

A Glu-urea-Lys Ligand-conjugated Lipid Nanoparticle/siRNA System Inhibits Androgen Receptor Expression *In Vivo*

Justin B Lee¹, Kaixin Zhang², Yuen Yi C Tam¹, Joslyn Quick¹, Ying K Tam¹, Paulo JC Lin¹, Sam Chen¹, Yan Liu¹, Jayaprakash K Nair³, Ivan Zlatev³, Kallanthottathil G Rajeev³, Muthiah Manoharan³, Paul S Rennie² and Pieter R Cullis¹

The androgen receptor plays a critical role in the progression of prostate cancer. Here, we describe targeting the prostate-specific membrane antigen using a lipid nanoparticle formulation containing small interfering RNA designed to silence expression of the messenger RNA encoding the androgen receptor. Specifically, a Glu-urea-Lys PSMA-targeting ligand was incorporated into the lipid nanoparticle system formulated with a long alkyl chain polyethylene glycol-lipid to enhance accumulation at tumor sites and facilitate intracellular uptake into tumor cells following systemic administration. Through these features, and by using a structurally refined cationic lipid and an optimized small interfering RNA payload, a lipid nanoparticle system with improved potency and significant therapeutic potential against prostate cancer and potentially other solid tumors was developed. Decreases in serum prostate-specific antigen, tumor cellular proliferation, and androgen receptor levels were observed in a mouse xenograft model following intravenous injection. These results support the potential clinical utility of a prostate-specific membrane antigen-targeted lipid nanoparticle system to silence the androgen receptor in advanced prostate cancer.

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Subject Category: Nanoparticles

Introduction

We have previously shown that inhibition of androgen receptor (AR) expression and reduction of prostate-specific antigen (PSA) serum levels in mouse models of human prostate cancer (PCa) can be achieved by intravenous (i.v.) administration of lipid nanoparticles (LNPs) containing small interfering RNA (siRNA) targeting the gene encoding the AR (LNP-AR-siRNA).¹ However, relatively high doses (six doses at 10 mg siRNA/kg body weight) were required to see appreciable effects. This is in significant contrast to the potency of LNP-siRNA systems that have achieved 50% silencing of a hepatic gene with a single dose of 0.005 mg siRNA/kg.^{2–4} This large-dose disparity is attributed to the liver's favorable physiology and to endogenous processes that result in targeting of LNPs to hepatocytes.^{3,5} In particular, LNPs associate with apolipoprotein E (ApoE)^{3,6} following i.v. administration and are taken into hepatocytes through the LDL receptor, the scavenger receptor, and the "LDL-like" receptor.^{3,7} Although it is unlikely that potencies equivalent to those seen for gene silencing in hepatocytes can be achieved in localized and disseminated PCa, reductions in current dose levels must be achieved for this approach to become a viable clinical strategy.

The objective of this study was to improve the potency of the LNP-siRNA system developed previously.¹ The primary techniques that we explored concerned using a different polyethylene glycol (PEG)-lipid to achieve longer circulation

lifetimes and facilitate higher levels of LNP accumulation at tumor sites, as well as the use of targeting ligands attached to the LNP-siRNA system to specifically enhance uptake into PCa cells following arrival at the tumor site. However, in order to build the most potent LNP system possible, two other LNP variables were investigated: first, the use of a more potent cationic lipid and second, a more potent AR-siRNA than used previously.¹

The cationic lipid, DLin-KC2-DMA, used in our previous study¹ was identified by screening a variety of cationic lipids in LNP-siRNA systems.² Recent advances in cationic lipid design have resulted in a number of more potent cationic lipids, including DMAP-BLP,⁸ which results in improved gene-silencing potency when used in LNP-siRNA formulations compared to DLin-KC2-DMA.⁴ Furthermore, the AR-siRNA used previously¹ was a 25-mer siRNA complementary to nucleotides 3542–3563 in the AR mRNA region that encodes the ligand-binding domain. Improvement in gene silencing can be expected with the use of other sequence-optimized siRNAs as it has been shown that siRNAs against different regions of an mRNA have drastically different silencing activities.^{9,10}

Additional factors that intrinsically influence the silencing activity of LNP-siRNAs are their ability to accumulate at the target site and to be taken up into the target cells. LNPs with long half-lives in circulation are likely to accumulate at tumors due to the impaired lymphatic drainage and leaky

The first two authors and the last two authors contributed equally to this work.

¹Department of Biochemistry and Molecular Biology at the University of British Columbia, Vancouver, British Columbia, Canada; ²Vancouver Prostate Centre, Vancouver, British Columbia, Canada; ³Department of Drug Discovery, Alnylam Pharmaceuticals, Cambridge, Massachusetts, USA. Correspondence: Pieter R Cullis, Department of Biochemistry and Molecular Biology, University of British Columbia, 2350 Health Sciences Mall, Vancouver, British Columbia, Canada V6T 1Z3. E-mail: pieterc@mail.ubc.ca

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vasculature at these sites.^{11,12} The PEG-lipid used in the previous studies was anchored into the LNP formulation by two C₁₄ alkyl chains; these lipids rapidly exchange out of the LNP with half-times on the order of minutes following i.v. injection.^{13,14} This loss of the PEG-lipid results in short (< 1 hour) LNP circulation half-times with substantial liver accumulation. In order to achieve longer circulation lifetimes, we examined here the properties of LNP systems containing PEG-lipid with long alkyl chains, which can reside in the LNP formulation for 24 hours or longer.

The type of targeting ligand to actively target LNP-siRNA systems to PCa cells is of interest. A small molecule-targeting approach has been previously demonstrated for anisamide,^{15–17} as well as the cardiac glycoside strophanthidin (STR).¹⁸ The prostate-specific membrane antigen (PSMA), a plasma membrane glycoprotein that is overexpressed in PCa cells as well as the neovasculature of many solid tumors (but not in healthy tissues),¹⁹ represents an attractive target for LNP systems. Binding to PSMA results in internalization through clathrin-mediated endocytosis and thus can potentially carry LNP into the cell.²⁰ *In silico* screening studies have identified the small molecule 2-(3-(1,3-dicarboxypropyl)-ureido)pentanedioic acid (DUPA),²¹ which binds specifically to PSMA with high affinity.^{21–23} Urea-based analogs of DUPA have served as the template for further development of various potent PSMA-targeting ligands and have been studied by the Kozikowski,^{24,25} Spiegel,^{26–28} and low groups,^{21–23} resulting in diagnostics and therapeutics that have considerable clinical potential.²⁹ In this manuscript, we achieved improved potency in AR silencing by incorporating a DUPA analog for PSMA-targeting in long-circulating LNP systems that contain optimized cationic lipid and siRNA against AR.

Results

Optimized LNP-AR21-siRNA silences AR expression *in vitro*

Our first optimization of the LNP-AR-siRNA employed the ionizable cationic lipid, DMAP-BLP,⁸ which is three times more potent in hepatic gene silencing than the DLin-KC2-DMA,⁴ the lipid used in earlier LNP-AR-siRNA studies.¹ Furthermore, the previously used AR siRNA (AR25-siRNA) was a 25-mer derived from an shRNA sequence that was shown to mediate silencing of AR and tumor growth delay *in vivo*.³⁰ In order to improve gene-silencing activity, a 21-mer siRNA (AR21-siRNA) was identified in a screen of siRNAs targeting the AR gene (data not shown). We incorporated two phosphorothioate linkages in order to reduce degradation by serum nucleases such as ribonuclease A (RNase A) and RNase A-like enzymes,³¹ as well as multiple 2'-OMe modifications to enhance stability in the presence of nucleases and to prevent undesired immune responses.³² To compare relative potencies of the 25-mer and 21-mer siRNAs, LNPs containing either AR21-siRNA or AR25-siRNA were incubated with LNCaP cells *in vitro* at siRNA concentrations of 0.5, 1.0, or 5.0 µg/ml for 48 hours and AR protein levels were analyzed by immunoblotting (Figure 1). Essentially, complete AR silencing was observed in cells treated with LNP containing AR21-siRNA at all dose levels tested, whereas AR protein knockdown was incomplete in cells treated with all

