Prostate-specific membrane antigen-targeted liposomes specifically deliver the Zn²⁺ chelator TPEN inducing oxidative stress in prostate cancer cells

Aim: To evaluate the potential use of zinc chelation for prostate cancer therapy using a new liposomal formulation of the zinc chelator, N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN). **Materials & methods:** TPEN was encapsulated in nontargeted liposomes or liposomes displaying an aptamer to target prostate cancer cells overexpression prostate-specific membrane antigen. The prostate cancer selectivity and therapeutic efficacy of liposomal (targeted and nontargeted) and free TPEN were evaluated *in vitro* and in tumor-bearing mice. **Results & conclusion:** TPEN chelates zinc and results in reactive oxygen species imbalance leading to cell death. Delivery of TPEN using aptamer-targeted liposomes results in specific delivery to targeted cells. *In vivo* experiments show that TPEN-loaded, aptamer-targeted liposomes reduce tumor growth in a human prostate cancer xenograft model.

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Background

The most widely used treatment for advanced prostate cancer (PCa) is androgen deprivation therapy, but disease that recurs is often unresponsive to repeated androgen deprivation therapy, resulting in castration-resistant prostate cancer (CRPC). CRPC is much more aggressive and carries a worse prognosis. Treatment of CRPC generally includes docetaxel, a taxane which binds to and stabilizes microtubules. Nonetheless, patients with advanced CRPC have a median survival of 9–30 months [1], in spite of several survival-enhancing novel drugs.

Modulation of Zn²⁺ is a possible therapeutic strategy for PCa treatment. In the normal prostate, Zn²⁺ can reduce citrate production and slow oxidative metabolism by inhibiting m-aconitase; this process requires tightly-regulated Zn²⁺ homeostasis [2]. Zn²⁺ concentrations are reduced in PCa tissue, and they decrease further as malignancy progresses [3]. However, supplementing PCa cells with Zn²⁺

induces apoptosis and reduces levels of pro-angiogenic and metastatic cytokines, suggesting that reduced Zn²⁺ concentrations are vital for PCa survival and may be involved in disease progression [4,5]. Treatment of PCa with the cell-permeable Zn²⁺ chelator N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN) reduces cell viability through apoptotic pathways, although the initiating factor(s) are unclear [6,7]. Previously, we demonstrated that TPEN can enhance apoptosis induced by other agents [8].

One possible mechanism for TPEN is dysregulation of reactive oxygen species (ROS) detoxification. TPEN decreases mitochondrial membrane potential and increases superoxide concentrations in breast cancer and leukemia cell lines [9,10]. Both cell types were also rescued from TPEN by the antioxidant N-acetyl cysteine (NAC), indicating that oxidative stress may be involved in TPEN-induced cytotoxicity. However, the effects of TPEN on oxidative stress in PCa, a

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malignancy with high oxidative stress and tight regulation of Zn^{2+} homeostasis, have not been reported previously [2,11].

Although TPEN is cytotoxic to cancer cells *in vitro*, it also causes neurotoxicity that was sometimes fatal when administered systemically in mice [12]. If Zn^{2+} chelation therapy is to have clinical potential, biodistribution must be restricted to malignant tissue to minimize potential side effects. Nanoparticle-based drug delivery systems, such as liposomes, may reduce side effects associated with parent drugs [13]. For example, liposomes have been used to deliver the iron chelator desferoxamine [14], complexes of radio-labeled metals [15] and for targeted drug delivery [16,17], but liposomal formulations of TPEN have not been previously reported. We recently reported a DNA aptamer (SZTI01) that specifically binds to prostate-specific membrane antigen (PSMA) [18]. Aptamers are DNA or RNA oligonucleotides that can be used to specifically bind to cellular targets. Unlike other targeting strategies such as antibodies or small molecules, aptamers have the advantages of nonimmunogenicity and scaling [19]. PSMA is a dimeric folate hydrolase expressed in prostate epithelia and proximal kidney tubules and is also upregulated in PCa [20]. Importantly for treating CRPC, PSMA is highly expressed in over 50% of recurrent cases of PCa, including nearly all metastases to lymph nodes and bone [21]. Our laboratory has demonstrated specific delivery of doxorubicin to PSMA⁺ C4–2 PCa cells using the SZTI01 aptamer and, in these studies, we investigate the potential of the SZTI01 aptamer for selective delivery of liposomal TPEN to PCa cells as a potential new approach for targeted therapy of CRPC.

