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Targeting the TMPRSS2/ERG fusion mRNA using liposomal nanovectors enhances docetaxel treatment in prostate cancer

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Abstract

Background.—The TMPRSS2/ERG (TE) fusion gene is present in half of prostate cancers. The TMPRSS2 and ERG junction of the fusion mRNA constitutes a cancer specific target. Although docetaxel-based chemotherapy is the second line of therapy following development resistance to androgen ablation therapies, it is not curative. Therefore, development of nontoxic novel monotherapies for targeting TE mRNA in prostate cancer patients and for increasing the clinical efficacy of docetaxel treatment are needed

Methods.—We evaluated multiple approaches to enhance delivery of TE siRNA containing liposomes including PEGylation,; topical treatment with nitroglycerin to increase permeability and retention: and three different PEG modifications: folate, RGD cyclic peptide and a bFGF fibroblast growth factor receptor-targeting peptide. The efficacy of the optimized TE siRNA liposome in combination with docetaxel was then evaluated in vivo with or without topical nitroglycerin in vivo using a VCaP xenograft model. TE fusion protein knockdown in residual tumors was assessed using Western blotting and immunohistochemistry.

Results.—In vivo therapeutic targeting of TE fusion gene by systemic delivery of RGD-peptide coated liposomal-siRNA nanovectors led to sustained target silencing, suppressed tumor growth in xenograft models and enhanced the efficacy of docetaxel chemotherapy. Simultaneous application of the vasodilator nitroglycerin to the skin further increased tissue the delivery of siRNA and enhanced target knockdown.

Conclusions.—TE targeted gene silencing therapy using liposomal nanovectors is a potential therapeutic strategy as a monotherapy and to enhance the efficacy of chemotherapy in patients with advanced prostate cancer.

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Keywords

prostate cancer; TMPRSS2/ERG fusion gene; docetaxel; liposomes

INTRODUCTION

Prostate cancer (PCa) is the second leading cause of cancer deaths in US men¹. The TMPRSS2/ERG (TE) fusion gene is present in approximately 50% of prostate cancer lesions²⁻⁵ and arises by fusion of the promoter and 5' portions of the TMPRSS2 gene with the coding sequence of the oncogenic ERG gene. This results in increased expression of an ERG oncogene in PCa, which has multiple biological activities that can promote tumor progression. We have shown using stable shRNA knockdown that targeting the TE fusion mRNA can markedly decrease tumor growth in vivo⁶. Thus, the TE fusion gene is an outstanding therapeutic target in prostate cancer.

The TE fusion mRNA junctions, where the TMPRSS2 and ERG mRNA sequences are contiguous, are present only in PCa cells since these two genes are not transcribed into a single mRNA in normal cells. We have developed non-toxic neutrally charged liposomal nanovectors that can target siRNA into various tumors, including PCa, in vivo⁷. In addition, we have designed highly effective siRNAs spanning the fusion junctions that specifically target the most common fusion mRNA junctions in PCa without impacting expression of native ERG protein, which is expressed at high levels in endothelial cells. Using VCaP PCa cells, the only PCa cell line expressing the TE fusion gene, we have shown significant antitumor effects of our TE fusion gene targeting nanoliposomal vectors in vivo⁸. Tumor growth was significantly inhibited, and treated tumors showed decreased proliferation, increased apoptosis and decreased angiogenesis. No toxicities were observed. However, enhanced ERG knockdown is needed in order to maximize therapeutic efficacy since the ERG oncoprotein was not completely eliminated and tumors continued to grow, although at slower rates, and final tumor size was proportional to residual ERG expression (8).

A number of therapeutic modalities are used in the treatment of PCa depending on disease stage and tumor aggressiveness. The current standard of care for men with castrate resistant PCa failing second line androgen ablation therapies is docetaxel chemotherapy⁹. In addition, docetaxel has been shown to be useful in metastatic hormone-sensitive PCa¹⁰. However, this therapy can extend survival but is not curative. Thus, nontoxic therapies to enhance the efficacy of docetaxel treatment are needed. Galletti et al¹¹ have shown that ERG expression inhibits response to taxanes such as docetaxel in vitro and in mouse xenograft models. In addition, several groups have shown an inverse correlation between ERG expression and response to taxane treatment¹¹⁻¹³. These findings suggest that suppression of ERG expression may enhance responses to docetaxel treatment.