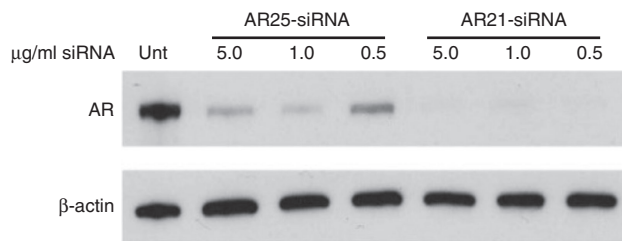


Figure 1 Lipid nanoparticle (LNP) encapsulating AR21-siRNA results in enhanced AR knockdown *in vitro*. AR21-siRNA was composed of two complementary RNA strands: sense strand (AR-S) 5'-cuGGGAAAGucAAGcccAudTsdT-3' and antisense strand (AR-AS): 5'-AUGGGCUUGACUUUCCcAGdTsdT-3'. LNCaP cells were incubated with 0.5, 1.0 or 5.0 µg/ml of LNP encapsulated with either AR25-siRNA or AR21-siRNA for 48 hours. Equal portions of protein samples were analyzed by immunoblotting to AR and β-actin, the latter serving as a loading control.

doses of AR25-siRNA. Untreated cells or cells treated with control siRNA (a scramble sequence or siRNA against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) showed no reduction of AR levels (Supplementary Figures S3 and S4). Alternatively, another siRNA sequence against the AR also showed appreciable AR knockdown (Supplementary Figure S4). These results indicate that the AR21-siRNA is a more potent sequence than AR25-siRNA for silencing the AR gene in LNCaP cells *in vitro*. In addition, an alternate siRNA against The AR21-siRNA was used in all subsequent experiments.

LNPs containing PEG-DSG accumulate in distal tumors

Previously reported studies of AR siRNA utilized LNPs containing PEG-DMG, a PEG-lipid with two C₁₄ alkyl chains.¹ PEG-lipids are required to produce LNP systems with defined sizes³³ and to prevent aggregation of the LNP after formation.³⁴ As noted elsewhere,^{13,14} PEG-lipids with C₁₄ chains rapidly dissociate from the LNP (dissociation halftimes of minutes or less) following *in vivo* administration and result in short circulation lifetimes, enhancing liver accumulation but reducing LNP accumulation at tumor sites. In order to improve tumor accumulation, LNP systems used in this work incorporated PEG-DSG, a lipid with C₁₈ alkyl chains. Previous work has shown that PEG-lipids with C₁₈ alkyl chains remain associated with LNP for days or longer,¹⁴ leading to extended circulation lifetimes following i.v. administration relative to those with shorter PEG chains.^{13,14,35} Consistent with these reports,³⁵ we found that LNP-siRNA, produced by microfluidic mixing incorporating 1.5% PEG-DSG, exhibited extended circulation properties compared to LNP with equivalent amounts of PEG-DMG.¹⁴ Increasing the total PEG-DSG lipid in the LNP from 2.5 to 5% resulted in a marked increase in circulation half-time from approximately 30 minutes to greater than 8 hours (Figure 5). To show that increased circulation lifetime translates to enhanced LNP accumulation in tumors, fluorescently labeled LNP-AR21-siRNA containing either 2.5 or 5 mol% PEG-DSG were prepared and administered intravenously once every day for 3 days at a dose of 10 mg siRNA/kg body weight in athymic nude mice bearing LNCaP tumors. Tumors were harvested at 4 and 24 hours following the final

injection of LNP formulations, fixed in 10% formalin, cryo-sectioned, and analyzed for LNP accumulation by confocal microscopy. Consistent with the enhanced circulation lifetime, tumor tissues from mice treated with LNP containing 5 mol% PEG-DSG showed significantly higher accumulation of fluorescence than those from mice injected with LNP containing 2.5 mol% PEG-DSG (Figure 2b). Fluorescent micrographs showed that LNPs accumulated in tumor tissues over time, with higher levels observed in tumors collected 24 hours postadministration than at 4 hours. Approximately fourfold more LNP containing 5 mol% PEG-DSG was observed in tumor tissues than LNP containing 2.5 mol% PEG-DSG (Figure 2b).

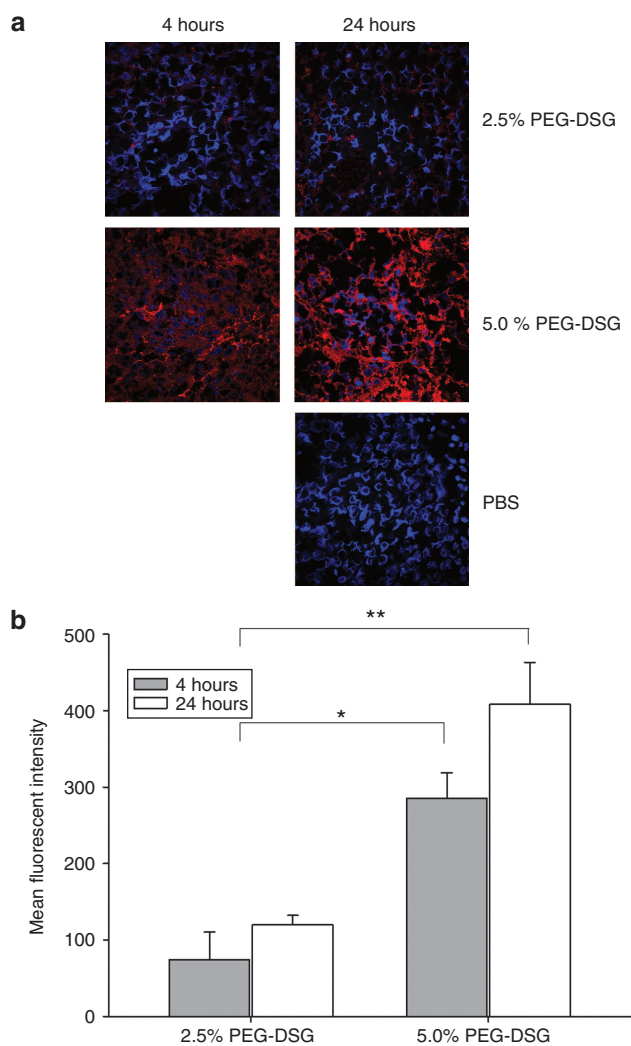


Figure 2 Systemic administration of lipid nanoparticle (LNP) containing 5 mol% PEG-DSG results in greater accumulation in LNCaP tumors compared to LNP containing 2.5 mol% PEG-DSG. (a) Mice were injected via the tail vein with fluorescently labeled LNP containing 5 or 2.5 mol% PEG-DSG (red) and were sacrificed 4 or 24 hours following the final i.v. injection. LNCaP tumors were harvested, cryo-sectioned, and analyzed under a confocal microscope. Representative images are shown. Nuclei were stained with Hoechst (blue). (b) Quantitation of uptake of fluorescent label into LNCaP tumor tissues was performed using ImageJ ($n = 5$) (<http://rsb.info.nih.gov/ij/>); * $P < 0.05$; ** $P < 0.01$.

Incorporation of (Glu-urea-Lys)-PEG-DSG into LNP systems results in increased cellular uptake and AR gene silencing in LNCaP cells and is enhanced via a PSMA-dependent endocytic mechanism

A durable PEG coating enhances LNP accumulation at sites of tumors, but also hinders uptake into cells and subsequent delivery of the siRNA payload to the cytoplasm. A strategy to overcome this problem is to incorporate into the LNP a small-molecule homing ligand that targets a cell surface receptor.^{15,18} As indicated under Methods, we synthesized a small-molecule PSMA-targeting ligand and chemically conjugated it to PEG-DSG, as shown in Figure 3.