Herein, we describe the synthesis and efficacy of a novel-targeted liposome which can selectively deliver TPEN to PSMA⁺ cells using the SZTI01 DNA aptamer. Once internalized by targeted PCa cells, liposomes release TPEN, which chelates Zn^{2+} and results in increased ROS levels leading to cell death. The targeted, TPEN-loaded liposomes enable killing of targeted PCa cells under treatment conditions where free TPEN is ineffective. Targeted liposomes are also nontoxic to nontargeted cells under these same conditions. We demonstrate that TPEN induces oxidative stress in PCa cells, which has not been previously reported. Cells can be rescued from TPEN by antioxidants or $\text{Zn}^{2+}/\text{Cu}^{2+}$ salts. Additionally, we demonstrate that targeted liposomes loaded with a fluorescent dye preferentially localize to C4–2 tumors, consistent with active targeting. Targeting of TPEN via aptamer-targeted liposomes (Ap-Lips) provides an opportunity to use Zn^{2+} chelation as a treatment for advanced CRPC, where traditional chemotherapy is ineffective.

Methods

Hydrogenated soy phosphatidylcholine (HSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (PEG2000 PE) and Maleimide-terminated PEG2000 PE (M-PEG2000 PE) were obtained from Avanti Lipids (AL, USA) as powders and dissolved in 3:1 chloroform:methanol and stored at -20°C until used. Dithiol/Quasar 670 terminated DNA was synthesized by the Wake Forest University DNA Core Lab. Tris(2-carboxyethyl)phosphine was obtained from Santa Cruz Biotech (TX, USA). Manganese (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) was obtained by Santa Cruz BioTech. All chemicals used in TPEN synthesis were obtained from Sigma Aldrich (MO, USA).

Cell maintenance

C4–2 cells were a gift from Dr Elizabeth M Wilson (UNC, NC, USA). PC3 cells were purchased from the cell and viral vector core laboratory at Wake Forest School of Medicine. All cells were maintained with RPMI 1640 (Gibco, NY, USA) with 10% fetal bovine serum (Gemini Bio-Products, CA, USA). All cells were kept at 5% CO_2 at 37°C .

Synthesis of tetrakis-(2-pyridylmethyl) ethylenediamine

The procedure used for synthesis of TPEN was modified from a previous publication [22]. Deionized water (10 ml) was cooled to 4°C and degassed with Argon for 30 min and stirred with a magnetic stir bar. 1 g (0.006 mol) of 2-chloromethyl-pyridine was added, followed by additional argon degassing for 10 min. Ethylenediamine (102 μl , 0.0015 mol) was mixed with 1 ml water and added over 10 min to the stirring solution. 1.2 ml of 10 M NaOH was dissolved in 10 ml water and added dropwise over 30 min, which produced a brown-red by-product that initially dissipated but that persisted after approximately 50% of the base had been added. After NaOH addition was complete, the reaction was warmed to room temperature while being degassed with Argon. The reaction vessel was sealed and heated to 40°C overnight. The reaction was extracted 3 $\times$ with 20 ml chloroform. The organic phase was washed 3 $\times$ with brine and dried over MgSO_4 . The organic phase was filtered and solvent was removed by rotary evaporation. The resulting brown oil was diluted with minimal chloroform (generally <0.5 ml), and precipitated with hexane. The solution was then refrigerated at 4°C for 1 h resulting in a dark precipitate. The target compound was present in the milky organic phase and was dried, dissolved in chloroform, and recrystallized using hexane.

