% of the tumors.[6]

The design of lipid nanoparticle (LNP) systems for *in vivo* delivery of siRNA is complicated by the need to employ cationic lipids to achieve efficient encapsulation of negatively

charged lipid polymers such as siRNA. The use of cationic lipids usually leads to a positive charge on the LNP carrier, which results in enhanced serum protein adsorption and rapid clearance from the circulation following intravenous (i.v.) injection, leading to lack of penetration to target tissue.[10] We have addressed this problem by designing ionizable cationic lipids that have pK_a values of 7 or below, enabling encapsulation of nucleic acid polymers at low pH values (*e.g.*, pH 4) but also allowing the LNP to exhibit a near neutral surface charge at physiological pH values.[11,12] Such LNP systems are of increasing utility for the *in vivo* delivery of siRNA.[13,14]

Recent work has shown that the *in vivo* gene silencing potencies of LNP siRNA systems following i.v. administration are sensitive to relatively small changes in the structure of the ionizable cationic lipids employed. For example, the *in vivo* (hepatocyte) gene silencing activity of LNP siRNA systems containing 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA), 1,2-dilinoleyloxy-ketal-*N*,*N*-dimethyl-3-aminopropane (DLinKDMA), 1,2-dilinoleyloxy-*N*,*N*-dimethyl-3-aminopropane (DLinDMA) and 1,2-dilineoyl-3-dimethylammonium-propane (DLinDAP) varies according to the relationship DLin-KC2-DMA > DLinKDMA > DLinDMA ≫ DLinDAP.[14] In this work, we compare levels of AR gene silencing induced *in vitro* employing LNP siRNA systems containing DLin-KC2-DMA, DLinKDMA, DLinDMA and DLinDAP and show that LNP siRNA systems containing DLin-KC2-DMA are the most effective systems for achieving intracellular delivery and gene silencing of AR in the LNCaP cell lines. They are also effective for silencing AR in LAPC-4 and CWR22Rv1 human prostate cancer cell lines. Furthermore, it is shown that LNP AR-siRNA systems containing DLin-KC2-DMA effectively silence the AR gene in distal LNCaP xenograft tumors following i.v. injection.

Material and Methods

Materials

Distearoylphosphatidylcholine (DSPC) and cholesterol (Chol) were purchased from Avanti Lipids, 1,1′-dilinoleyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and 3,3′-dioctadecyl-5,5′-di(4-sulfophenyl) oxacarbocyanine (SP-DiOC$_{18}$) was obtained from Invitrogen (Burlington, ON, Canada). The ionizable cationic lipids 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA), 1,2-dilinoleyloxy-keto-*N*,*N*-dimethyl-3-aminopropane (DLinKDMA), 1,2-dilinoleyloxy-*N*,*N*-dimethyl-3-aminopropane (DLinDMA) and 1,2-dilineoyl-3-dimethylammonium-propane (DLinDAP) were provided by Tekmira Pharmaceuticals (Burnaby, BC, Canada) or were synthesized using established methods.[14] PEG-S-DMG (3-*N*-[(ω-methoxypoly(ethyleneglycol)2000)carbamoyl]-1,2-dimyristyloxypropylamine) and PEG-C-DOMG (R-3-[(ω-methoxy-poly(ethyleneglycol)2000)carbamoyl)]-1,2-dimyristyloxypropyl-3-amine) were provided by Alnylam Pharmaceuticals (Boston, MA). All other chemicals were of reagent or analytical grade.

Cell culture, cell lines and reagents

The LNCaP, LNCaP-eGFP, LAPC-4 and CWR22Rv1 human prostate cancer cell lines were used in all *in vitro* experiments.[15–18] LNCaP, LAPC-4 and CWR22Rv1 cells were originally obtained from ATCC and were not passaged beyond 6 months after receipt or resuscitation. Cells maintained in RPMI 1640 (Invitrogen, Burlington, ON, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and incubated at 37°C and 5% CO_2. The LNCaP and LNCaP-eGFP cell line was supplemented with 0.1 nM R1881 (Sigma-Aldrich, Oakville, ON, Canada).

siRNA sequences

Sequence of the human *AR* gene (Genbank accession no. NM_000044) was extracted from the NCBI Entrez nucleotide database. The 25-mer AR-siRNA was purchased as Stealth RNAi siRNA (Invitrogen): 5′-AGCACUGCUACUCUUCAGCAUUAUU-3′ (AR sense) and 5′-AAUAAUGCUGAAGAGUAGCAGUGCU-3′ (AR antisense). An additional scrambled (SC) LoGC negative control was purchased as a Stealth RNAi siRNA (Invitrogen, Burlington, ON, Canada). Luciferase (LUC) siRNA was a gift from Alnylam Pharmaceuticals (Boston, MA): 5′-cuuAcGcuGAGuAcuucGAdTsdT-3′ (LUC sense) and 5′-UCGAAGuACUcAGCGuAAGdTsdT-3′ (LUC antisense). Lower case letters = 2′-OMe; Upper case = RNA; s = phosphothioate backbone.

Encapsulation of siRNA into LNPs

Lipid stocks [DSPC, PEG-S-DMG, PEG-C-DOMG, cholesterol, SP-DiOC$_{18}$ and a cationic lipid (DLin-KC2-DMA, DLinKDMA, DLinDMA and DLinDAP)] were co-dissolved in ethanol at appropriate molar ratios (10 mol % DSPC, 10 mol % PEG-S-DMG, 39.8 mol % Chol, 0.2 mol % SPDiOC$_{18}$ or DiI and 40 mol % cationic lipid). In some cases, other molar ratios of lipids [10 mol % DSPC, 2.5 (or 10) mol % PEG-C-DOMG, 47.5 (or 40) mol % cholesterol and 40 mol % DLin-KC2-DMA] were constructed to examine the effect of PEG on *in vitro* transfection. Multilamellar vesicles (MLVs) were generated by adding the lipid in ethanol to an aqueous buffer (50 mM citrate, pH 4.0) to achieve a final concentration of 40% ethanol. Large unilamellar vesicles (LUVs) were generated by extruding the MLVs through two nuclepore polycarbonate filters (80 nm, 10 passes) using an extrusion device from Northern Lipids (Vancouver, BC, Canada) at ~300 psi.