Based on the considerations above, we have now systematically optimized methods for in vivo targeting of TE fusion gene and further decreasing ERG expression in PCa tumors using nanoliposomal vectors. We then evaluated the impact of ERG knockdown using these improved approaches on response to combination treatment with docetaxel. We have found

that using combined therapy with TE targeting nanoliposomal vectors enhances response to docetaxel treatment and can completely suppress tumor growth in vivo.

MATERIALS AND METHODS

Cell lines and tissue culture.

VCaP-Luciferase (VCaP-Luc) cells were grown in DMEM with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (Invitrogen). VCaP-Luciferase cells, which were engineered to express luciferase, have been described previously⁶.

siRNA design and evaluation.

The TMPRSS2/ERG targeting siRNA, Si14, targets the fusion junction of the most common TE fusion gene mRNA isoform and has been described previously⁸. Control non-silencing siRNA (scramble) corresponded to sequence 5'AATTCTCCGAACGTGTCAC GT-3' and TE targeting Si14 were purchased from Sigma Life Science.

The siRNA targeting the luciferase gene was purchased from Dharmacon (GI3 Duplex, D-001400-01-200). The stock siRNAs were adjusted to 100 uM in Ultrapure DNase, RNase free water (Invitrogen). To evaluate its efficacy, VCaP-Luciferase cells were culture overnight and the next day the cells with 60–70% confluency were transfected with siRNAs using Lipofectamine™ RNAiMAX (Cat. No. 13778–150, Thermo Fisher Scientific) according to the manufacturer's protocol. Opti-MEM® Reduced Serum was used to dilute the siRNAs. The final concentration of siRNA used was 10 nM or 25 nM. The cells were culture for another 48 hours. In vitro luciferase activity was measured using Luciferase Assay System (Cat# E1500, Promega) on a Veritas Microplate Luminometer (Turner Biosystems). The medium was removed and the attached cells were washed with cold phosphate buffered saline and Reporter buffer (Part#E397A, Promega) pre-equilibrated at room temperature was added. The 100 uL lysates were transferred to a microplate (675074, Greiner-Bio-One) containing 100µl of the Luciferase Assay Reagent per well and read. The assays were performed in duplicate. Luciferase activity was decreased 75% at 10 nM and 86% at 25 nM compared to control (data not shown).

Western blotting and immunohistochemistry.

Western blots⁸ and immunohistochemistry with anti-ERG antibody¹⁴ were performed as described previously.

Preparation of liposomal-siRNA nanovectors.

For in vivo delivery, siRNA was mixed with 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) from Avanti Polar Lipids Inc in the presence of excess tertiary butanol at a ratio of 1:10 siRNA:DOPC (weight: weight) as described previously¹⁵. PEG-modified nanoliposomes were prepared using DOPC and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) with various PEG linker lengths, including PEG 1000, PEG 2000 and PEG 5000 (Avanti Polar Lipids Inc). DOPC was mixed with DSPE-PEG (10:1) and later mixed with Si14 or luciferase targeting siRNA at 10:1 lipid to siRNA ratio in excess tertiary butanol and lyophilized overnight. The mean size of the liposomes was about

78 nm by Zetasizer Nano (Malvern). Prior to in vivo administration, the lyophilized mixture was reconstituted with saline at a concentration of 1.5 mg/ml to achieve the desired dose in 100 μ l per i.v. injection via tail vein. Folate bound DSPE-PEG- was purchased from Avanti Polar Lipids Inc. Preparation of folate or peptide coated-PEG-modified liposomal-siRNA nanovectors were done using 10:1 ratio of DOPC:DSPE-PEG-linker containing maleimide functional group (Avanti polar lipids Inc) that was used for conjugation with RGD (Arg-Gly-Asp-D-Phe-Cys), or bFGFR1 binding peptide (Lys-Arg-Thr-Gly-Gln-Tyr-Lys-Leu-Cys¹⁶; Peptide International Inc)

In vivo treatment of VCaP xenograft models.