Formation of liposomes

Lipid films were formed by mixing hydrogenated soy phosphatidylcholine, cholesterol, PEG2000 PE and M-PEG2000 PE in a 2:1:0.08:0.02 molar ratio. Chloroform and methanol were removed by rotary evaporation under reduced pressure. Lipid films were further dried under an Argon stream for 30 min. Liposomes were formed by reconstituting lipid films with 1.5 ml of a 50 mM pH 5.5 citrate buffer that contained 5% dextrose and 1 mM TPEN at 55°C. 10 mg/ml 70 kDa fluorescein isothiocyanate (FITC)-dextran or 100 nM IRDYE 800CW were included in the buffer for select fluorescence microscopy studies. The suspension was pipetted thoroughly until a cloudy solution formed. The liposomes were then sonicated at 37°C for 5 min, which resulted in a slight clearing of the liposome solution. The liposomes were then extruded through a 0.2 µm syringe filter. Finally, liposomes were purified using 100 kDa Amicon centrifugal filtration devices. Solutions were spun at 2000 × g for 15 min, or until 90% of the solution had passed through the filter. The filtrate was resuspended with 50 mM citrate buffer pH 5.5 with 5% dextrose. This process was repeated twice to ensure no free TPEN or dye remained in solution. This same process was used to purify fluorescent liposomes as well, which results in the removal of all molecules not contained in the liposomes.

Aptamer targeting of liposomes

Aptamers were synthesized by The Wake Forest University DNA Core Lab with 3' terminal dithiols and 5' terminal Quasar 670 dye (Biosearch Technologies, CA, USA). Aptamers were folded by heating a 500 µm solution of DNA to 95°C for 5 min and slow sequential cooling to 80°C for 5 min, 70°C for 10 min, 37°C for 30 min, 23°C for 1 h and 4°C for at least 1 h. The dithiol was reduced by adding a 10× excess of tris(2-carboxyethyl)phosphine in 50 mM tris buffer (pH 7.4) and incubating for 30 min before adding to a solution of liposome (2× excess of activated aptamer versus M-PEG2000 PE used to form liposomes). The reaction proceeded overnight prior to purifying the liposomes as described above.

Characterization of liposomes

TPEN loading into liposomes was determined by preparing a standard curve of absorbance at 260 nm. Liposomes were diluted 1:10 in methanol and sonicated 5 min before measuring the absorbance at 260 nm. The concentration of TPEN in liposomes was then determined by comparing the absorbance to the standard curve, which was also obtained in 90% methanol. Aptamer concentration was determined in a similar fashion, except the absorbance at 630 nm, the

maximum absorbance of Quasar 670 and the extinction coefficient of the dye were used to determine the concentration of aptamers bound to liposomes. Liposome size was measured with a Malvern Zetasizer ZS90 (Malvern Instruments, Malvern, UK), and data was analyzed using Malvern software. A Nanosight NS500 (Nanosight, Salisbury, UK) and nanotracking analysis software provided by Nanosight were used to determine both liposome size and the number of liposomes produced. Transmission electron microscopy (TEM) images of liposomes were acquired using an FEI Tecnai-Spirit TEM (FEI, OR, USA) using a phosphotungstic acid counterstain. A small drop of liposome containing 2% phosphotungstic acid as counterstain was placed on a formvar plate and allowed to rest for 2 min. The remaining liquid was wicked away, and the plate was allowed to dry completely before imaging.

Fluorescence microscopy

For all microscopy experiments, cells were seeded at 35,000 cells/well in 8-well Lab-Tek II chambered #1.5 German Coverglass System (Thermo Fisher Scientific, MA, USA), and incubated at 37°C under 5% CO₂ for 24 h. For targeted liposomes, cells were incubated with 5 µm (as measured by aptamer concentration) of Ap-Lip that contained FITC dye as well as TPEN, in RPMI medium with 10% fetal bovine serum for 2 h at 37°C. For all microscopy experiments, cells were washed after incubation with dyes using fresh media and Dulbecco's phosphate-buffered saline (PBS) prior to imaging. Cells were visualized using a Zeiss LSM510 confocal microscope (Carl Zeiss, Oberkochen, Germany). For studies with dichloro-dihydro-fluorescein diacetate (DCFH), cells were treated with 8 µm TPEN for 6 h prior to addition of 50 nM of the dye. The cells were allowed to incubate for 30 min before imaging. Experiments utilizing Bodipy C11 (5 µm; Invitrogen), and Mito-Tracker Green® (100 nM; Invitrogen) were performed in a similar manner to methods used for DCFH, but were treated for 7 h with TPEN prior to imaging.