Our PSMA-targeting analog is based on a Glu-urea-Lys scaffold and was designed according to previously published data.^{21–23} The PSMA-targeting lipid (13, Figure 3) is composed of a Glu-urea-Lys moiety tethered to the PEG lipid through a phenyl ring. We first synthesized the appropriately protected Glu-urea-Lys carboxylic acid 5 (Figure 3) from 2-[3-(5-amino-1-*tert*-butoxycarbonyl)pentyl]-ureido]pentanedioic acid di-*tert*-butyl ester (4)²⁵ and from the carboxylic acid 3 under peptide coupling conditions, followed by selective deprotection of the benzyl ester as shown in Scheme 1. The carboxylic acid 3 was synthesized from benzyl 5-hexynoate (1)³⁶ and (*p*-iodophenyl)acetic acid (2) by standard Sonogashira coupling.³⁷ Covalent attachment of the protected carboxylic acid (5) under peptide coupling conditions with the amino-PEG-lipid (11) followed by deprotection of functional groups afforded the desired (Glu-urea-Lys)-PEG-DSG lipid (13), used for PSMA-targeting LNP formulation of the siRNA. The experimental details and compound characterization are included in the **Supplementary Materials and Methods**.

To evaluate the effects of incorporating (Glu-urea-Lys)-PEG-DSG on cell uptake, fluorescently-labeled LNP systems were utilized. A PSMA-targeted LNP-AR-siRNA system containing 1 mol % (Glu-urea-Lys)-PEG-DSG and 1.5% PEG-DSG was compared to an untargeted LNP system containing a total of 2.5% PEG-DSG lipid. Uptake into LNCaP cells was analyzed by fluorescence microscopy. At 24 hours, fluorescence was approximately fourfold higher in cells treated with the PSMA-targeted LNP-AR-siRNA compared to those treated with the nontargeted LNP (Figure 4a).

To verify that uptake was via a PSMA-dependent mechanism, LNCaP cells were treated with (Glu-urea-Lys)-LNP in the presence or absence of the competitive reagent, 2-PMPA, at 100-fold molar excess to (Glu-urea-Lys)-PEG-DSG. The addition of 2-PMPA caused a substantial inhibition of (Glu-urea-Lys)-LNP uptake in LNCaP cells and little or no effect on the uptake of nontargeted LNP (Figure 4a). Uptake of PSMA-targeted and untargeted LNPs was also measured in the PSMA-negative PCa cell line PC-3 (ref. 38); no enhancement in LNP uptake due to the presence of the (Glu-urea-Lys) ligand in the LNP would be expected. In these cells, significantly greater LNP uptake was observed for nontargeted LNP compared to PSMA-targeted LNP (Figure 4b), possibly due to charge repulsion between the plasma membrane and the negatively charged (Glu-urea-Lys) targeting ligand.

We next evaluated whether the presence of the (Glu-urea-Lys)-PEG-DSG would lead to enhanced target gene silencing in LNCaP cells. In LNCaP cells treated with PSMA-targeted

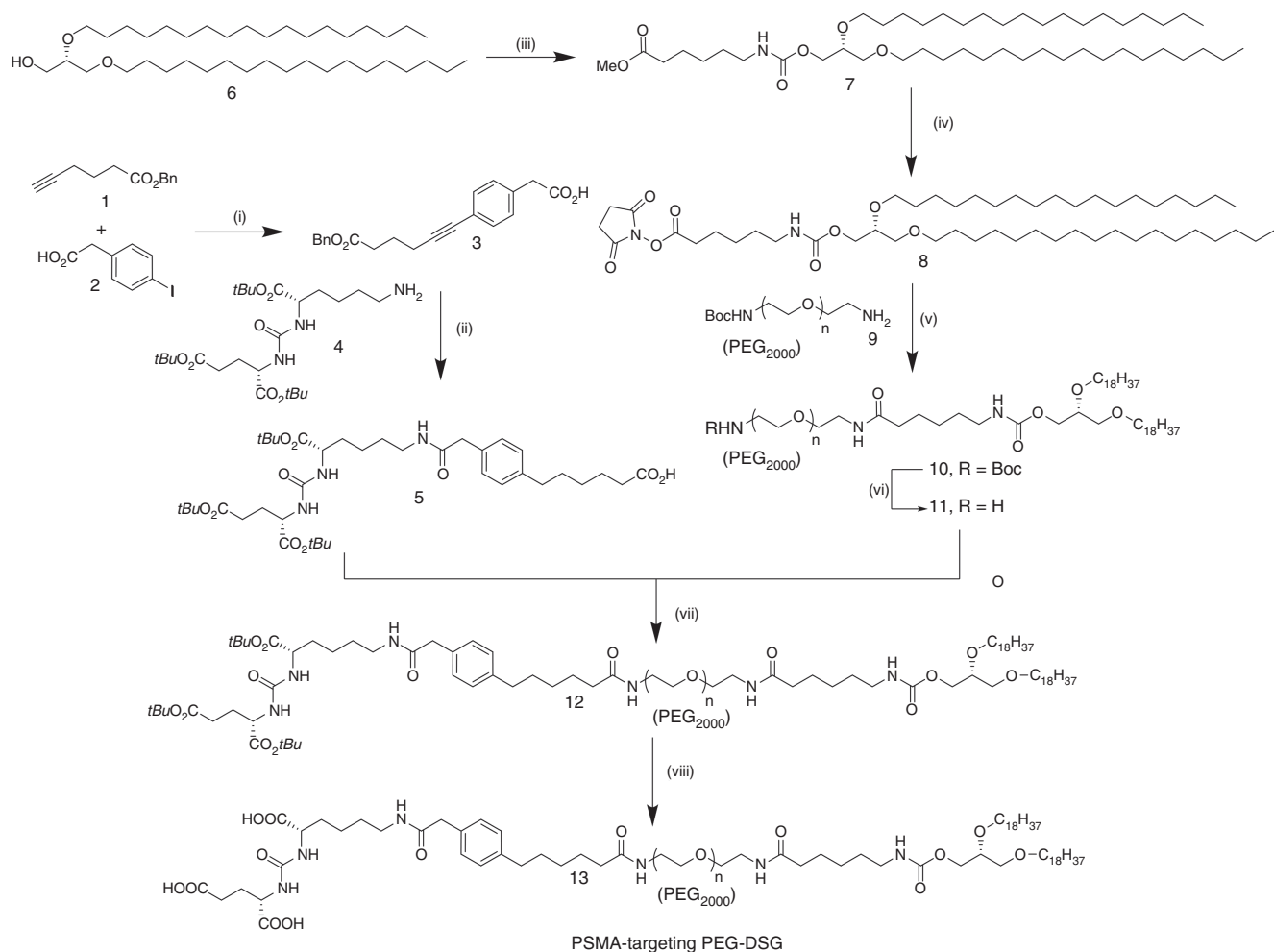


Figure 3 Reagents and conditions for synthesis of (Glu-urea-Lys)-PEG-DSG: (i) $(\text{PPh}_3)_2\text{PdCl}_2$, CuI, triethylamine/acetonitrile, 80 °C, 3 h, 95%; (ii) a. *N,N,N',N'*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), *N,N*-diisopropylethylamine (DIEA)/dichloromethane (DCM), rt, overnight 93% and b. H_2 , Pd-C/ methanol, rt, overnight, 92%; (iii) a. *N,N'*-disuccinimidyl carbonate (DSC), triethylamine, DCM, 0 °C to room temperature, overnight, and b. methyl 6-aminocaproate hydrochloride, pyridine/DCM, 0 °C to rt, overnight, 88%; (iv) a. LiOH, methanol/water/THF, 0 °C to rt, overnight, 96% and b. *N*-hydroxysuccinimide, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDAC), 4-(dimethylamino)pyridine (DMAP)/DCM, rt, overnight; (v) Triethylamine (TEA)/DCM, rt, overnight, 66%; (vi) formic acid/DCM (2:1), rt, overnight, 91%; (vii) HBTU, DIEA / *N,N*-dimethylformamide (DMF), rt, overnight, 43%; (viii) neat formic acid, 48%.