All animal procedures were approved by the Baylor College of Medicine Institutional Animal Use and Care Committee. Male SCID Beige male mice, 6–8 weeks of age, were obtained from the Baylor College of Medicine Patient Derived Xenograft Core. Subcutaneous tumors were established by injecting 1 million VCaP-Luc subcutaneously over the left and right flanks. Body weights and tumor volume were monitored weekly. Tumor luciferase imaging was performed using an IVIS imaging system (Xenogen, Alameda, CA) after intraperitoneal injection of D-Luciferin (Caliper Life Sciences) at 150 mg/kg body weight concomitant with isoflurane anesthesia. After 2 –3 weeks, when maximal tumor diameter was approximately 5–7 mm, mice were randomized and treatment initiated. The siRNA nanoliposomal complexes were freshly prepared and lyophilized, re-suspended in sterile saline on the day of treatment at the concentration of 400 μ g/ml. Red heat light (200 watts) was used to dilate the animal tail blood vessels and 100 μ L siRNA solution was intravenously injected. The siRNA dosage was 4 μ g per injection, equivalent to 0.15 mg/kg body weight.

In vivo evaluation of PEGylated nanoliposomal-siRNA nanovectors.

Three different of molecular weights of PEG were used to make the PEGylated DOPC-liposomes containing luciferase-targeting siRNA: PEG 1000; PEG 2000 and PEG 5000, which were compared with unmodified DOPC liposomes containing luciferase targeting siRNA. An additional control was unmodified DOPC liposomes containing scrambled siRNA. A total of 25 mice bearing 5–7 mm xenografts were randomly divided into 5 groups and each group treated with one of the liposomal preparations. After a single i.v. injection, the luciferase activities were measured at 48 and 144 hours post siRNA injection using an IVIS imaging system after D-luciferin injection.

Nitroglycerin treatment studies.

In studies in which nitroglycerin (NG) was applied, Nitro-bid (Nitroglycerin Ointment USP, 2%, NDC 0281-0326-60, Savage Laboratories) was squeezed from the tube as a 25–30 mm plug (equivalent to 80–100 mg NG) and applied to each xenograft surface 5 minutes before siRNA injection; the similar amount of Jelly (petroleum jelly, 100% pure, L1115, White Rose) was used as control.

In vivo analysis of PEG modified nanoliposomal siRNA vectors.

A total of 19 mice bearing 5–7 mm xenografts over each flank were randomly divided into 4 groups and each group treated with DOPC: DSPE PEG 5000 liposomes with no modification, folate modified, RGD peptide modified or bFGFR1 binding peptide modified liposomes containing luciferase targeting siRNA. The luciferase activities of each xenograft were measured after D-luciferin injection using an IVIS imaging system on days 0, 2, 3, 4 and 5 after a single i.v. injection of liposomes on Day 0.

Combined therapy with optimized TE fusion targeting liposomal nanovectors and docetaxel.

Docetaxel (Cat# 114977-29-5, LC Laboratories) was diluted to 75 mg/mL stock in DMSO, and further diluted to 0.75 mg/mL in double distilled sterile H₂O for intraperitoneal injection. A pilot experiments were carried out using 1, 3, 6 and 9 mg/kg, twice a week intraperitoneally using 4 mice per group two weeks after injection of VCaP cells over each flank. By two weeks, tumor growth was 50% compared to vehicle control in the 3 mg/kg group without toxicity (data not shown).

For combination treatment studies RGD peptide coated DOPC:DSPE PEG 5000-DOPC liposomes incorporating Si14 or control siRNA were used. For the first study a total of 38 mice were injected with VCaP-Luciferase cells in both flanks. When tumors reach 5–7 mm in diameter, the mice were divided into 5 groups: Control group 1: scrambled siRNA with petroleum jelly; Control group 2: scrambled siRNA with nitroglycerin; Group 3: docetaxel (3 mg/kg) and nitroglycerin; Group 4: siRNA-TE with nitroglycerin and Group 5: siRNA-TE plus docetaxel and nitroglycerin. The nitroglycerin, siRNA and docetaxel treatments were performed twice per week, with docetaxel treatment 48 hr after siRNA injection for Group 5. The treatments lasted 3 weeks. Tumor size and body weight were measured weekly. There was no apparent toxicity.