Liposome cytotoxicity assays

PC3 and C4-2 cells were seeded at a density of 5000 cells/well in 96-well plates and incubated at 37°C under 5% CO₂. The following day, cells were treated with Ap-Lips, nontargeted liposomes or TPEN, at equivalent levels of TPEN. After 4 h, the media was removed, cells were washed with media, and fresh media was added to the cells incubated for 68 h. For coculture, 2500 C4-2 luciferase-expressing cells were plated with 2500 PC3 cells and treated as described above. For aptamer competition experiments, 25 µm aptamer was added to each well before adding either aptamer-targeted or nontargeted liposomes. In Zn²⁺

rescue experiments, twice the molar amount of ZnSO_4 was added concurrently with the drugs being tested. For PC3 cells, viability was assessed indirectly by measuring the ATP levels using CellTiter-Glo luminescent cell viability assay (Promega). Because the C4–2 cells expressed luciferase, the luciferase-Glo assay (Promega) was used to determine viability, as was done in our previous publication [8]. Statistical significance for *in vitro* studies was determined by using Student's *t*-test, with significance indicated at either $p < 0.01$ or $p < 0.05$. All experiments were performed in triplicate.

TPEN cytotoxicity rescue experiments

For rescue with NAC and MnTBAP, PC3 and C4–2 cells were seeded at a density of 5000 cells/well in 96-well plates and incubated at 37°C under 5% CO_2 . The following day, cells were treated with either NAC or MnTBAP 1 h prior to TPEN treatment. Eight hours after treatment, the drug was removed and fresh media was added to the cells. The cells were allowed to grow for 72 h before viability was assayed using a Cell-Titer Glo assay kit. For metal ion rescue experiments, PC3 and C4–2 cells were cultured in an identical manner and allowed to incubate overnight. The following day, cells were treated with 8 μM TPEN, and 16 μM of either ZnSO_4 or CuSO_4 was added at the indicated time. At the end of 8 h, media containing drugs and metal ion salts was removed and replaced with fresh media. The cells were incubated for 72 h before viability was measured using a Cell-Titer Glo assay kit. Statistical significance for *in vitro* studies was determined by using Student's *t*-test, with significance indicated at either $p < 0.01$ or $p < 0.05$. All experiments were performed in triplicate.

In vivo experiments

In vivo studies were approved by the Institutional Animal Care and Use Committee at Wake Forest School of Medicine. All animal experiments at our institution are regulated by and conform to requirements of the US Public Health Service Policy on the Humane Care and Use of Laboratory Animals, the National Research Council's Guide for the Care and Use of Laboratory Animals, and the US Department of Agriculture's Animal Welfare Act & Regulations.

For fluorescent biodistribution studies, liposomes were synthesized as described above, with the inclusion of 25 nmol of IRDYE 800CW per mouse. Eight mice were injected with 2×10^6 C4–2 cells on the right flank in 200 μL of Matrigel®. Studies were initiated when tumors reached 100 mm^3 . Four mice were then injected with a single dose of 4 mg/kg TPEN via dye-labeled liposomes while four received 25 nmol of free

dye and an equivalent dose of TPEN. After 48 h, mice were sacrificed and organs and tumors were imaged using a Li-Cor Pearl Trilogy imaging system (Li-Cor, NE, USA).

A second imaging study was performed with three mice injected with 2×10^6 C4–2 cells on the right flank and 2×10^6 PC3 cells on the left flank, both in 200 μL of Matrigel. Studies were initiated when tumors reached 100 mm^3 . Mice were then injected with a single dose of 4 mg/kg TPEN via labeled liposomes and sacrificed after 48 h for tumor excision and imaging.

For efficacy studies, male nude mice were implanted with permanent jugular vein catheters for drug delivery. After 2 weeks of recovery from the surgery, mice were injected with 2×10^6 C4–2 cells on the right flank and 2×10^6 PC3 cells on the left flank, both in 200 μL of Matrigel. Studies were initiated when tumors reached 100 mm^3 . Six mice per group were injected twice weekly with liposomes that resulted in a dosage of 4 mg/kg of TPEN (general injection volume ≈ 200 –250 μL , depending on batch-to-batch variability) for 30 days. Tumor size and animal weight were measured concurrently with drug treatments. For *in vivo* studies, statistical significance was determined using a two-way repeated measures analysis of covariance (ANCOVA) model, with significance at $p < 0.05$.