The siRNA was dissolved in 10 mM citrate, 30 mM NaCl, pH 6.0, quantified by measuring absorbance at OD_{260} and added to the LUV dispersion dropwise under constant vortex at an siRNA:lipid ratio of 1:10 (wt/wt). The resulting LNP siRNA systems were then dialyzed in PBS overnight to increase the pH to ~7.4. The mean diameter of the vesicles was determined using a NICOMP370 particle sizer (Nicomp Particle sizing, Santa Barbara, CA) using the intensity mode. For LNP containing PEG-S-DMG diameters were 75 ± 12 nm (DLinDAP), 73 ± 6 nm (DLinDMA), 71 ± 7 nm

(DLinKDMA) and 70 ± 8 nm (DLin-KC2-DMA). LNP containing PEG-C-DOMG had slightly larger diameters, 86 ± 9 nm (DLin-KC2-DMA, 2.5% PEG-C-DOMG) and 82 ± 10 nm (DLin-KC2-DMA, 10% PEG-C-DOMG). Lipid concentrations were measured by total cholesterol (T-CHO) using the cholesterol E enzymatic assay from Wako Chemicals (Richmond, VA). Free siRNA was removed using VivaPureD MiniH columns (Sartorius Stedim Biotech GmbH, Goettingen, Germany). The elutants were then dissolved in 75% ethanol and siRNA was quantified by measuring absorbance at OD_{260}. The siRNA encapsulation efficiency was 80% or greater for all formulations.

Western blotting and immunofluorescence

LNCaP, LAPC-4 and CWR22Rv1 cells were plated in 12-well plates (2.0×10^5 cells per well). Cells were washed in PBS and lysed in RIPA buffer (1% NP-40, 0.25% deoxycholic acid) with protease inhibitor tablets (Roche Diagnostics, Indianapolis, IN). Total protein (10 μg) quantified by Bradford Assay was analyzed by immunoblotting. Antibodies to AR from Santa Cruz Biotechnology (AR-441) (Santa Cruz, CA). Antibodies to GAPDH from Abcam (Cambridge, MA). Antigen–antibody complexes were detected using Millipore Immobilon Western Chemiluminescent HRP Substrate (Billerica, MA).

Confocal microscopy

LNCaP cells were seeded onto poly-D-lysine cover slips (2.0×10^5 cells) per well in a 12-well plate. Cells were treated with 5 μg/ml of AR-siRNA encapsulated in LNPs containing DLin-KC2-DMA, DLinKDMA, DLinDMA and DLinDAP for 48 hr. Cells were then fixed in 3% PFA with Hoechst's stain and examined under an Olympus FV1000 (Center Valley, PA) laser scanning microscope.

Fluorescent microscopy

LNCaP cells were seeded (2.0×10^4 cells) per well in a 96-well plate. Cells were treated with 1 μg/ml of AR-siRNA encapsulated in LNPs containing DLin-KC2-DMA, DLinKDMA, DLinDMA and DLinDAP for 4, 12, 24 and 48 hr. Cells were fixed in 3% PFA with Hoechst's stain and examined using a Cellomics ArrayScan VTI HCS Reader (Thermo Scientific, Pittsburgh, PA).

Flow cytometry

LNCaP-eGFP cells were treated with DiI-labeled LNPs encapsulating either AR- or LUC-siRNA. Cellular uptake and AR knockdown were then assessed using a LSRII flow cytometer and FACS Diva Software (BD Bioscience, Billerica, MA) by measuring the fluorescence intensity of DiI and GFP, respectively.

Assessing effect of siRNA to knockdown AR in vivo

LNCaP xenograft prostate tumors were established as described previously.[6] When tumors became palpable, volumes were measured (width × length × depth × 0.5236) and blood was collected from the tail vein to assess serum prostate specific antigen (PSA) by ELISA (ClinPro International, Union City, CA). Once PSA values reached 50–100 ng/ml, animals were randomized into three groups. LNP containing AR-siRNA or LNP scrambled siRNA (SC-siRNA) and DLin-KC2-DMA were i.v. administered through the lateral tail vein. PBS was injected as a baseline control. Doses of LNP siRNA were administered three times on consecutive days at a dose level of 10-mg siRNA/kg of mouse body weight. Further injections were made an additional three times on Days 7, 9 and 11. Animals were sacrificed at Day 14. Serum PSA and protein levels of AR in the xenograft tumors were analyzed. All animal procedures were done according to the guidelines of the Canadian Council of Animal Care with appropriate institutional certification.

5′-RNA-linker-mediated rapid amplification of cDNA ends PCR

5′-RNA-linker-mediated rapid amplification of cDNA ends (5′-RLM-RACE) was performed using a GeneRacer kit according to the manufacturer's protocol (Invitrogen, Burlington, ON, Canada). LNCaP cells were transfected with 10 nM of AR-siRNA with oligofectamine.[19] After 72 hr, LNCaP cells were harvested. Total RNA from LNCaP cells after siRNA treatment or from tumor tissues collected from mice treated with AR-siRNA, scrambled siRNA or PBS was isolated using the Trizol method (Invitrogen, Burlington, ON, Canada). Hundred nanograms of total RNA was linked to an RNA oligonucleotide with T4 RNA ligase. Purified RNA after DNAse I digestion were reverse transcribed. PCR was performed with primers specific for the RNA linker and for the targeted gene with the following conditions: 94°C, 2 min; 54°C, 1 min and 72°C, 1 min; then 35 cycles of 94°C, 30 sec; 54°C, 1 min and 72°C, 30 sec, and finally an extension period at 72°C, 7 min. Five microliters of this product was taken for nested PCR using the same conditions, but with the annealing temperature decreased to 50°C. To use as a control, GAPDH primers were used to amplify the cDNA transcribed with random hexamers. Ten microliters of each reaction was analyzed on a 2% agarose gel visualized with SybrSafe (Invitrogen, Burlington, ON, Canada). The band corresponding to the predicted size of the amplicon was excised and purified using QIAquick Gel Extraction kit (Qiagen, Mississauga, ON, Canada). Subsequently, the fragment was cloned using a TA Cloning kit supplied with the GeneRacer kit and sequenced.

Statistical analyses

All statistical analyses were performed using GraphPad. Initially, a one-way ANOVA was used to statistically evaluate the differences between treatment groups. In the case of statistically significant results, the differences between treatment groups were assessed through the use of the Tukey–Kramer multiple comparisons test unless otherwise stated. Probability (p) values less than 0.05 were considered significant.

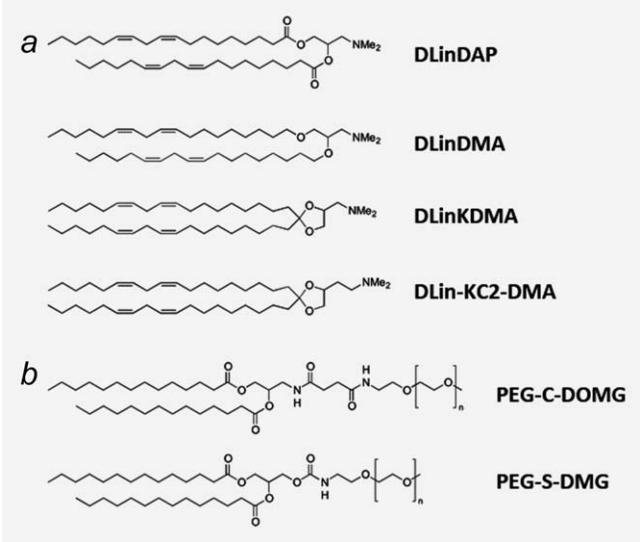

Figure 1. (*a*) Structures of the ionizable cationic lipids employed. Distinguishing features are that DLinDAP contains ester acyl chain linkages; DLinDMA contains ether linkages; DLin-KC2-DMA and DLinKDMA contains ketal linkages. (*b*) Structures of PEG–lipids are employed. Note that PEG-S-DMG contains ester linkages; PEG-C-DOMG contains ether linkages.