In a second experiment, VCaP xenograft tumors were established in 20 mice and treated with RGD-coated DOPC:DSPE PEG 5000 nanoliposomes containing scrambled siRNA or RGD-coated DOPC:DSPE PEG 5000 nanoliposomes containing Si14 and docetaxel (3 mg/kg) twice per week, with docetaxel treatments 48 hr after siRNA injection. The treatments lasted 3 weeks. Tumor size and body weight were measured weekly. There was no apparent toxicity.

RESULTS

PEGylation of liposomal siRNA nanoparticles increases target knockdown in vivo

To develop an effective therapy targeting the TE fusion gene, as both a monotherapy and as an adjunct to standard of care therapies we utilized siRNA nanovectors⁷. Although liposomes are an established nanoplatform, with several-FDA approved formulations for cancer treatment, unmodified liposomes are limited by their short blood circulation time due to elimination by the reticuloendothelial system¹⁷. To increase in vivo efficacy of target knockdown liposomal nanoparticles with polymer modifications such as polyethyglycol (PEGylation) is commonly used. PEGylated liposomes with longer circulation times provide

increased accumulation in tumor tissues and enhanced therapeutic efficacy^{7,18}. PEGylated liposomes evade detection and destruction by phagocytes and are not immunogenic.

We therefore tested various sizes of PEG length to determine the effect on in vivo target knockdown. We took advantage of the engineered expression of luciferase in our VCaP cells⁸ to rapidly assess the optimal PEG size by incorporating a luciferase targeting siRNA into the liposomes and directly assessing luciferase activity using an IVIS imager after D-luciferin injection. Representative results from these studies are shown in Figure 1. Subcutaneous VCaP luciferase (VCaP-Luc) tumors were established and matched for luciferase activity. Mice were then treated by intravenous injection with DOPC liposomes with scrambled siRNA, unmodified liposomes with siRNA targeting luciferase or DOPC liposomes modified with PEG of different sizes (1000, 2000 and 5000) containing luciferase targeting Luc siRNA G13. This siRNA had potent knockdown activity for luciferase in vitro of more than 75% on transient transfection, as described in Materials and Methods. At 48 hrs, unmodified DOPC liposomes and DOPC liposomes modified with PEG 5000 had equivalent knockdown, both of which were highly statistically significant by analysis of variance on ranks (to compensate for multiple testing). At 144 hrs (6 days) only the PEG 5000 modified liposomes maintained statistically significant knockdown. Thus, PEGylation with PEG 5000 optimally knocked down target gene expression in vivo and were used for subsequent studies.

Augmentation of TE gene knockdown by enhanced permeability and retention effect

The enhanced permeability and retention (EPR) effect is well established and underlies the ability of nanoliposomal particles to be delivered to tumors with high efficacy⁷. The basis for this effect is the abnormal and leaky vasculature of tumors which allows uptake and retention of particles that will not permeate normal vasculature. There are a number of approaches which have been used to enhance delivery of nanoliposomal particles into tumor tissues by manipulating the EPR effect^{18–21}. One relatively easy approach is use of the cardiac drug isosorbide dinitrate, i.e. nitroglycerin (NG). Application of topical NG on the skin can significantly enhance delivery of macromolecular drugs to tumors by enhancing tumor blood flow by vasodilation²².

We therefore evaluated the efficacy of treatment with topical nitroglycerin to enhance luciferase knockdown after injection. VCaP-Luc cells were used to establish tumors in SCID mice. A total of 15 mice bearing 5–7 mm xenografts over each flank were randomly divided into 3 groups and each group treated with DOPC:DSPE PEG 5000 liposomes containing scrambled siRNA (5 mice) or luciferase targeting siRNA. The latter group was treated with petroleum jelly (5 mice) or nitroglycerin (5 mice) 5 min before a single i.v. injection. The luciferase activities were measured at 2, 4 or 8 days post siRNA injection using an IVIS imaging system after D-luciferin injection. As shown in Figure 2, treatment with nitroglycerin increased luciferase knockdown relative to vehicle control (petroleum jelly), particularly at 48 hrs after injection, with persistent effects out to 8 days after injection of PEGylated liposomes containing luciferase targeting siRNA.