Results

TPEN induces oxidative stress & mitochondrial damage in PCa cells

We investigated whether TPEN induced oxidative damage in PCa cells using Bodipy C11, an ROS-sensitive dye that changes emission from ≈ 590 to ≈ 511 nm upon oxidation (Figure 1A). PCa cells treated with TPEN for 7 h showed a large increase in green fluorescence consistent with ROS-mediated damage, indicating TPEN increases oxidative stress. Cells treated with TPEN also showed an increase in DCHF fluorescence. DCHF is a sensor for peroxynitrite and other oxidative species, and was used as an additional sensor of oxidative stress [23] (Figure 1B). To evaluate changes in mitochondrial morphology due to TPEN treatment, PCa cells were stained with the mitochondrial-specific dye MitoTracker Green. In contrast to untreated PC3 cells in which mitochondria are localized near the nucleus, mitochondria in TPEN-treated PC3 cells delocalize and form large, punctate aggregates. C4–2 mitochondria do not demonstrate an altered size, but do display altered distribution in the cell upon treatment with TPEN (Figure 1C, Supplementary Figure 1). Further, PCa cells treated with TPEN showed altered morphology, including large membrane blebs and increased granularity consistent with apoptosis.

TPEN-mediated damage rapidly becomes irreversible in PCa cells

Because TPEN has a high affinity for both Zn^{2+} and Cu^{2+} , we investigated at what time PCa cells could no longer be rescued from TPEN with Zn^{2+} or Cu^{2+} .

Inability to rescue TPEN cytotoxicity with exogenous $\text{Zn}^{2+}/\text{Cu}^{2+}$ could signify irreversible cellular damage. Both Zn^{2+} (Figure 2A) and Cu^{2+} (Figure 2B) could completely rescue both cell lines up to 3 h after treatment with 8 μM TPEN, with decreasing ability to rescue at

Figure 1. N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine induces significant oxidative stress in advanced prostate cancer cells. (A) Confocal microscopy of C4-2 and PC3 cells treated with or without TPEN for 7 h and imaged with Bodipy C11. Images denoted by i, ii, iii or iv are phase, unoxidized Bodipy, oxidized Bodipy and merged images, respectively. **(B)** Fluorescent microscopy demonstrating increased DCFH oxidation after 6 h of TPEN treatment. **(C)** Fluorescent microscopy of C4-2 and PC3 cells using MitoTracker® Green after 7 h of treatment with 8 μM TPEN. Large granular bodies can be identified in TPEN-treated cells, which colocalize with MitoTracker Green.
TPEN: N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine.

Figure 2. Time-dependence of Cu^{2+} and Zn^{2+} rescue. (A) Viability of PCa cells after 8 h of 8 μ M TPEN treatment followed by 72 h of drug-free incubation with Zn^{2+} rescue at the indicated times. (B) The same as A, but with Cu^{2+} rescue. (C) Viability of cells treated with 8 μ M of TPEN for 8 h rescued with N-acetyl cysteine 1 h before and concurrent with TPEN. * $p \leq 0.05$ between TPEN-only and TPEN+N-acetyl cysteine. (D) The same as C except cells were rescued with 250 μ M MnTBAP. * $p \leq 0.05$ between aptamer-targeted liposomes only and + rescue. Error bars represent standard deviations.

PCa: Advanced prostate cancer; MnTBAP: Manganese (III) tetrakis (4-benzoic acid) porphyrin chloride; TPEN: N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine.

5 h and with minimal rescue at 7 h. These results demonstrate that TPEN initiates cell death within 3 h and irreversible cellular damage occurs within 7 h.