Results

LNP AR-siRNA systems formulated with DLin-KC2-DMA exhibit maximum levels of AR gene silencing in LNCaP cells

Previous studies have shown that upregulation of the AR occurs at both the mRNA and protein levels and causes hypersensitivity to androgens even at small concentrations.[20] We selected an siRNA sequence that has been demonstrated to effectively silence AR and halt tumor progression in a study using inducible plasmid shRNA to knockdown AR expression *in vivo*.[6] The AR-siRNA and SC-siRNA was encapsulated as indicated in Material and Methods section using four species of LNPs containing DLin-KC2-DMA, DLinKDMA, DLinDMA or DLinDAP (Fig. 1). Essentially complete AR knockdown was seen in cells incubated with DLin-KC2-DMA LNP encapsulating AR-siRNA at a concentration of 5 μg/ml (Fig. 2*a*). Significant AR knockdown was also detected when LNP AR-siRNA systems containing DLinKDMA or DLinDMA were used; however, low amounts of protein still remained (Figs. 2*a*–2*c*). Cells treated with LNP containing SC-siRNA showed normal expression levels of AR.

The results shown in Figure 2*a* suggest that LNP AR-siRNA containing DLin-KC2-DMA are more potent than LNP containing DLinKDMA, DLinDMA or DLinDAP. However, the nearly complete knockdown of AR for the DLin-KC2-DMA system implies that saturating levels of LNP-siRNA are present, which makes comparative estimates of relative potency difficult. When the experiments were repeated using siRNA at a concentration of 1 μg/ml, there

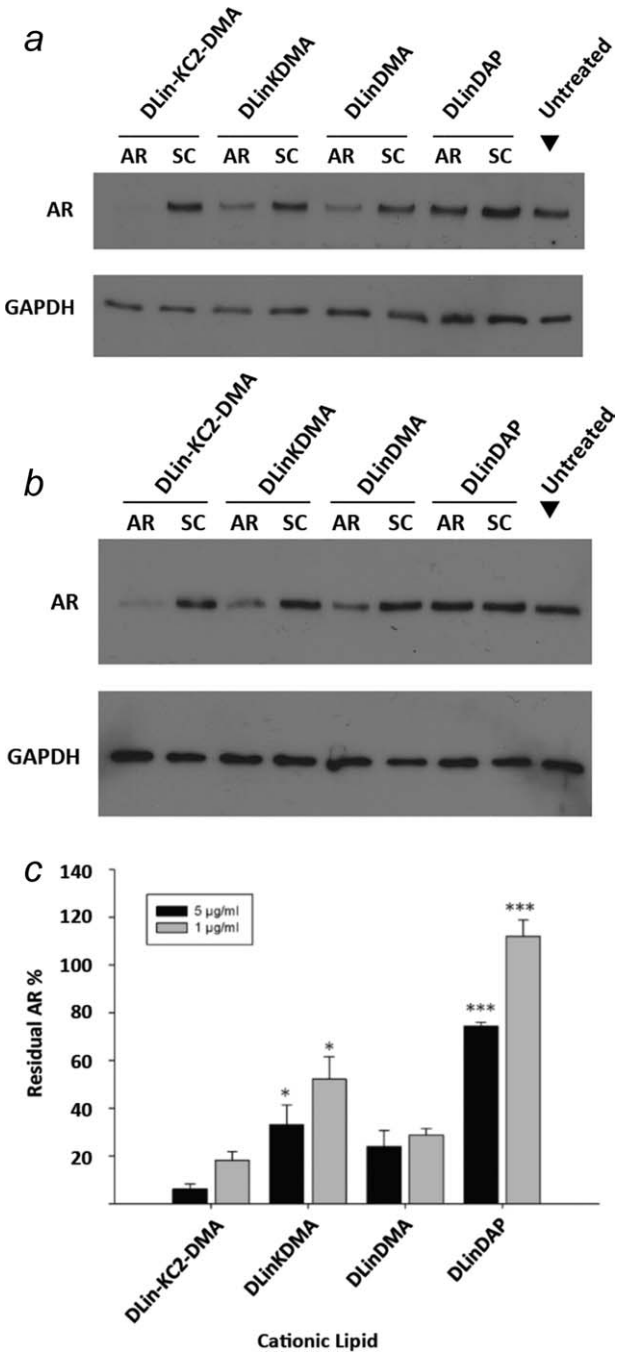

Figure 2. Silencing of the AR gene in LNCaP prostate cancer cells following incubation with LNP AR-siRNA systems. (*a*) LNCaP cells were incubated with 5 μg/ml or (*b*) 1 μg/ml of AR-siRNA or SC-siRNA encapsulated in either DLin-KC2-DMA, DLinKDMA, DLinDMA or DLinDAP LNP siRNA systems for 48 hr as described in Material and Methods section. Protein expression was analyzed by Western immunoblotting. (*c*) Quantification of AR in LNCaP prostate cancer cells. Data points are expressed as the residual percentage of AR, which was normalized to GAPDH as a ratio to the scrambled control and represent group mean ($n = 3$) ± SE. Statistical significance is determined between DLin-KC2-DMA *versus* the other three cationic lipids (DLinKDMA, DLinDMA and DLinDAP). *$p < 0.05$; ***$p < 0.001$.

again was substantial knockdown of AR with the LNP AR-siRNA system containing DLin-KC2-DMA, with LNP systems containing DLinKDMA or DLinDMA being less effective (Fig. 2*b*). Quantification of the residual AR normalized to GAPDH expression and relative to the scrambled control (Fig. 2*c*) revealed a dose-dependent drop in residual AR levels for DLin-KC2-DMA and DLinKDMA. The relative potency of the LNP formulations was DLin-KC2-DMA > DLinDMA > DLinKDMA ≫ DLinDAP (Fig. 2*c*).