Peptide-targeted PEG modified nanoliposomes enhance in vivo uptake

Ligands can be linked to the surface of the nanoparticle together via PEG-linker to promote tumor-targeting capacity and/or uptake of liposomes. These ligands can be antibodies to cell surface proteins or small molecules known to bind to surface proteins expressed on target cells²³. We evaluated three different approaches in vivo: folate, RGD cyclic peptide and a bFGF fibroblast growth factor receptor-targeting peptide. Folate is an attractive ligand for targeting PCa due to the widespread expression of folate hydrolase, also known as prostate specific membrane antigen (PSMA) in PCa. PSMA is expressed at increased levels in almost all PCas including advanced cancers and its expression is associated with aggressive disease^{24,25} with minimal expression in normal tissues²⁶. PSMA binds folate and Hattori and Maitani²⁷ have shown that folate enhances targeting of a different form of liposomal nanoparticles in the LNCaP PCa cell line, which expresses PSMA.

RGD peptide binds to $\alpha v \beta 3$ integrin receptors. PCa cell lines express $\alpha v \beta 3$ integrin receptors²⁸ and recent bioinformatics analysis has confirmed expression of $\alpha v \beta 3$ integrins in PCa tissues²⁹. Moreover, recent studies suggest that $\alpha v \beta 3$ integrins play an important role in PCa progression and bone metastasis by mediating PCa cell adhesion and migration³⁰ so that the most aggressive cancers will be preferentially targeted using this approach.

Our group and others have shown that basic (bFGF or FGF2) binding FGFRs such as FGFR-1 and FGFR-4 are highly expressed on PCa cells^{31–33}. Terada et al have identified a peptide that binds bFGF and in particular, binds avidly to bFGF bound to cellular FGF receptors^{16,34}. They have shown this peptide can promote uptake of liposomes via cellular FGFRs when conjugated to liposomes via PEG and have optimized this formulation to decrease non-specific uptake in vivo. We thus developed nanoliposomes targeted to FGFRs through conjugation of this bFGF binding peptide (KRTGQYKLC³⁴) to a PEGylated (PEG) arm containing a maleimide functional group.

We compared the impact of various modifications on luciferase activity in vivo using liposomes with PEG 5000 alone or with modified with folate, RGD peptide, and bFGF binding peptide conjugated to PEG 5000 using liposomes containing luciferase targeting siRNA at days 2 through 5 after a single intravenous injection (Figure 3). Both folate and RGD modifications showed statistically significant decreases in luciferase activity compared to PEG 5000 unmodified liposomes at day 3. However, only RGD peptide conjugation led to a consistent and significant decrease in luciferase activity at days 4 and 5. The bFGF binding peptide conjugated liposomes did not decrease luciferase activity at any time point compared to PEG alone. Thus, the RGD modification is most potent modification in vivo.

Combination therapy with optimized RGD-peptide targeted liposomes and docetaxel

To evaluate the efficacy of combination treatments targeting the TE fusion mRNA in combination with docetaxel, we established subcutaneous VCaP-Luc xenografts and treated mice with 1) DOPC- PEG 5000-RGD-peptide conjugated - liposomes containing scrambled siRNA (SCR), 2) SCR and cutaneous nitroglycerin (SCR+ NG), 3) SCR liposomes, docetaxel and NG (Docetaxel + NG), or 4) DOPC-PEG 5000 RGD Si14 liposomes and NG (Si14+NG) or 5) PEG 5000 RGD Si14 liposomes, cutaneous NG and docetaxel (Docetaxel

+Si14+NG). Tumor volumes were measured weekly. The experiment was stopped after 3 weeks of treatment since the mice in control groups required euthanasia due to the tumor size. Results are shown in Figure 4A. Docetaxel and NG and Si14 liposome treatment with NG both significantly decreased tumor growth relative to controls, by 84% and 92% at 3 weeks, respectively. The combination of docetaxel with Si14 liposomes and cutaneous NG was even more effective and mean tumor volume did not increase over the treatment period; and 7 of 10 xenografts showed a decreased volume by week 3 compared to week 0. The three treatments were all significantly different when analyzed across the 4 weeks of treatment by 2-way ANOVA followed by Holm-Sidak multiple comparisons. Thus, combination therapy with optimized TE targeting liposomes with docetaxel is effective at decreasing tumor growth of VCaP tumors in vivo.