LNP containing 0.5 or 1 mol% (Glu-urea-Lys)-PEG-DSG, AR protein levels were significantly lower than in cells incubated with nontargeted LNP (Figure 4c). In addition, a greater inhibition of AR expression was observed with the formulation containing 1 mol% (Glu-urea-Lys)-PEG-DSG than 0.5 mol% (Glu-urea-Lys)-PEG-DSG.

LNP-AR-siRNA systems containing the PSMA-targeting Glu-urea-Lys ligand exhibit long circulation lifetimes

As described previously, LNP systems exhibiting long circulation characteristics are essential to achieving enhanced accumulation at tumor sites. In this context, it was important to establish that the presence of the (Glu-urea-Lys)-PEG-DSG did not negatively impact the circulation lifetime of the PEG-DSG LNP systems employed here. This is of potential concern since (Glu-urea-Lys)-PEG-DSG contains three carboxylic acid chemical groups at its hydrophilic terminal end (Figure 3). The pK_a values of these carboxylic acid groups

are predicted to be 3.11, 3.69, and 3.99 (Marvin, ChemAxon, <http://www.chemaxon.com/products/marvin/>), indicating that this PSMA-targeting ligand will possess a strong negative charge at physiological pH. Consistent with these estimates, the zeta-potential of LNP containing 1 mol% of (Glu-urea-Lys)-PEG-DSG was determined to be -14.97 ± 9.34 mV, whereas nontargeted LNP exhibited a zeta-potential of -4.91 ± 11 mV. LNPs exhibiting negative charges can be rapidly cleared from the bloodstream via opsonization by serum proteins and subsequent accumulation in the reticuloendothelial cells of the liver and spleen.^{39,40}

The circulation lifetime of the PSMA-targeting LNP was determined following i.v. administration of tritiated (^3H) PSMA-targeted or nontargeted LNP to mice at 1 mg siRNA/kg body weight (see Materials and Methods). Blood was collected via intracardiac sampling at 0.5, 2, 8, 24 hours postinjection and the percentage of the injected LNP remaining in the circulation was determined (Figure 5). Importantly, the

PSMA-targeted and nontargeted LNPs exhibited very similar circulation properties; both formulations had $t_{1/2}$ values of approximately 10–12 hours. Since it was previously shown that LNP-siRNA systems incorporating PEG-DSG accumulate in tumors (Figure 2),¹¹ these data support the probability that (Glu-urea-Lys)-LNP-siRNA will exhibit similar accumulation at distal tumor sites.

PSMA-targeted (Glu-urea-Lys)-LNPs enhance AR knockdown in mice bearing LNCaP tumors

The potency of long-circulating, PSMA-targeted (Glu-urea-Lys)-LNP systems was evaluated in athymic nude mice bearing LNCaP tumors. When serum PSA levels reached 50–75 ng/ml, mice were randomly assigned to three experimental groups and treated i.v. with phosphate buffered saline (PBS) or PSMA-targeted or nontargeted LNP at a dose of 5 mg siRNA/kg body weight. While serum PSA levels rose to 40% above the pretreatment levels by Day 14 in the control group, PSA levels remained relatively unchanged compared to baseline in mice treated with nontargeted LNP (Figure 6a). This represents a significant improvement over previous studies, since similar effects on PSA levels were observed at half the dose used for the first generation LNP-AR-siRNA.¹ The enhanced potency can be attributed to the combined effects of a more potent cationic lipid and a more active siRNA payload, as well as greater LNP accumulation at the distal tumor site due to enhanced circulation characteristics. Even more promising were the results from the PSMA-targeted (Glu-urea-Lys)-LNP group; in these mice, a 45% reduction in the serum PSA levels was observed at day 14 compared to the control group (Figure 6a). In mice treated with siRNA formulated in the PSMA-targeted (Glu-urea-Lys)-LNP, a decrease in serum PSA levels relative to pretreatment levels was observed. To directly verify AR gene silencing, levels of AR and PSA mRNAs were assessed in samples from LNCaP tumors at day 14 via quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). In the PSMA-targeting (Glu-urea-Lys)-LNP group, there was a significant reduction in AR mRNA transcript levels compared to mice treated with the nontargeted LNP or PBS (Figure 6b). The siRNA formulated in the nontargeted LNP did not cause a significant reduction in mRNA transcript levels compared to the PBS control (Figure 6b). These results are consistent with AR protein levels (Supplementary Figure S1). Similar results were obtained when levels of PSA mRNA transcript were evaluated. There was a significant decrease in levels of PSA mRNA in the PSMA-targeted LNP treatment group compared to mice treated with nontargeted LNP-AR-siRNA (Figure 6c).

Intravenous administration of PSMA-targeted (Glu-urea-Lys)-LNP reduces cellular proliferation, but does not enhance apoptosis

The ultimate goal in cancer treatment is to induce tumor regression; this has never been observed in the LNCaP xenograft model using gene-silencing strategies, even in conjunction with complete androgen ablation via castration.^{1,30}

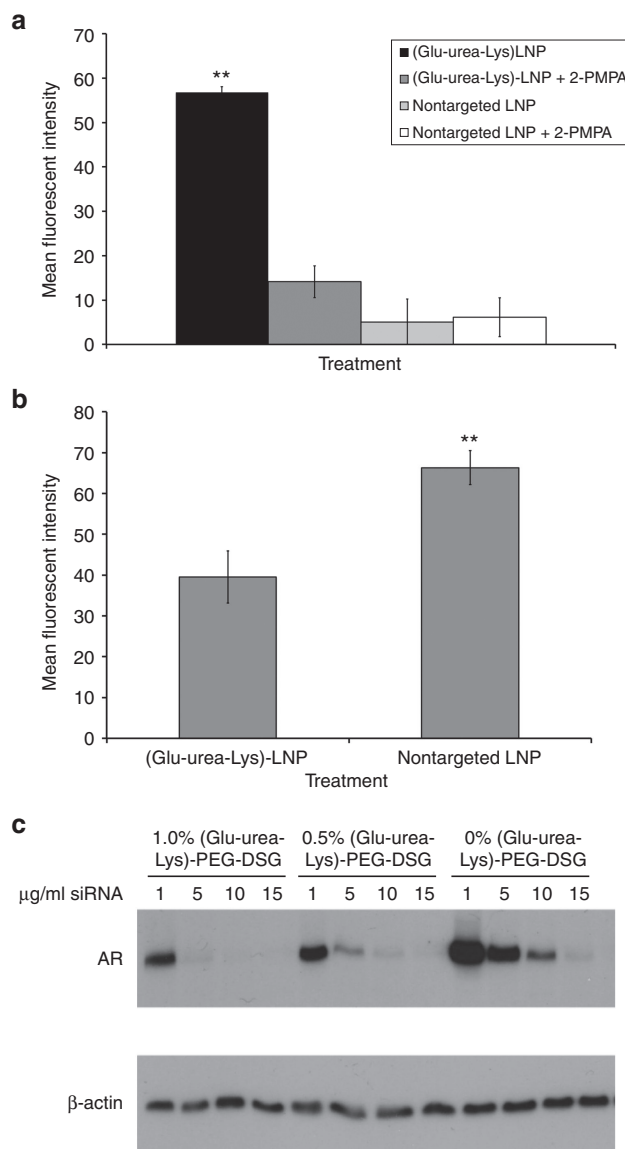


Figure 4 Lipid nanoparticle (LNP) formulations containing (Glu-urea-Lys)-PEG-DSG enhances cellular uptake and inhibition of AR expression in AR-positive LNCaP cells *in vitro*. (a) LNCaP cells were incubated for 24 hours with fluorescently labeled AR-targeting LNP at an siRNA concentration 5 μ g/ml. Cellular uptake was quantified using Cellomics ArrayScan and is expressed as mean fluorescent intensity per cell. Approximately 400 cells were measured in four individual wells ($n = 4$). 2-PMPA was added to compete with AR-dependent uptake. Statistical significance was determined for (Glu-urea-Lys)-LNP in comparison to all the other groups; $**P < 0.01$. (b) PC-3 (AR-negative) PCa cells were incubated with 5 μ g/ml of fluorescently labeled (Glu-urea-Lys)-LNP for 24 hours. 2-PMPA was added as a competitor. Cellular uptake was quantified using Cellomics ArrayScan and expressed as mean fluorescent intensity per cell. Approximately 400 cells were measured in four individual wells ($n = 4$). Statistical significance was determined between non-targeted LNP versus PSMA-targeted (Glu-urea-Lys)-LNP groups. $**P < 0.01$. (c) LNCaP cells were incubated with 1, 5, 10, or 15 μ g/ml siRNA formulated as indicated for 48 hours. Levels of AR protein were analyzed by immunoblotting, with β -actin as the loading control.