The potency of LNP siRNA systems containing DLin-KC2-DMA can be attributed to improved siRNA uptake and endosome release properties

The superior potency of LNP AR-siRNA systems containing DLin-KC2-DMA could arise due to increased uptake into cells and/or improved release from endosomes following uptake. To better understand how these factors contribute, we first compared the potency of these lipids with their uptake into cells. Higher positive surface charges would be expected to lead to greater uptake into cells due to enhanced potential for association with the negatively charged cell exterior. In this regard, LNPs containing DLin-KC2-DMA, DLinKDMA, DLinDMA or DLinDAP will exhibit different surface charges due to the differing pK_a values of these ionizable cationic lipids. Previous work[14] has shown that DLin-KC2-DMA, DLinKDMA, DLinDMA or DLinDAP exhibit pK_a values of 6.7, 5.9, 6.8 and 6.2, respectively. These results suggest that LNP containing DLin-KC2-DMA or DLinDMA should exhibit higher levels of uptake than those observed for LNPs containing DLinKDMA or DLinDAP, which have lower pK_a values. Uptake experiments employed LNCaP cells incubated with AR-siRNA LNP containing DLin-KC2-DMA, DLinKDMA, DLinDMA or DLinDAP, as well as the SPDiOC$_{18}$ fluorescent lipid label, for 4, 12, 24 and 48 hr. Uptake was measured as the mean fluorescence intensity per cell as determined employing the Cellomics ArrayScan apparatus, which averaged fluorescent intensity of over ~400 LNCaP cells. LNP containing DLin-KC2-DMA exhibited the highest level of uptake at 48 hr, followed closely by DLinDMA (Fig. 3*b*). Confocal microscopy images taken at 48 hr are consistent with increased uptake in LNCaP cells when DLin-KC2-DMA is used in the LNP formulation (Fig. 3*a*). The cellular uptake data correlates with the extent of AR knockdown (Figs. 2*b* and 2*c*) and suggests that uptake is a major contributing factor to the differential knockdown profiles observed for the four cationic lipids (Fig. 3).

To further assess the correlation between knockdown, LNP uptake and endosomal release, flow cytometry studies were performed using an LNCaP cell line that stably expresses an AR-responsive promoter linked to an eGFP reporter. As shown elsewhere, in the absence of AR expression no GFP expression is observed[18] in this cell line. Cells were incubated (1-μg siRNA/ml for 48 hr) with LNP containing DLin-KC2-DMA, DLinKDMA, DLinDMA or DLinDAP and the fluorescent lipid analogue (DiI). These LNP contained either AR-siRNA or control (Luc)-siRNA. Uptake was measured by quantifying DiI (red) fluorescence, and GFP expression was used as a surrogate for AR expression. The GFP expression was normalized to cellular uptake (*i.e.*, the ratio of GFP fluorescence to DiI fluorescence) for both AR and control-siRNA sequences. The ratio of normalized GFP expression for LNP containing AR siRNA to normalized GFP expression for LNP containing control siRNA then gives a measure of the relative potencies of the different cationic lipids for inducing endosomal escape. As shown in Figure 3*c*, DLin-KC2-DMA showed the highest potency, followed closely by DLinDMA, indicating that DLin-KC2-DMA and DLinDMA have an increased ability to facilitate endosomal release of siRNA as compared to DLinKDMA or DLinDAP.

The species of PEG–lipid influences LNP siRNA-induced AR silencing

The LNP siRNA systems used above contain 10 mol % PEG-S-DMG, which contains labile ester linkages between the acyl chains and the headgroup. To achieve more chemically stable systems on storage, and to make *in vivo* results more comparable with recent *in vivo* studies using related LNP systems,[14] we employed PEG-C-DOMG in place of PEG-S-DMG for *in vivo* studies. PEG-C-DOMG is more chemically stable than PEG-S-DMG due to the presence of ether bonds linking the acyl chains to the headgroup (Fig. 1*b*). This substitution required crossover studies to determine the relative effects of PEG-C-DOMG and PEG-S-DMG on LNP potency.

As shown in Figure 4*a*, LNP AR-siRNA systems containing 10 mol % PEG-C-DOMG exhibited reduced silencing of the AR as compared to systems containing 10 mol % PEG-S-DMG. These results could be explained by a slower rate of release of PEG-C-DOMG from the LNP surface as compared to PEG-S-DMG. As discussed elsewhere, the PEG–lipids have been engineered to exhibit rapid leaving rates from the LNP surface following i.v. injection as the presence of PEG–lipids can inhibit interaction with target cells.[21] The inhibitory effects of a slower dissociation rate of PEG-C-DOMG from the LNP systems can be most easily corrected by starting with a lower PEG–lipid content in the LNP siRNA system. Thus, the *in vitro* AR silencing properties of AR-siRNA systems containing PEG-C-DOMG were tested at 2.5 mol % as opposed to 10 mol %. As shown in Figure 4*a*, substantial AR silencing is observed for systems containing 2.5 mol % PEG-C-DOMG. LNP that contained 2.5 mol % PEG-C-DOMG and the most potent cationic lipid, DLin-KC2-DMA, were used in all subsequent LNP formulations.

LNP AR-siRNA induces AR silencing in vitro in LAPC-4 and CWR22Rv1 cell lines

LNCaP cells contain a point-mutated AR within the ligand-binding domain. It is important to examine the potency of LNP AR-siRNA in other prostate cancer cell lines such as LAPC-4 (expressing wild-type AR), and CWR22Rv1 (expressing a variant AR).[16,17,22] The potency of LNP AR-siRNA