To confirm ERG knockdown in this experiment we performed Western blots of tumor protein extracts with anti-ERG antibody. As shown in Figure 4B, the DOPC PEG 5000 RGD Si14 liposomes with topical NG potently knocked down ERG expression compared to scrambled control scrambled siRNA liposomes with or without topical nitroglycerin. Examination of ERG immunohistochemistry confirmed a marked decrease in expression of ERG in tumors treated with siRNA liposomes and nitroglycerin with only small residual foci of ERG expression with decreased staining intensity (Figure 4C).

To determine if the nitroglycerin treatment was critical for inhibiting tumor growth we carried out treatments with DOPC-RGD PEG 5000 Si14 liposomes and docetaxel without NG treatment and compared to scrambled controls (Figure 5). This combination treatment was also effective at inhibiting mean tumor volume and 5 of 10 tumors and led to significantly reduced tumor volume over the two weeks of treatment. Thus, NG treatment was not essential to obtain potent effects with combination therapy with optimized TE targeting liposomes and docetaxel.

DISCUSSION

We have previously shown that nanoliposomal siRNAs targeting the TE fusion mRNA junction can decrease ERG oncoprotein expression without systemic toxicity⁸. However, there was variability in ERG oncoprotein knockdown and decreased tumor growth was correlated with the extent of ERG oncoprotein decrease. In the current studies we have shown that PEGylation with PEG 5000 and modification with RGD peptide significantly increases knockdown efficiency of liposomal siRNA. The target knockdown can be further increased by enhancing the EPR effect with topical nitroglycerin. In combination, these modifications can markedly decrease ERG oncoprotein and can inhibit tumor growth by more than 90%. Our studies suggest that use of optimized TE nanoliposomes may be effective as a monotherapy in TE expressing PCa. The approach described using RGD-peptide modified nanoliposomes with or without EPR enhancement is applicable to other potential targets in PCa which can be knocked down using siRNAs such as androgen receptor. However, it should be noted nanoliposomal particles can be taken up by normal tissues as well as tumors³⁵ so potential targets must be chosen with care to minimize off target toxicities in normal tissues. Use of a cancer specific siRNA, such as one targeting the TE fusion mRNA junction as in this study, is thus an attractive approach.

Our studies also show that combination therapy with optimized TE fusion gene RGD-peptide targeted DOPC liposomes with docetaxel can completely inhibit tumor growth in the PCa models, with the majority of tumors decreasing in size during treatment. It should be noted that docetaxel dosage was titrated to be submaximal so it is likely that higher docetaxel dosage would be even more effective in combination with optimized TE fusion gene targeting nanotherapy. We saw a similar effect without nitroglycerin treatment, suggesting that there is a threshold level of ERG knockdown that is sufficient to lead to complete tumor growth inhibition by docetaxel treatment. Our studies support the further development of combination treatments with docetaxel and agents targeting the ERG oncoprotein. The efficacy other taxanes, such as cabazitaxel, may also be increased by similar TE-targeted approaches¹¹.

These studies also show that combination therapy with optimized TE fusion gene targeting DOPC liposomes with docetaxel can completely inhibit tumor growth in the VCaP model, with the majority of tumors decreasing in size during treatment. It should be noted that docetaxel dosage was titrated to be submaximal, so it is likely that higher docetaxel dosage would be even more effective in combination with optimized TE fusion gene targeting liposomes. We saw a similar effect without nitroglycerin treatment, suggesting that there is a threshold level of ERG knockdown that is sufficient to lead to complete tumor growth inhibition by docetaxel treatment. Our studies support the further development of combination treatments with docetaxel and agents targeting the ERG oncoprotein. The efficacy other taxanes, such as cabazitaxel, may also be increased by similar approaches¹¹.