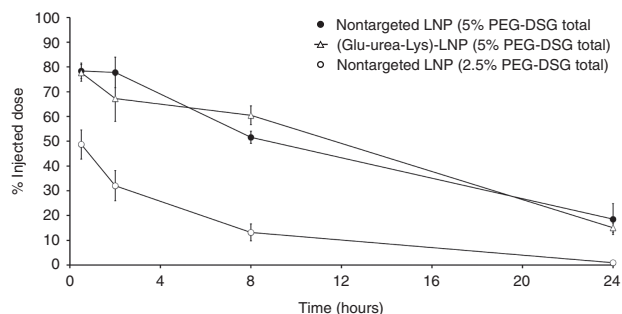


Figure 5 PSMA-targeted (Glu-urea-Lys)-LNP and non-targeted LNP exhibit similar pharmacokinetics. PSMA-targeted (Glu-urea-Lys)-LNP and nontargeted lipid nanoparticle (LNP) were synthesized with trace amounts of [^3H] CHE. The LNP formulations contained DMAP-BLP/DSPC/cholesterol/(Glu-urea-Lys)-PEG-DSG/PEG-DSG molar ratios of 50/10/35/1/4 or 50/10/35/0/5 or DMAP-BLP/DSPC/cholesterol/PEG-DSG at molar ratios of 50/10/37.5/2.5. Mice treated by tail vein injection with 1 mg/kg siRNA formulated in PSMA-targeted (Glu-urea-Lys)-LNP or non-targeted LNP. Plots show percentage of the total injected dose remaining as a function of time. Each data point represents the mean \pm SD ($n = 4$). Circulation half-time ($t_{1/2}$) for nontargeted 2.5% PEG-DSG LNP, nontargeted 5% PEG-DSG LNP and (Glu-urea-Lys)-LNP was ~ 0.5 , 8.7, and 11.6 hours, respectively. Area under the curve (AUC) for nontargeted 2.5% PEG-DSG LNP, nontargeted 5% PEG-DSG LNP and (Glu-urea-Lys)-LNP was $309.18 \pm 35.22\%$ -hours, $1,066.57 \pm 57.21\%$ -hours, and $1,095.90 \pm 45.65\%$ -hours, respectively.

LNCaP tumors were isolated from mice treated with PBS as a control or AR21-siRNA formulated in PSMA-targeted or nontargeted LNPs on day 14. Tumors were sectioned and analyzed by immunohistochemistry for cellular proliferation as indicated by staining for Ki67. Ki67 is a marker that is detected during all phases of the cell cycle, but is absent in quiescent cells.⁴¹ Data indicated that treatment with AR21-siRNA formulated in the PSMA-targeted (Glu-urea-Lys)-LNP caused an approximate 50% decrease in Ki67-positive cells relative to levels in tumors from PBS-treated mice (Figure 7a,b). No effect on apoptosis was observed based on TUNEL staining of LNCaP tumors from treated and untreated animals (Figure 7c,d). The lack of apoptotic cells may be due to the short duration of this study (14 days).

Discussion

In previous work, we showed that AR25-siRNA formulated in LNP containing DLin-KC2-DMA and PEG-DMG (a PEG-lipid that rapidly dissociates from the LNP following i.v. injection) inhibited expression of the AR gene in an animal model of PCa. The potential for translating this work into the clinic is limited by the high doses necessary to achieve an appreciable pharmacological effect. Here, we describe the optimization of properties of the LNP-AR-siRNA systems to achieve improvements in the *in vivo* gene-silencing potency. An improved cationic lipid, DMAP-BLP, was used and the siRNA sequence and chemistry were optimized. In addition, a PEG-lipid (PEG-DSG) that does not readily dissociate from LNP systems and a PSMA-targeting lipid were incorporated, resulting in a longer lifetime in circulation, improved accumulation at distal tumor sites, and PSMA-mediated uptake into prostate tumor cells.

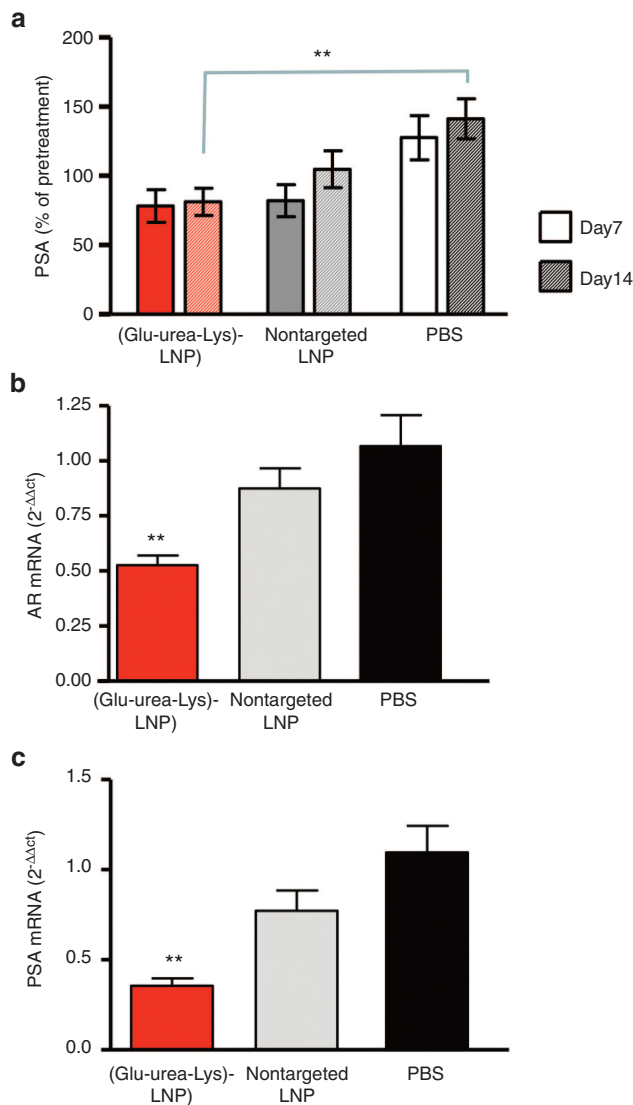


Figure 6 Systemic administration of siRNA formulated in PSMA-targeted (Glu-urea-Lys)-lipid nanoparticle (LNP) lowers serum PSA levels and enhances AR gene silencing. Mice were treated via tail vein injection with PBS as a control or with the 5 mg/kg of siRNA formulated in PSMA-targeted (Glu-urea-Lys)-LNP or nontargeted LNP. (a) Percentages of serum PSA levels are relative to PSA levels one day prior to treatment. Serum PSA levels were measured on days 7 and 14. (b) Quantitative real-time PCR was used to assess AR mRNA levels from tumor tissue on day 14. (c) Quantitative real-time PCR was used to assess PSA mRNA levels from tumor tissue on day 14. Data points are the means \pm SE ($n = 6-7$); $**P < 0.01$.