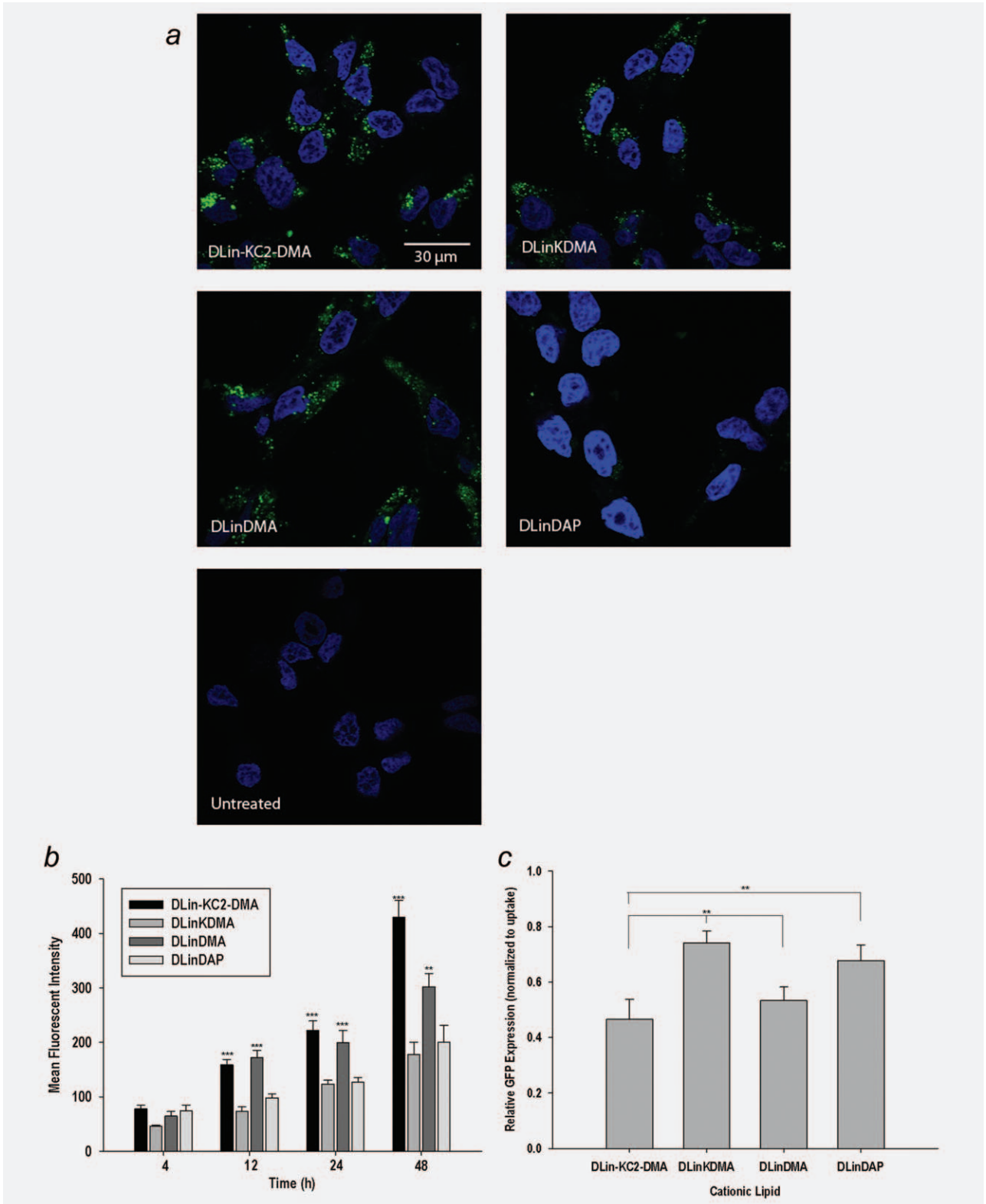

Figure 3. Influence of cationic lipid species on LNP uptake and knockdown in LNCaP and LNCaP-eGFP. (*a*) LNCaP cells were incubated with 5 µg/ml of LNP AR-siRNA formulated using either DLinDAP, DLinDMA, DLinKDMA or DLin-KC2-DMA for 48 hr. Representative images are shown. Nuclei were stained with Hoechst's dye (blue) and SPDiO-C_{18} fluorescence (green). Scale bar = 30 µm. (*b*) LNCaP cells were incubated with 1 µg siRNA/ml of LNP AR-siRNA formulated using either DLinDAP, DLinDMA, DLinKDMA or DLin-KC2-DMA for 4, 12, 24 and 48 hr. Cellular uptake was quantified using Cellomics ArrayScan, expressed as mean fluorescent intensity per cell. Approximately 400 cells were measured in four individual wells ($n = 4$) ± SD. Statistical significance is determined between DLin-KC2-DMA or DLinDMA *versus* the other two cationic lipids (DLinKDMA and DLinDAP) $^{**}p < 0.01$; $^{***}p < 0.001$. (*c*) LNCaP-eGFP cells were incubated with 1 µg siRNA/ml of LNP AR-siRNA or LNP LUC-siRNA formulated using either DLinDAP, DLinDMA, DLinKDMA or DLin-KC2-DMA for 48 hr and analyzed *via* flow cytometry. Bar graph shows the relative GFP expression compared to LUC-siRNA control normalized to uptake *via* DiI (red). Statistical significance is indicated between treatment groups ($^{**}p < 0.01$).

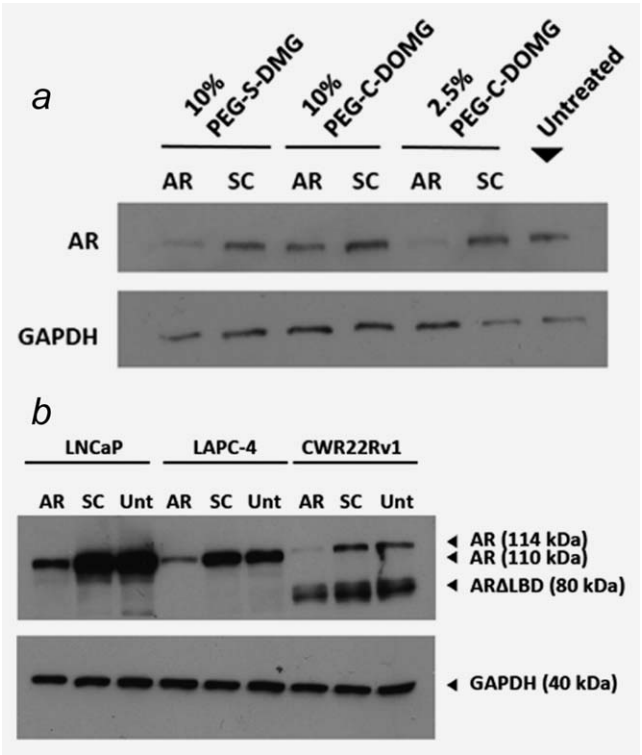

Figure 4. Influence of PEG–lipid species and concentration on silencing of the AR gene in LNCaP, LAPC-4 and CWR22Rv1 cells. (*a*) LNCaP cells were incubated with 5 μg/ml of LNP AR-siRNA encapsulated in DLin-KC2-DMA formulated with different types and amounts of PEG–lipids (10 mol % PEG-S-DMG, 10 mol % PEG-C-DOMG and 2.5 mol % PEG-C-DOMG) for 48 hr. (*b*) LNCaP, LAPC-4 and CWR22Rv1 cells were incubated with 5 μg/ml of LNP AR-siRNA encapsulated in DLin-KC2-DMA and 2.5 mol % PEG-C-DOMG formulated with AR and SC-siRNA. AR expressed in LNCaP and LAPC-4 is 110 kDa, and CWR22Rv1 expresses AR at 114 kDa and an additional ARΔLBD at 80 kDa. Levels of AR protein were analyzed by Western immunoblotting with GAPDH (loading control).

systems (40 mol % DLin-KC2-DMA and 2.5 mol % PEG-C-DOMG) for silencing the AR in LNCaP, LAPC-4 and CWR22Rv1 cell lines is shown in Figure 4*b*. Substantial knockdown is observed in both LNCaP and LAPC-4 cell lines as indicated by the reduced intensity in the band at 110 kDa when using LNP AR-siRNA compared to the LNP SC-siRNA and untreated controls. In the case of the CWR22Rv1 cell line,[17] the full-length AR band is located at 114 kDa and significant AR knockdown is also observed for this band in addition to some knockdown at 80 kDa, representing the truncated AR lacking the ligand-binding domain (ARΔLBD) (Fig. 4*b*).