Mechanistically, cellular damage induced by high levels of ROS may partially explain this short window in which cells can be rescued from Zn^{2+} chelation via TPEN. Treating PCa cells with the ROS scavenger NAC 1 h before, or concurrent with TPEN treatment (8 h treatment followed by 64 h in drug-free medium), significantly reduced TPEN cytotoxicity (Figure 2C). TPEN may increase oxidative stress and induce cytotoxicity through inhibiting Cu^{2+} -/ Zn^{2+} -dependent proteins such as the ROS-detoxifying enzyme superoxide dismutase 1 (SOD1). Consistent with this, treatment with MnTBAP, a putative SOD1 mimetic and peroxynitrite scavenger, or NAC, 1 h before TPEN

treatment rescued cells from TPEN (Figure 2D) [24,25]. However, neither NAC nor MnTBAP completely rescued either cell line, consistent with alternative Zn^{2+} / Cu^{2+} chelation effects also being exerted by TPEN.

Characterization of SZTI01-targeted TPEN liposomes

We hypothesized that encapsulation of TPEN in liposomes that target PSMA could be used to selectively deliver TPEN to PSMA⁺ PCa cells. We developed a liposomal formulation of TPEN that incorporates the PSMA-specific SZTI01 aptamer. Liposomes were washed extensively using an Amicon centrifugal filter to remove free TPEN. Because TPEN absorbs at 260 nm, the A_{260} of the final wash was measured to demonstrate the liposome solutions did not contain free TPEN

(Supplementary Figure 2D). Dialysis of liposomes at 37°C against PBS showed that TPEN was half-life of ≈ 8 h under these conditions (Supplementary Figure 2B). The content of TPEN in purified liposomes was calculated by measuring the A_{260} of a solution after liposome dissolution relative to a standard curve of TPEN absorbance (Supplementary Figure 2A). The loading efficiency of TPEN was generally $\geq 10\%$, that is, using 1 ml of a 1 mM TPEN solution yields 1 ml of 100 μM of liposomal TPEN after purification. However, when TPEN concentration in the buffer was increased beyond 1 mM, liposomes failed to form. A general scheme of liposome formation is depicted in Supplementary Figure 3.

The average diameter of liposomes was 109 ± 1.0 nm by dynamic light scattering (DLS) (Figure 3A), and 97.2 ± 9.3 nm by nanoparticle tracking analysis (NTA) (Figure 3B). Liposomes prepared using 1 mM TPEN were spherical (Figure 3C), with a slightly negative zeta potential, -37 mV (Figure 3D). In order to assess the stability of TPEN-loaded liposomes over time, liposomes

were dialyzed against PBS at 37°C between 0–48 h and the liposome size was followed by DLS. The average diameter of liposomes was relatively unchanged over 48 h of incubation, with an average size of 146.8 nm for undialyzed liposomes, and 138.7 nm after 48 h; however, the size distribution of the particles does shift to smaller particles at times >4 h (Supplementary Figure 4). We estimated the number of liposomes in our preparations using NTA, with approximately 350 billion liposomes in a typical preparation. For liposomes formed with Quasar 670 modified aptamers, dye absorbance was also quantified and was used to determine the number of aptamers attached to each liposome using Beer's law. This was divided by the number of particles counted with NTA. The number of aptamers per liposome was typically ≈ 100 .

Specificity of SZTI01-targeted TPEN liposomes

We have previously demonstrated that the SZTI01 aptamer can specifically deliver doxorubicin to PSMA⁺ PCa cells [18], an approach that resulted in reduced cyto-

Figure 3. Physical characterization of aptamer-targeted liposomes. (A) Aptamer-targeted liposome sizes as determined by dynamic light scattering. (B) Aptamer-targeted liposome sizes as determined by nanoparticle tracking analysis; red bars represent standard deviation. (C) Dehydrated aptamer-targeted liposomes imaged with transmission electron microscopy. (D) Zeta potential of aptamer-targeted liposomes.

toxicity to nontargeted cells. However, the ability of SZTI01 to deliver larger particles, such as liposomes, has not been evaluated. We prepared liposomes conjugated with the SZTI01 aptamer, and followed binding of the aptamer–liposome complex to prostate cancer cells that differed in PSMA expression. The Ap-Lips included a Quasar670 dye covalently bound to the aptamer, and liposomes were loaded with FITC-dextran to track delivery of the liposomal cargo to cells. We used PSMA⁺ C4–2 as our targeted cells and PSMA[−] PC3 as nontargeted controls, as in our previous studies [18]. After 1 h treatment, confocal microscopy revealed a marked difference in binding and cargo delivery between C4–2 cells, which displayed strong uptake, and PC3 cells, which displayed minimal binding and cargo delivery (Figure 4). Additionally, nontargeted liposomes demonstrated little to no FITC delivery to either PC3 or C4–2 cells. These data demonstrate that Ap-Lips bind and deliver liposomal cargo selectively to PSMA⁺ cells.