Inclusion of 5 mol% PEG-DSG clearly resulted in improved LNP accumulation at the tumor site compared to LNP with lower amounts of the PEG-lipid (Figure 2). This had a minimal impact on LNP-siRNA potency, as judged by PSA levels, compared to the previously reported work.¹ However, although the more stable PEG coatings lead to improved tumor accumulation, the PEG coat can impede cellular uptake, reducing the activity of the LNP payload. Ideally, the PEG-lipid would remain associated with the LNP until arrival at the tumor site, after which it would dissociate. For actively targeted systems

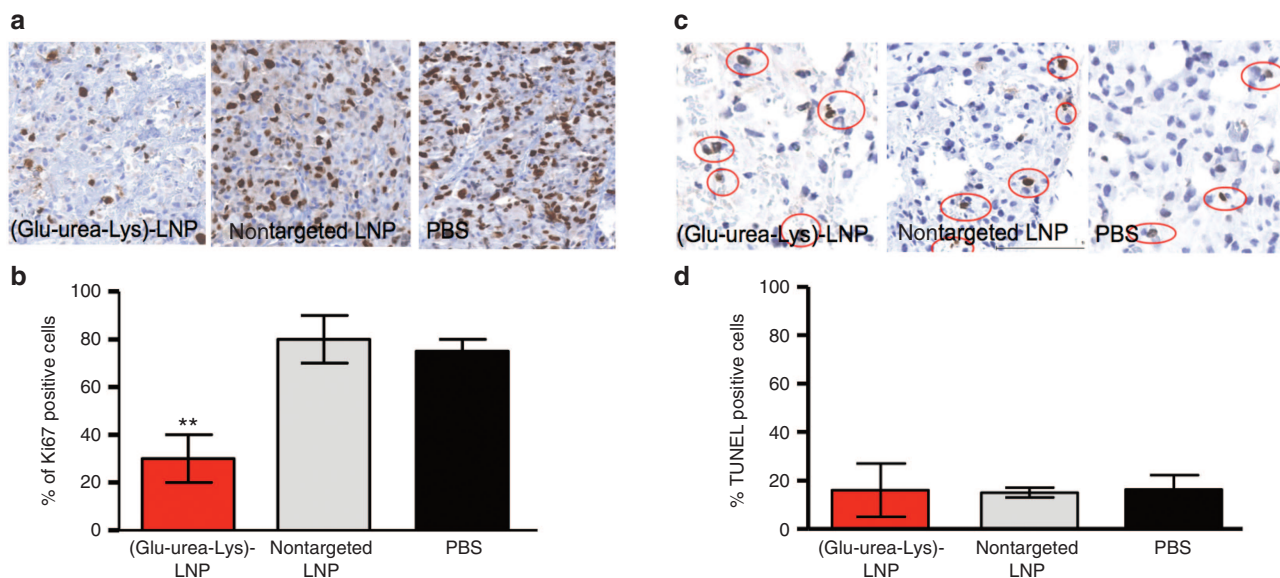


Figure 7 Systemic administration of PSMA-targeted (Glu-urea-Lys)-lipid nanoparticle decreases cellular proliferation but does not induce apoptosis in tumor cells. Tissues from the mice described in **Figure 6** were analyzed. **(a)** Representative images of Ki67 (cell proliferation marker) stained samples. **(b)** Quantitation of Ki67 stained tumor sections plotted as means \pm SD ($n = 6$); $**P < 0.01$. **(c)** Representative images of TUNEL (cell apoptosis marker) stained samples, with red circles highlighting stained cells. **(d)** Quantitation of TUNEL stained tumor sections plotted as the means \pm SD ($n = 6$). No significant difference was observed for TUNEL-stained tumor sections.

such as those containing the PSMA-targeted (Glu-urea-Lys)-PEG-lipid, it is possible that the presence of the PEG-lipid may reduce the endosomolytic properties of the LNP following ligand-dependent uptake.

The results presented demonstrate significantly greater reductions in AR (~40%) and PSA (~50%) mRNA when the AR21-siRNA was formulated in the PSMA-targeted (Glu-urea-Lys)-LNP system compared to the nontargeted LNP system (**Figure 6**). The use of the small molecule targeting ligand has a number of advantages compared with use of larger entities such as antibodies; most notably, the small molecule targeting ligands can be incorporated at the time of LNP manufacture rather than after the LNP is formed and well-defined, scalable systems are more readily achieved. It is perhaps surprising that the highly negatively charged PSMA-targeting Glu-urea-Lys ligand adopted a configuration when incorporated into the LNP in which it is available on the external surface for ligand binding. A concern was that the targeting ligand could become associated with the cationic lipid during formulation and is buried in the interior of the LNP as a result. It is possible that the PEG tether limits internal localization of the (Glu-urea-Lys)-PEG-DSG due to polarity and/or steric effects.

The data presented here demonstrate that the potency of LNP siRNA systems to silence the AR following i.v. administration can be improved from dose levels of 10 mg/kg siRNA to 5 mg/kg when more potent cationic lipids, higher levels of tumor accumulation and the PSMA targeting ligand is employed. However, the dose levels of 5 mg siRNA/kg body weight required for AR silencing are still approximately an order of magnitude too high for clinical applications to be envisaged, and further work is required to improve potency. This is especially important for achieving tumor growth regression, as we observed no statistically significant difference

in the tumor sizes between PSMA-targeted LNP treatment mice and control groups, despite appreciable knockdown of the AR and PSA in the treatment group (**Supplementary Figure S2**). Ways forward include higher levels of external PSMA-targeting lipid or extending the PSMA-targeting moieties beyond the PEG coat to improve targeting could be envisioned. It has been noted that use of a PEG5000 tether to extend the targeting ligand further from the LNP surface resulted in a 160-fold improvement in targeting capability *in vitro*.⁴² Incorporation of an additional ligand that targets another cell surface factor, such as the prostate stem cell antigen,⁴³ may synergistically enhance the LNP uptake. Finally, nanoparticle systems smaller than 50 nm exhibit significantly improved delivery to tumor cells by virtue of their ability to achieve improved tumor penetration.⁴⁴ Of relevance to this approach, the microfluidic mixing technique does offer the possibility of manufacturing LNP with sizes as small as 20 nm in diameter,³³ and efforts will be directed toward reductions in the size of the LNP-siRNA system.

The potential of targeting ligands is demonstrated by recent highly encouraging clinical data utilizing siRNAs directly conjugated to a *N*-acetylgalactosamine (GalNAc) targeting ligand which have demonstrated efficient systemic delivery allowing robust and durable mRNA knockdown of various targets in hepatocytes.⁴⁵ The GalNAc ligand targets the highly expressed ASGPR receptor in hepatocytes—which presents the important advantages of exhibiting high copy number and a quick turnover. In this aspect, the selection of efficient receptors for targeting is a key parameter for the success of targeted siRNA systems. In addition, recently developed biodegradable lipids enabling rapidly eliminated LNPs that display improved tolerability and safety profiles can be incorporated into potent and safe LNP-siRNA systems.⁴⁶ It is therefore expected that this continuous progress in the field

of siRNA medicines will lead to LNP-AR-siRNAs targeting PCas that have direct clinical utility.

Materials and methods

Materials. 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) was purchased from Avanti Lipids (Alabaster, AL), cholesterol (Chol) was purchased from Sigma (St. Louis, MO). 1,1'-Dilinoylel-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil) was bought from Invitrogen (Burlington, ON, Canada). The ionizable cationic lipid DMAP-BLP and PEG-lipids (R)-2,3-bis(octadecyloxy)propyl-1-(methoxy poly(ethylene glycol) 2000) carbamate (PEG-DMG) and (R)-2,3-bis(stearoyloxy)propyl-1-(methoxy poly(ethylene glycol)2000) carbamate (PEG-DSG) were synthesized at Alnylam Pharmaceuticals (Cambridge, MA). The PSMA inhibitor 2-(phosphonomethyl)-pentanedioic acid (2-PMPA) was purchased from Cedarlane (Burlington, ON, Canada).