Intravenous administration of LNP AR-siRNA can reduce serum PSA levels in mice bearing LNCaP tumors

The results summarized to this point indicate that an LNP siRNA system containing DLin-KC2-DMA and 2.5 mol % PEG-C-DOMG exhibits optimized activity *in vitro*. However, these systems are primarily designed to enable the long circulation lifetimes required to result in accumulation at tumour sites following i.v. injection (diameter < 100 nm and little positive surface charge). It is therefore of interest to determine the effectiveness of this formulation for *in vivo* AR knockdown. The LNCaP xenograft tumor model generated in athymic nude mice has previously been shown to provide a reproducible *in vivo* experimental system for monitoring antitumor therapeutics.[6,7,23–25] Although androgen ablation does not generally result in significant tumor regression in this model, it does result in a drop in serum PSA, which can serve as an index for impacting androgen signaling. Consistent with this, we have previously reported that AR knockdown in these tumors is correlated with the extent that serum PSA is reduced.[6,7]

A few weeks after inoculation with LNCaP cells, when serum PSA levels reached 50–75 ng/ml, mice were treated i.v. with 10-mg siRNA/kg of DLin-KC2-DMA LNP AR-siRNA or LNP SC-siRNA on Days 1, 2 and 3 (Fig. 5*b*) and then again on Days 7, 9 and 11. A second set of injections was made on Days 7, 9 and 11. Serum PSA levels were measured daily to gauge the effectiveness of these treatments.

While the serum PSA continued to increase in both control (PBS) and LNP SC-siRNA-treated mice, mice treated with LNP AR-siRNA showed no increase in serum PSA over levels observed at the initiation of treatment (Day 0) and at least 40% lower than that measured in the controls by Day 7 ($p < 0.05$) (Fig. 5*a*). Significantly, the second round of treatment with LNP-AR-siRNA on Days 7, 9 and 11 was sufficient to maintain suppression of serum PSA whereas PSA levels continued to rise in animals treated with either LNP SC-siRNA or the PBS (Fig. 5*a*). This result suggests that repeated LNP AR-siRNA injections could be used for long-term treatment. No obvious toxic side effects of such treatment were observed. Immunoblotting analyses revealed that protein levels of AR dropped in xenograft tumor tissues collected from mice treated with LNP AR-siRNA, whereas in tumor tissues from control mice treated with LNP SC-siRNA or PBS, the AR levels remained relatively unchanged (Fig. 5*b*).

AR-siRNA-induced specific cleavage in AR mRNA

To confirm that knockdown of the AR was RNAi mediated, a RLM RACE-PCR was conducted to amplify the mRNA cleaved by AR-siRNA in LNCaP cells treated with free AR-siRNA and in tumor tissues collected from mice treated with LNP AR-siRNA. As shown in Figure 6, there were amplifications of the cleaved mRNA with the predicted size in LNCaP cells treated with free AR-siRNA and also in tumor tissues collected from mice treated with LNP AR-siRNA. Sequencing of cDNAs reverse transcribed from cleaved mRNA confirmed that those cDNAs are authentic to the cDNA encoding *AR*. Further analysis demonstrated that the cleaved sites were

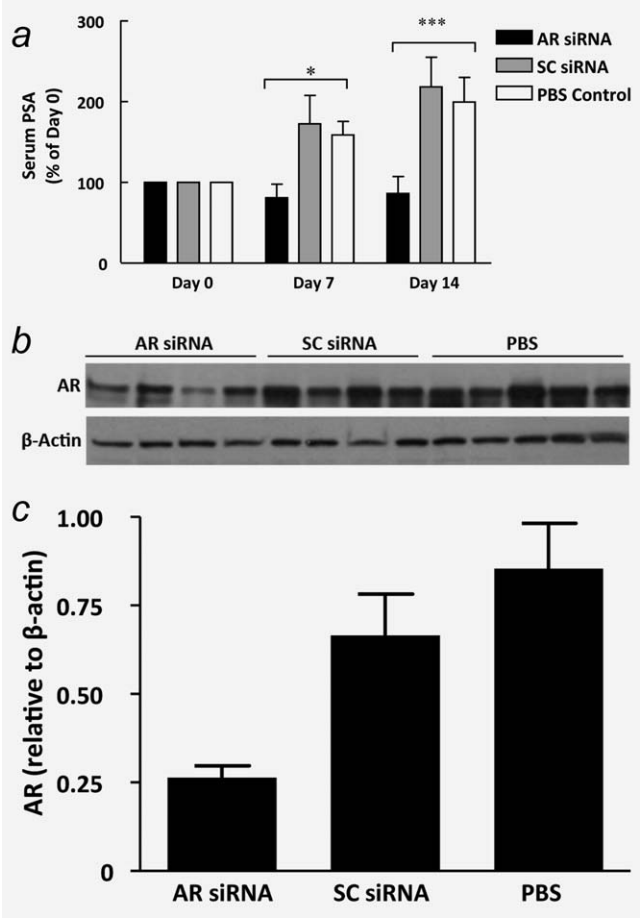

Figure 5. Systemic administration of LNP AR-siRNA results in decreased serum PSA levels. Mice were i.v. injected *via* tail vein with LNP AR and SC-siRNA (10 mg/kg) as described in Material and Methods section. (*a*) Percentages of serum PSA levels are relative to PSA levels at 1 day before treatment. $*p < 0.05$; $***p < 0.001$. Bonferroni post-tests followed by two-way ANOVA. Data points are the mean of one representative experiment ($n = 6–7$) ± SD. (*b*) Western blot of AR from all tumor tissues in each group of animals with β-actin as loading control. (*c*) Western-blot quantification of AR from all tumor tissues in each group of animals, normalized to levels of β-actin (loading control). Protein extracts were isolated from tumor tissues at Day 14. Data points are the mean of one representative experiment ($n = 6–7$) ± SE. $**p < 0.01$; $***p < 0.001$.

within the sequence recognized by the siRNA against the *AR* (Supporting Information Fig. S1).

Discussion

To our knowledge, the results presented here constitute the first demonstration of the feasibility and efficacy of using a LNP siRNA delivery system to knockdown the AR in a human prostate tumor *in vivo* and thereby significantly inhibit any subsequent increase in serum PSA. Three points of interest concern the reasons why the LNP containing DLin-KC2-DMA are the most active *in vitro*, comparison to other systems for silencing AR *in vivo* and ways in which the potency of these LNP siRNA systems may be improved.