If the toxicity of TE targeted gene silencing therapy using liposomal nanovectors is low in PCa patients, as it is in mice, it could be used as a low toxicity monotherapy in men with locally advanced or metastatic cancers that contain the TE fusion gene who have failed standard of care therapies and are castration resistant (Figure 6). While this approach is unlikely to be curative in this context, it may offer significant benefit to patients. Combination of TE targeting nanoliposomes with docetaxel in the treatment of locally advanced or metastatic cancers that contain the TE fusion gene (Figure 6) will potentially significantly enhance the efficacy of docetaxel therapy and is likely to increase survival of such patients. It should be noted that androgen deprivation alone will decrease TE fusion gene expression but TE re-expression is seen in castration resistant prostate cancer³⁶ due to a variety of alterations such as androgen receptor mutation and amplification or intratumoral androgen synthesis which re-establish androgen receptor signaling. Thus it would presumably not be useful to use TE targeted therapies during the initial phases of androgen deprivation therapy but they should be useful after failure of such therapies. Similarly, TE targeted therapy is unlikely to be effective in neuroendocrine prostate cancers, which generally have little or no TE fusion gene expression since they do not have androgen receptors.

Several approaches to targeting ERG activity are currently in the early phases of development. Urbinati et al³⁷ have used TE fusion junction targeted siRNAs, similar to our approach, delivered via conjugation to squalene and self-organization into nanoparticles. Treatment with these nanoparticles resulted in decreased VCaP tumor growth in vivo. A peptidomimic inhibitor of ERG fusion protein has been recently described which inhibits

VCaP tumor growth in vivo with no apparent toxicity³⁸. Another small molecule, that decreases ERG expression by inhibition of the ribosomal biogenesis atypical kinase R1OK2, has been reported which decreases VCaP tumor growth in vivo³⁹. YK-4-279 is a small molecule which inhibits ERG transcriptional activity and decreases VCaP invasion⁴⁰. However recent studies have shown that only one of three ERG expressing patient derived xenografts was inhibited by this drug in vivo⁴¹. Studies by Yu et al⁴² showed synergistic effects of YK-4-279 and docetaxel in LNCaP and PC3 PCa cells in vitro. While LNCaP express ETV1 (which is another ETS transcription factor inhibited by YK-4-279⁴⁰), the PC3 cells do not express ERG or ETV1. This suggest the effects seen in these combination treatments are not specific to inhibition of ETS transcription factors. The best approach to combining ERG inhibitors with docetaxel in PCa will ultimately need to be assessed by appropriate clinical trials.

CONCLUSIONS

TE fusion targeting therapy with optimized liposomal nanovectors shows antitumor efficacy as a monotherapy or in combination with docetaxel in PCa expressing the TE fusion mRNA with no apparent toxicity. These findings suggest that patients with TE fusion gene expressing castration resistant prostate cancer may be benefit from treatment with such nanovectors alone or in combination with docetaxel.

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Figure 1. PEGylation of siRNA containing DOPC liposomes enhances target knockdown in vivo. To evaluate the impact of different sizes of PEG-linkers on in vivo siRNA knockdown efficiency, 3 different of molecular weights of PEG were used to make the PEGylated DOPC- liposomes containing luciferase-targeting siRNA: PEG 1000; PEG 2000 and PEG 5000 and were compared with unmodified DOPC liposomes containing luciferase targeting siRNA. An additional control was unmodified DOPC liposomes containing scrambled siRNA. A total of 25 mice bearing 5–7 mm xenografts were randomly divided into 5 groups and each group treated with one of the liposomal preparations. After a single i.v. injection, luciferase activities were measured at 48 and 144 hours post siRNA injection using an IVIS imaging system after D-luciferin injection. SCR control group readings average was assigned as 100% at each time point. Mean $\pm$ SEM is shown. Statistically significant differences by ANOVA compared to scrambled control at each time point are indicated * $p < .05$; ** $p < .01$.

Figure 2. Topical treatment with isosorbide dinitrate enhances target knockdown by siRNA containing DOPC liposome in vivo.

A total of 15 mice bearing 5–7 mm xenografts over each flank were randomly divided into 3 groups and each group treated with PEG 5000 liposomes containing scrambled SiRNA (5 mice) or luciferase targeting siRNA. The latter group was treated with petroleum jelly (5 mice) or nitroglycerin (5 mice) 5 min before a single i.v. injection. The luciferase activities were measured at 2, 4 or 8 days post siRNA injection using an IVIS imaging system after D-luciferin injection. Values are mean $\pm$ SEM normalized to control at Day 0 (100%). Asterisks indicate statistically significant differences between targeted siRNA and scrambled control. * $p < .05$; *** $p < .001$, Mann Whitney. The # indicates statistically significant difference between vehicle treated versus topical nitroglycerin, $p < .05$, Mann Whitney.