Cell culture, cell lines, and reagents. LNCaP and PC-3 human PCa cell lines were used in all *in vitro* experiments.^{38,47} LNCaP and PC-3 cells were obtained from ATCC and were not passaged beyond 6 months after receipt or resuscitation. LNCaP cells were maintained in RPMI 1640 (Life Technologies, Burlington, ON, Canada), supplemented with 10% heat-inactivated fetal bovine serum. PC-3 cells were maintained in DMEM (Life Technologies, Burlington, ON, Canada) supplemented with 5% heat-inactivated fetal bovine serum. Both cell lines were incubated at 37 °C with 5% CO₂.

siRNA sequences. The sequence of the human AR gene (GenBank accession no. NM_000044) was extracted from the NCBI Entrez nucleotide database. The AR21-siRNA was composed of two complementary RNA strands: sense strand (AR-S) 5'-cuGGAAAGucAAGcccAudTsdT -3' and antisense strand (AR-AS): 5'-AUGGGCUUGACUUUCCcAG-dTsdT-3'. The two strands of the AR21-siRNA are modified 21-nt oligoribonucleotides that contain phosphorothioate linkages (indicated as "s") between the 3'-deoxythymidine (dT) overhangs and that include multiple 2'-OME modifications (indicated by lower-case letters).

Oligonucleotide synthesis. Oligonucleotides were synthesized using an ABI-394 DNA/RNA synthesizer. Solvents/reagents, solid-supports and phosphoramidites were all purchased from Glen Research or ChemGenes and used as received. Oligonucleotides were synthesized using modified synthesis cycles provided with the instrument. After solid phase synthesis, the oligonucleotides were deprotected and released from the support. The crude oligonucleotides were purified by anion-exchange high performance liquid chromatography (HPLC) to >85% (260 nm) purity and then desalted by size exclusion chromatography. The isolated yields for the final oligonucleotides were calculated based on the respective ratios of measured to theoretical 260 nm optical density units (ODUs) and their identity was confirmed by LC/MS. Hybridization to generate double-stranded siRNA duplexes was performed by mixing equimolar amounts of purified complementary strands to a final concentration of 20 μmol/l in 1×

PBS buffer pH 7.4, and by heating the solution over a water bath at 95 °C for 5 minutes and cooling it to room temperature over a period of approximately 12 hours.

Chemical synthesis. Detailed chemical synthesis procedures and characterization data of all intermediates (according to **Figure 3**) is given as **Supplementary Materials and Methods** only.

Preparation of tris-(t-Butyl) protected (Glu-urea-Lys)-PEG-DSG (12). Compound **5** (see **Supplementary Materials and Methods**, 1.00g, 0.37 mmol) and compound **11** (see **Supplementary Materials and Methods**, 0.35g, 0.48 mmol) were dissolved in DMF (10ml) under argon atmosphere. HBTU (0.22g, 0.58 mmol) and DIEA (0.250ml, 1.46 mmol) were added to the mixture, and the mixture was stirred overnight. The solvents were removed under reduced pressure, and the residue was purified by silica gel chromatography (5–20% MeOH in DCM) to yield compound **12** (0.53g, 43%) as a white solid. ¹H NMR (400 MHz, DMSO-*d*₆): δ 7.78 (t, *J* = 5.7 Hz, 2H), 7.14–7.05 (m, 3H), 6.26 (m, 2H), 4.11–3.83 (m, 5H), 3.75–3.63 (m, 3H), 3.60–3.30 (m, OCH₂- and O-CH-protons, PEG, O-alkyl and glycerol), 3.27–3.12 (m, 5H), 3.07–2.85 (m, 4H), 2.30–2.15 (m, 3H), 2.03 (t, *J* = 7.4, 2H), 1.59–1.33 (m, 23H), 1.30–1.18 (m, 60H), 0.84 (t, *J* = 6.7 Hz, 6H). MS calc. Av. MW. ~ 3,343; MALDI Av. MW found: 3,342.

Preparation of (Glu-urea-Lys)-PEG-DSG (13). Formic acid (20ml) and anisole (0.5ml) were added to compound **12** (0.50g, 0.14 mmol), and the mixture was stirred at room temperature for 24 hours. The solvents were removed under reduced pressure, and the residue was coevaporated with toluene (2×50ml). The crude compound was purified by flash silica gel column chromatography using a gradient of 10–50% MeOH in DCM followed by MeOH to yield compound **13** as a white solid (230mg, 48%). ¹H NMR (400 MHz, DMSO-*d*₆): δ 7.96 (bs, 2H), 7.80–7.77 (m, 3H), 7.22–6.95 (m, 6H), 4.09–3.80 (m, 6H), 3.68–3.60 (m, 2H), 3.59–3.40 (m, OCH₂- and O-CH-protons, PEG), 3.39–3.08 (m, 33H), 3.05–2.87 (m, 5H), 2.76–2.57 (m, 4H), 2.36–2.14 (m, 3H), 2.03 (t, *J* = 7.5 Hz, 5H), 1.78 (brs, 2H), 1.65–1.60 (m, 2H), 1.55–1.30 (m, 15H), 1.29–1.13 (m, 61H), 1.10–1.00 (m, *J* = 6.5 Hz, 19H), 0.84 (t, *J* = 6.7 Hz, 6H). MS calc. Av. MW. ~ 3,190; MALDI Av. MW found: ~3,193.

Encapsulation of siRNA into LNP using microfluidic mixing. LNP formulations were constructed using a microfluidic staggered herringbone micromixer (SHM) provided by Precision Nanosystems (Vancouver, BC) as described previously.³³ The siRNA solutions were prepared in 25 mmol/l acetate buffer at pH 4.0. For comparison studies between AR21-siRNA and AR25-siRNA, lipid stocks were co-dissolved in ethanol at molar ratio of 40% DMAP-BLP, 17.5% DSPC, 40% Chol, and 2.5% PEG-DMG. For PEG-lipid comparison studies containing 2.5 mol% total PEG-lipid, lipid stocks were codissolved in ethanol at appropriate molar ratios: 50 mol% DMAP-BLP, 10 mol% DSPC, 37.3 mol% Chol, 0/0.5/1 mol% (Glu-urea-Lys)-PEG-DSG, 2.5/2/1.5 mol% PEG-DSG, and 0.2 mol% Dil. For cationic LNPs encapsulating AR21-siRNA containing 5 mol% total PEG-lipid used for *in vivo* studies,

lipid stocks were codissolved in ethanol at the following molar ratios: 50 mol% DMAP-BLP, 10 mol% DSPC, 34.8 mol% Chol, 0/1 mol% (Glu-urea-Lys)-PEG-DSG, 5/4 mol% PEG-DSG, and 0.2 mol% DiI. The siRNA to lipid ratio for all formulations was kept at 0.067 (wt/wt). The siRNA and lipid ethanol solutions are mixed at a 1:3 ratio, respectively. Total formulation volume ranges from 4 to 40 ml depending on experiment size.