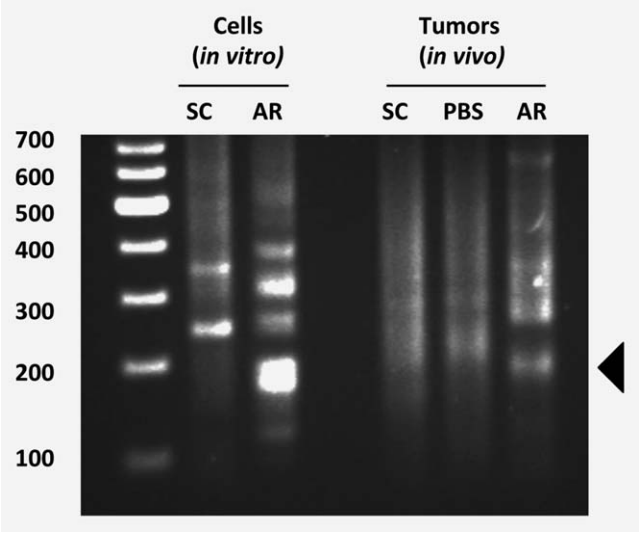

Figure 6. AR-siRNA induces specific cleavages in *AR* mRNA. Total RNA from LNCaP cells after siRNA transfection and from tumor tissues collected from mice treated with AR-siRNA (AR), scrambled siRNA (SC) and PBS. A 5′-RLM-RACE PCR was performed on RNAs collected using a GeneRacer kit. Ten microliters of each 5′-RLM-RACE PCR reaction was analyzed on a 2% agarose gel visualized with SybrSafe. The band corresponding to the predicted size of the amplicon is indicated (arrow).

LNP containing DLin-KC2-DMA gave rise to the greatest level of gene silencing in human prostate cancer cells (Fig. 2) of the four cationic lipids tested (Fig. 1). To determine whether the superior activity was due to increased uptake of LNPs, uptake experiments were performed in which LNCaP cells were incubated with LNP AR-siRNA containing DLin-KC2-DMA, DLinKDMA, DLinDMA or DLinDAP as well as a fluorescent label at 4, 12, 24 and 48 hr time points (Fig. 3*b*). As expected on the basis of surface charge, LNP containing DLin-KC2-DMA or DLinDMA exhibited higher levels of uptake (Fig. 3*b*). DLin-KC2-DMA and DLinDMA were also found to have increased AR knockdown compared to DLinKDMA and DLinDAP when normalized to cell uptake (Fig. 3*c*). As suggested elsewhere,[26] the ability of cationic lipids to facilitate intracellular delivery of nucleic acid polymers can be attributed to an ability to form ion pairs with endogenous anionic lipids in the endosome following uptake. These ion pairs promote endosomolytic nonbilayer hexagonal (H_{II}) phase structures, potentially resulting in the cytoplasmic release of the RNA or DNA. An enhanced ability to disrupt endosomes would be consistent with a greater ability of DLin-KC2-DMA and DLinDMA to induce H_{II} phase structure in the presence of anionic lipids as compared to DLinKDMA or DLinDAP as demonstrated elsewhere.[14]

Intravenous administration of the LNP AR-siRNA formulation containing DLin-KC2-DMA is effective in knocking down the AR in distal LNCaP xenograft tumors, which is also reflected by reduced serum PSA levels as compared to

controls (Fig. 5). This provides a proof-in-principle for developing a therapeutic strategy to treat advanced prostate cancers. Furthermore, our results indicate that AR knockdown and inhibition of serum PSA increases is sustainable with repeat i.v. injections of these DLin-KC2-DMA siRNA formulations (Fig. 5). These results are consistent with previous studies using a LNCaP-derived cell line engineered to express an inducible shRNA targeting the AR, which showed that extensive knockdown of the AR in all the cells inhibits tumor growth and decreases serum PSA levels.[6] Moreover, in a castration-resistant (hormone refractory) prostate cancer model, shRNA silencing of the AR was equally effective and even caused regression in 50% of the tumors,[7] suggesting that anti-AR therapy could be effective against both androgen-dependent and independent prostate cancers. To achieve a comparable impact on both tumor volume and serum PSA levels may require a substantial increase in the potency of *in vivo* LNP siRNA delivery systems.

It has been reported[27] that free siRNA (daily i.p. injections of 125 μg/kg AR-siRNA) can lower AR expression and inhibit prostate tumor growth. We were unable to repeat these results following their exact protocols (Supporting Information Materials and Methods and Supporting Information Fig. S2). This was not unexpected since *in vivo* therapy with free siRNA is problematic due to rapid clearance of siRNA from the bloodstream, degradation by serum nucleases, poor distribution to the target tumor tissue and an inability to penetrate target cell membranes.[28]

While the potential utility of the LNP AR-siRNA systems as agents to treat prostate cancer is supported by the clinical use of lipid-based delivery systems for small molecule applications[29] and the early stage clinical development of LNP siRNA systems aimed at hepatocyte targets (www.alnylam.com), improvements in *in vivo* potency are likely required before such systems can become viable therapies for prostate cancer therapy. That such improvements are possible is indicated by the potency of LNP siRNA systems developed for silencing FVII in hepatocytes, where dose levels of approximately 30-μg siRNA/kg body weight are required to achieve 50% gene silencing.[14] This contrasts with the 10-mg siRNA/kg body weight doses employed here. The remarkable potency of the hepatocyte LNP siRNA system is a consequence of the targeting effects of ApoE, which becomes associated with the LNP following i.v. administration[30] and leads to uptake into hepatocytes *via* the LDL receptor, the scavenging receptor and the "LDL-like" receptor.[31] Likewise, other *in vivo* studies have revealed that rHDL nanoparticles incorporated with siRNA are readily taken up into ovarian and colorectal cancer cells *via* the scavenger receptor type B1 (SR-B1) and are very effective at silencing cancer promoting genes at dose levels of 0.2 mg/kg.[32] This clearly suggests that strategies to improve targeting to and uptake of LNP AR-siRNA systems into prostate cancer cells *in vivo* should lead to more efficient AR knockdown and potentially better therapeutic control of advanced prostate cancer.

Acknowledgements

We thank Fariba Ghaidi, Jessica Firus, Anthony Chan (Vancouver Prostate Centre) and Sam Chen (University of British Columbia) for technical assistance. P.R.C. has a financial interest in Tekmira and received grant support from Alnylam.