To demonstrate that selective delivery translated into target-specific cell killing, C4–2 and PC3 cells were treated with: Ap-Lips; nontargeted liposomes; or, free TPEN. Cells were also treated with liposomes that contained no drug, which resulted in no significant difference from control cells (not shown). Because of this, only treatments that contained TPEN were tested exhaustively. All treatments delivered equal amounts of TPEN (normalized to 5 μ M free TPEN concentration). Our previous studies showed C4–2 and PC3 cells were equally sensitive to TPEN following 72 h treatment while a 3-h treatment with 5 μ M free TPEN was nontoxic to both cell lines [8]. PSMA⁺ C4–2 cells, but not PC3 cells,

displayed significantly reduced viability upon treatment with Ap-Lips. Nontargeted liposomes and free TPEN had lesser effects, comparable to untreated controls, in both cell lines (Figure 5A). The specificity of Ap-Lips was maintained when C4–2 and PC3 cells were cocultured, indicating the robustness of our targeting approach (Figure 5B). Further, adding exogenous aptamer to C4–2 cells treated with Ap-Lips reduced cytotoxicity by 30% (Figure 5C), consistent with an aptamer-mediated targeting effect. These results show that our Ap-Lips can selectively kill targeted PSMA⁺ PCa cells at concentrations that leave nontargeted cells unaffected. Such selective targeting may reduce the risk of neurotoxicity associated with TPEN treatment *in vivo*.

To confirm that the cytotoxic mechanism for TPEN was not altered by liposomal delivery, C4–2 cells were cotreated for 3 h with exogenous Zn²⁺ and Ap-Lips at 5 μ M TPEN, which was shown to be the point at which cells respond to TPEN in our previous publication. Addition of TPEN beyond 5 μ M does not enhance cytotoxicity, with exposure time playing a larger role. Cytotoxicity of Ap-Lips was completely rescued by exogenous Zn²⁺ (Figure 6A), demonstrating that Ap-Lips induce cell death via Zn²⁺ chelation selectively in PSMA⁺ cells. Additionally, when C4–2 cells were treated with Ap-Lips, nontargeted liposomes, or free TPEN for 2 h, only cells treated with Ap-Lips containing TPEN showed signs of oxidative damage when imaged with Bodipy (Figure 6B), consistent with Ap-Lips causing oxidative damage. C4–2 cells treated with Ap-Lips could also be rescued by cotreating the cells with the antioxidants MnTBAP or NAC (Figure 6C).

Figure 4. Targeted cell binding and delivery of aptamer-targeted liposomes. Confocal microscopy images of C4–2 and PC3 cells treated with aptamer-targeted liposomes with FITC along with TPEN or nontargeted liposomes (right).

TPEN: N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine.

Figure 5. Selective cytotoxicity of aptamer-targeted liposomes to C4-2 cells and rescue by exogenous targeting aptamer or exogenous Zn^{2+} . (A) Viability of C4-2 and PC3 cells when treated with Ap-Lips, nontargeted Lip or TPEN for 3 h. * $p \leq 0.01$ between Ap-Lip and Lip treatment; * $p \leq 0.01$ between C4-2 and PC3 cells treated with Ap-Lip. (B) Coculture of C4-2 and PC3 cells treated with Ap-Lip or Lip only for 3 h. (C) C4-2 cells treated with Ap-Lip or Ap-Lip plus exogenous aptamer. Error bars represent standard deviation. Ap-Lip: Aptamer-targeted liposome; Lip: Liposome; TPEN: N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine.