Characterization of LNP. The mean diameter of the vesicles was determined using a NICOMP370 particle sizer (Nicomp Particle Sizing, Santa Barbara, CA). Intensity-weighted size and distribution data was used. LNPs utilized for AR21-siRNA and AR25-siRNA comparisons were 56.5 ± 17.6 nm and 55.9 ± 19.61 nm in size, respectively. The size of LNP containing a total of 2.5 mol% PEG-lipid, (Glu-urea-Lys)-LNP-AR21-siRNA (1 mol% (Glu-urea-Lys)-PEG) was 84.5 ± 32.5 nm, (Glu-urea-Lys)-LNP-AR21-siRNA (0.5 mol% (Glu-urea-Lys)-PEG) was 77.5 ± 23.6 nm, and the non-targeted LNP-AR21-siRNA (0 mol% (Glu-urea-Lys)-PEG) was 73.6 ± 31.6 nm. For LNPs containing a total of 5.0 mol% PEG-lipid: (Glu-urea-Lys)-LNP-AR21-siRNA (1 mol% (Glu-urea-Lys)-PEG-DSG) the size was 55.9 ± 22.5 nm, and the non-targeted LNP-AR21-siRNA (no targeting PEG-lipid) exhibited a size of 45.3 ± 16.5 nm. Zeta potentials of LNPs were measured using the Malvern Nano ZS (Worcestershire, UK). Lipid concentrations were determined based on total cholesterol content determined using the Cholesterol E enzymatic assay from Wako Chemicals (Richmond, VA). Concentrations of siRNA were measured using Quant-iT RiboGreen RNA Reagent and Kit (Life Technologies) according to the manufacturer's protocol. Encapsulation efficiency was determined by analysis of siRNA concentrations after addition of 1% Triton-X-100 (Sigma) to intact LNPs.

Western blotting. LNCaP cells were plated in 12-well plates (2.0×10^5 cells per well). Cells were washed with PBS and lysed with RIPA buffer (1% NP-40, 0.25% deoxycholic acid) supplemented with protease inhibitors (Roche Diagnostics, Laval, Quebec, Canada). Aliquots of 10 μ g of total protein, as quantified by Bradford Assay, were analyzed by immunoblotting. Antibodies to AR were purchased from Santa Cruz Biotechnology (AR-441) (Santa Cruz, CA). Antibodies to β -Actin were purchased from Abcam (Cambridge, MA). Antigen-antibody complexes were detected using Millipore Immobilon Western Chemiluminescent HRP Substrate (Billerica, MA).

Confocal microscopy of tumor sections. Excised LNCaP tumors from groups of three mice each were maintained in 10% buffered formalin and then cryo-sectioned by Wax-IT Histology Services (Vancouver, BC). Tissue sections were fixed onto glass cover slips and examined under an Olympus FV1000 (Center Valley, PA) laser-scanning microscope. For each mouse xenograft, 5 tissue sections and 20 fields of view were examined. LNP fluorescence was quantified using Image J (v1.50b, <https://imagej.nih.gov/>).

Fluorescent microscopy. LNCaP cells were seeded at 2.0×10^4 cells per well and PC-3 cells were seeded at 1.5×10^4 cells per well in a 96-well format. Cells were treated with 5 μ g/ml (based on AR21-siRNA weight) (Glu-urea-Lys)-LNP-AR21-siRNA (1

mol% (Glu-urea-Lys)-PEG-DSG) or LNP-AR21-siRNA for 24 hours. 2-PMPA was added as a competitive reagent at 100-fold molar excess to (Glu-urea-Lys)-PEG-DSG. Cells were fixed in 3% PFA with Hoechst's stain and examined using a Cellomics ArrayScan VTI HCS Reader (Thermo Scientific, Pittsburgh, PA).

Pharmacokinetics of PSMA-targeted and nontargeted LNP-AR-siRNAs. Female CD1 outbred mice (6 to 8 weeks old) were obtained from Charles River Laboratories (Wilmington, MA) and acclimated for one week prior to use. Mice were given 1 mg/kg of either PSMA-targeted or nontargeted LNP-AR21-siRNA, containing trace amounts of [3 H]-cholesteryl hexadecylether (CHE), via the lateral tail vein injection. At 0.5, 2, 8, and 24 hours postinjection, mice were euthanized. Blood was collected via intracardiac sampling in Vacutainer tubes containing EDTA (BD Biosciences, Canada) and was chemically digested at room temperature using Solvable (Perkin-Elmer, Wellesley, MA) followed by decolorization with hydrogen peroxide (30% w/w). The amount of LNP in blood was determined by liquid scintillation counting in Pico-Fluor 40 (Perkin-Elmer). All procedures involving animals were approved by the Animal Care Committee at the University of British Columbia and performed in accordance with the guidelines established by the Canadian Council on Animal Care.

Treatment of mice with PSMA-targeted and non-targeted LNP-AR21-siRNAs. Xenograft prostate tumors were established as described.³⁰ Briefly, LNCaP cells (5×10^6) in 0.1 ml Matrigel (Becton Dickinson Labware, Mississauga, Ontario, Canada) were inoculated subcutaneously in two flank regions of 6- to 8-week-old male athymic nude mice (Harlan Sprague Dawley, Indianapolis, IN) under halothane anesthesia using a 27-gauge needle. When the tumors became palpable, volumes were measured, and blood was collected from the tail vein to assess serum PSA by ELISA (ClinPro International, Union City, CA). Once PSA values reached 50–75 ng/ml, animals were randomized into three groups and were treated with (Glu-urea-Lys)-LNP-AR21-siRNA, nontargeted LNP-AR21-siRNA, or saline (PBS) via the tail vein. Animals treated with PBS were given one injection on day 1. Animals given (Glu-urea-Lys)-LNP-AR21-siRNA and non-targeted LNP-AR21-siRNA were treated on days 1, 2, 3, 7, 9, and 11 with 5 mg siRNA/kg of mouse body weight. Mice were sacrificed on day 14. Serum PSA levels and levels of mRNA encoding AR and PSA were determined, and immunohistochemical analyses of xenograft tumors were performed. All animal procedures were performed according to the guidelines of the Canadian Council of Animal Care and with appropriate institutional certification.

qRT-PCR. Total RNA from mouse tissue was isolated using Trizol according to the manufacturer's protocol (Life Technologies, Burlington, ON, Canada). RNA extracts were reverse transcribed using random hexamers (Applied Biosystems, Foster City, CA) and MMLV reverse transcriptase (Invitrogen). Triplicates of the resulting cDNA were used as templates for quantitative real-time PCR on the Applied Biosystems 7900HT Fast Real-Time PCR System following the SYBR Green PCR

Master Mix protocol as described previously.⁴⁸ 18S rRNA was used as an endogenous control and relative quantitation was determined using the comparative Ct ($2^{-\Delta\Delta C_t}$) method. Primer sequences for AR were: sense 5'-GCAGGCAAGAGCACT GAAGATA-3' and antisense 5'-CCTTTGTGTAACCTCCCTT GA-3'. Primers for PSA were: sense 5'-TGTGCTTCAAGG TATCACGTCAT-3' and antisense: 5'-TGTACAGGGAAGG CCTTTCG-3'. Primers for 18S rRNA were: sense 5'-CGA TGCTCTTAGCTGAGTGT-3' and antisense 5'-GGTCCAAG AATTCACCTCT-3'.

Immunohistochemistry of tumor tissues. Immunohistochemistry (IHC) staining was conducted using a Ventana auto-stainer model Discover XT (Ventana Medical System Oro Valley, AZ) with an enzyme-labeled biotin streptavidin system and solvent-resistant DAB Map kit. The antibody used for Ki67 was from Lab Vision Corporation, and was diluted 1:500 in 1X PBS. The TUNEL, or apoptosis, study was done using a TdT enzyme kit (Roche, Indianapolis, IN). IHC slides were scanned on a Leica Digital Imaging System. Images were viewed using Digital Image Hub, (SlidePath, Dublin, Ireland). The proliferation factor is defined as the average number of Ki67 positive cells per core, or per section. The apoptotic factor is the average number of apoptotic positive cells per core, or per section.

Statistical analyses. All statistical analyses were performed using GraphPad. Initially, a one-way analysis of variance 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.

Supplementary material

Figure S1. Systemic administration of siRNA formulated in PSMA-targeted (Glu-urea-Lys)-LNP enhances AR protein knockdown in vivo

Figure S2. Effect of systemically administered LNP formulations on tumor growth.

Figure S3. LNP encapsulating scramble (SC)-siRNA does not result in AR knockdown in vitro.

Figure S4. siRNA against GAPDH does not result in AR knockdown in vitro

Materials and Methods

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