References

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics. *CA Cancer J Clin* 2010;60:277–300.
2. Niu Y, Altuwaijri S, Lai K, Wu C, Ricke WA, Messing, EM, Yao J, Yeh S, Chang C. Androgen receptor is a tumor suppressor and proliferator in prostate cancer. *Proc Natl Acad Sci USA* 2008; 105:12182–7.
3. Kent EC, Hussain MH. The patient with hormone-refractory prostate cancer: determining who, when, and how to treat. *Urol J* 2003;62: 134–40.
4. Petrylak DP. Future directions in the treatment of androgen-independent prostate cancer. *Urol J* 2003;65:8–12.
5. Iorns E, Lord CJ, Turner N, Ashworth A. Utilizing RNA interference to enhance cancer drug delivery. *Nat Rev Drug Discov* 2006;6: 556–68.
6. Cheng H, Snoek R, Ghaidi F, Cox ME, Rennie PS. Short hairpin RNA knockdown of the androgen receptor attenuates ligand-independent activation and delays tumor progression. *Cancer Res* 2006;66:10613–20.
7. Snoek R, Cheng H, Margiotti K, Wafa LA, Wong CA, Wong EC, Fazli L, Nelson CC, Gleave ME, Rennie PS. In vivo knockdown of the androgen receptor results in growth inhibition and regression of well-established, castration-resistant prostate tumors. *Clin Cancer Res* 2009;15:39–47.
8. Rennie, PS, Nelson CC. Epigenetic mechanisms for progression of prostate cancer. *Cancer Metastasis Rev* 1998;17:401–9.
9. Liao X, Tang S, Thrasher JB, Griebling TL, Li B. Small-interfering RNA-induced androgen receptor silencing leads to apoptotic cell death in prostate cancer. *Mol Cancer Ther* 2005;4: 505–15.
10. Chonn A, Semple SC, Cullis PR. Separation of large unilamellar liposomes from blood components by a spin column procedure: towards identifying plasma proteins which mediate liposome clearance in vivo. *Biochim Biophys Acta* 1991;1070:215–22.
11. Semple SC, Klimuk SK, Harasym TO, Dos Santos N, Ansell SM, Wong KF, Maurer N, Stark H, Cullis PR, Hope MJ, Scherrer P. Efficient encapsulation of antisense oligonucleotides in lipid vesicles using ionizable aminolipids: formation of novel small multilamellar vesicle structures. *Biochim Biophys Acta* 2001;1510: 152–66.
12. Maurer N, Fenske DB, Cullis PR. Developments in liposomal drug delivery systems. *Expert Opin Biol Ther* 2001;1:923–47.
13. Zimmermann TS, Lee AC, Akinc A, Bramlage B, Bumcrot D, Fedoruk MN, Harborth J, Heyes JA, Jeffs LB, John M, Judge AD, et al. RNAi-mediated gene silencing in non-human primates. *Nature* 2006;441:111–4.
14. Semple SC, Akinc A, Chen J, Sandhu AP, Mui BL, Cho CK, Sah DW, Stebbing D, Crosley EJ, Yaworski E, Hafez IM, Dorkin JR, et al. Rational design of cationic lipids for siRNA delivery. *Nat Biotechnol* 2010;28:172–6.
15. Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, Mirand EA, Murphy GP. LNCaP model of human prostatic carcinoma. *Cancer Res* 1983;43:1809–18.
16. Klein KA, Reiter RE, Redula J, Moradi H, Zhu XL, Brothman AR, Lamb DJ, Marcelli M, Belldegrun A, Witte ON, Sawyers CL. Progression of metastatic prostate cancer to androgen independence in immunodeficient SCID mice. *Nat Med* 1997;3:402–8.
17. Tepper CG, Boucher DL, Ryan PE, Ma A, Xia L, Lee L, Pretlow TG, Kung H. Characterization of a novel androgen receptor mutation in a relapsed CWR22 prostate cancer xenograft and cell line. *Cancer Res* 2002;62:6606–14.
18. Abdelbaqi K, Lack N, Guns ET, Kotha L, Safe S, Sanderson JT. Antiandrogenic and growth inhibitor effects of ring-substituted analogs of 3,3′-diindolylmethane (ring-DIMs) in hormone-responsive LNCaP human prostate cancer cells. *Prostate* 2011;71:1401–12.
19. Zhang K, Matsui Y, Hadaschik BA, Lee C, Jia W, Bell JC, Fazli L, So AI, Rennie PS. Down-regulation of type I interferon receptor sensitized

bladder cancer cells to vesicular stomatitis virus-induced cell death. *Int J Cancer* 2010;127: 830–8.
20. Kokontis J, Takakura K, Hay N, Liao S. Increased androgen receptor activity and altered c-myc expression in prostate cancer cells after long-term androgen deprivation. *Cancer Res* 1994;54: 1566–73.
21. Mori A, Chonn A, Choi LS, Israels MA, Monck MA, Cullis PR. Stabilization and regulated fusion of liposomes containing a cationic lipid using amphipathic polyethyleneglycol derivatives. *J Liposome Res* 1998;8:195–211.
22. Veldscholte J, Berrevoets CA, Brinkmann AO, Grootegoed JA, Mulder E. Anti-androgens and the mutated androgen receptor of LNCaP cells: differential effects on binding affinity, heat-shock protein interaction, and transcriptional activation. *Biochemistry* 1992; 31:2393–99.
23. Miyake H, Nelson C, Rennie PS, Gleave ME. Acquisition of chemoresistant phenotype by overexpression of the antiapoptotic gene testosterone-repressed prostate message-2 in prostate cancer xenograft models. *Cancer Res* 2000;60:2547–54.
24. Sato N, Gleave ME, Bruchovsky N, Rennie PS, Goldenberg SL, Lange PH, Sullivan LD. Intermittent androgen suppression delays progression to androgen-independent regulation of prostate-specific antigen gene in the LNCaP prostate tumour model. *J Steroid Biochem Mol Biol* 1996;58:139–46.
25. Zhang K, Moussavi M, Kim C, Chow E, Chen IS, Fazli L, Jia W, Rennie PS. Lentiviruses with trastuzumab bound to their envelopes can target and kill prostate cancer cells. *Cancer Gene Ther* 2009;16:820–31.
26. Hafez I, Maurer N, Cullis PR. On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. *Gene Ther* 2001;8: 1188–96.
27. Compagno D, Merle C, Morin A, Mathieu JR, Bozec A, Mauduit C, Benahmed M, Cabon F. siRNA-directed in vivo silencing of androgen receptor inhibits the growth of castration-resistant prostate carcinomas. *PLoS One* 2007;2: 1–9.
28. Xie FY, Woodle MC, Lu PY. Harnessing *in vivo* siRNA delivery for drug discovery and therapeutic development. *Drug Discov Today 2006*;11:67–73.
29. Allen TM, Cullis PR. Drug delivery systems: entering the mainstream. *Science* 2004;303: 1818–22.
30. Akinc A, Querbes W, De S, Qin J, Frank-Kamenetsky M, Jayaprakash KN, Jayaraman M, Rajeev KG, Cantley WL, Dorkin JR, Butler JS, Qin L, et al. Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms. *Mol Ther* 2010;18: 1357–64.
31. Mahley RW, Huang Y. Atherogenic remnant lipoproteins: role for proteoglycans in trapping, transferring, and internalizing. *J Clin Invest* 2007; 117:94–8.
32. Shahzad MM, Mangala LS, Han HD, Lu C, Bottsford-Miller J, Nishimura M, Mora EM, Lee JW, Stone RL, Pecot CV, Thanapprapasr D, Roh JW, et al. Targeted delivery of small interfering RNA using reconstituted high-density lipoprotein nanoparticles. *Neoplasia* 2011;13:309–19.