Figure 3. Modification of PEGylated DOPC nanoliposomes enhances uptake and target knockdown.

A total of 19 mice bearing 5–7 mm xenografts over each flank were randomly divided into 4 groups and each group treated with PEG 5000 liposomes with no modification, folate modified, RGD peptide modified or bFGFR1 peptide modified liposomes containing luciferase targeting siRNA. The luciferase activities of each xenograft were measured after D-luciferin injection using an IVIS imaging system on days 0, 2, 3, 4 and 5 after a single i.v. injection of liposomes on Day 0. Mean luciferase flux $\pm$ SEM is shown. Asterix indicates statistically significant difference between modified and unmodified liposomes at that time point ($p < .05$; Mann Whitney)

Figure 4. Combination treatment of VCaP xenografts in vivo with optimized DOPC liposomes and/or docetaxel and/or topical nitroglycerin.

A. RGD peptide coated DSPE-PEG 5000-DOPC liposomes incorporating Si14 or control siRNA were used. A total of 38 mice were injected with VCaP-Luc cells in both flanks.

When tumors reach 5–7 mm in diameter, the mice were divided into 5 groups: Control group 1: scrambled siRNA with petroleum jelly; Control group 2: scrambled siRNA with nitroglycerin; Group 3: docetaxel (3 mg/kg) and nitroglycerin; Group 4: siRNA-TE with nitroglycerin and Group 5: siRNA-TE plus docetaxel and nitroglycerin. The nitroglycerin, siRNA and docetaxel treatments were performed twice per week, with docetaxel applied 48 hr after siRNA injection for Group 5. Tumor volumes (mm³) were measured pretreatment and post-treatment weekly with electronic caliper (volume = 0.5*Length*width squared). Values shown are mean tumor volume (in mm³) ± SEM. The three treatments were all significantly different when analyzed across the 4 weeks of treatment by 2-way ANOVA followed by Holm-Sidak multiple comparisons. Asterisks indicate statistically significant differences between treatment and scrambled control with nitroglycerin at that time point by ANOVA; * p < .05; *** p < .001; docetaxel with nitroglycerin at week 2 was p = .05.

B. Western blot of tumor protein extracts with anti-ERG and anti-β-actin antibodies. CON are extracts from mice treated with liposomes with scrambled siRNA and petroleum jelly. Blot on the left shows extracts from tumors in mice treated with scrambled siRNA liposomes and nitroglycerin. The blot on the right uses extracts from tumors in mice treated with Si14 TE targeting liposomes and nitroglycerin.

C. Immunohistochemistry with anti-ERG antibody. CON tumor is from a mouse treated with liposomes with scrambled siRNA and petroleum jelly. The tumor on the right is from a mouse treated with Si14 TE targeting liposomes and nitroglycerin.

Figure 5. Combination treatment of VCaP xenografts in vivo with optimized DOPC liposomes and docetaxel.

VCaP-Luc xenograft tumors were established in 20 mice. The mice treated with RGD-coated PEG-5000 nanoliposomes containing scrambled siRNA or PEG-5000 RGD liposomes containing Si14 and docetaxel (3 mg/kg) twice per week, with docetaxel treatments 48 hr after siRNA injection. The treatments lasted 3 weeks. Values are mean tumor volume (in mm³) $\pm$ SEM. Asterisks indicate statistically significant differences between tumors in PEG-5000 RGD Si14 plus docetaxel treated mice and scrambled control at the same time point. * $p < .01$; *** $p < .001$, Mann Whitney.

Figure 6. Treatment of prostate cancer with TE targeting siRNA containing nanoliposomes. Men with advanced castration resistant prostate cancers (CRPC) containing the TE fusion gene who have reactivation of androgen receptor (AR) signaling after androgen deprivation therapy express the TE fusion mRNA. The TE fusion mRNA activates multiple targets that promote tumor progression and enhance resistance to docetaxel therapy. Targeting the TE fusion mRNA with siRNA spanning the TMPRSS2/ERG fusion junction decreases ERG protein and results in decreased tumor progression and enhanced sensitivity to docetaxel therapy.