In vivo targeting & efficacy of SZTI01-targeted TPEN liposomes

We first sought to determine if liposomal delivery had an effect on the accumulation of a near IR dye, IR Dye800CW, in organs and tumors compared with free dye. For all imaging studies, liposomes were formed with both the dye and TPEN. Mice that received dye delivered via liposomes displayed higher fluorescence in tumors, kidney, spleen and liver than mice dosed with free dye (Figure 7A–C), 48 h after injection. Organs and tumors from mice injected with free dye had no fluorescent signal after 48 h, consistent with rapid clearance of the small molecule via the kidneys. We then designed a follow-up experiment to examine possible toxicity associated with TPEN and liposomal TPEN. Mice were treated with either TPEN, or liposomal

TPEN. After 48 h, mice were sacrificed and the livers were examined for potential toxicity (Figure 7E). Interestingly, mice treated with TPEN-loaded liposomes showed no visible signs of toxicity, while livers of mice treated with free TPEN show lipid accumulation, consistent with free TPEN causing acute hepatotoxicity. Liposomal delivery may ameliorate this effect.

After establishing that our liposomes altered tissue retention and potential side effects of TPEN, we tested if dye delivered via Ap-Lips partitioned preferentially to C4-2 or PC3 tumors in the same animal. Mice were injected with either free dye or dye loaded into targeted liposomes. After 48 h, mice were sacrificed and tumors were excised for imaging. Tumors from mice that received liposomal dye showed fluorescence in both tumors, with greater fluorescence in the targeted

Figure 6. N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine delivered via aptamer-targeted liposomes induces cell death in a similar manner as free N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine. (A) C4-2 cells were treated with Ap-lip for 3 h and were rescued by addition of zinc salts (ZnSO_4). **(B)** C4-2 cells treated with Ap-lips display stress from reactive oxygen species after 2 h, compared with cells treated with untargeted liposomes or free TPEN. **(C)** C4-2 cells treated in a similar manner with Ap-lip or Ap-lip + MnTBAP or N-acetyl cysteine show rescue by antioxidants. Error bars represent standard deviation. Ap-lip: Aptamer-targeted liposomes; MnTBAP: Manganese (III) tetrakis (4-benzoic acid) porphyrin chloride; TPEN: N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine.

C4-2 tumors (Figure 7D), demonstrating that our Ap-Lips deliver their payload to tumors and preferentially to targeted C4-2 tumors.

For *in vivo* efficacy studies, we utilized a double xenograft model in male nude mice, where each mouse had both a PSMA⁺ C4-2 tumor and PSMA⁻ PC3 tumor established on opposite flanks. Mice were also implanted with permanent jugular vein catheters, to allow more consistent drug delivery and mimic how patients often receive treatment. We then sought to determine if our targeted liposomes displayed selective efficacy for targeted tumors. Tumors were measured and drugs administered twice per week for 5 weeks. This dosing regimen was chosen to maximize the drug given to mice without requiring high levels of

TPEN in a single dose, which might be toxic. After 30 days, group-by-time analysis indicated that both treated C4-2 and PC3 tumors were statistically different from untreated tumors ($p = 0.0008$ and $p = 0.009$). Treatment with Ap-Lips reduced C4-2 tumor growth by 51% compared with untreated controls (80.3 vs 163% change in tumor volume for Ap-Lips vs control after 30 days; Figure 8A). In contrast, treatment of PC3 tumors with Ap-Lips reduced tumors by only 33% compared with untreated controls (87 vs 130.2% change in tumor volume for Ap-Lips vs control after 30 days; Figure 8B). These data demonstrate that SZTI01-targeted liposomes increase the specificity and therapeutic efficacy of the Zn^{2+} chelator TPEN for treatment of PSMA⁺ tumors versus nontargeted

tumors. Targeted delivery resulted in reduced tumor growth compared with controls, with no adverse events or weight loss in treated animals.

Discussion

Zn²⁺ plays a key role in the development and progression of PCa [2,3,26]. Either removal or addition of Zn²⁺ to PCa cells can result in cell death, with apoptosis being attributed as the mode of cell death induced by Zn²⁺ chelation. TPEN, a zinc chelator, is cytotoxic to multiple cell types including PCa, and causes caspase activation and XIAP degradation within 3 h of admin-

istration [6,27,28]. Recent studies from our lab also showed that TPEN enhanced the cytotoxicity of other drugs [8]. Oxidative damage has been reported to play a role in TPEN-induced cell death, but this has not been previously investigated in PCa [10,11]. Modulation of oxidative stress is emerging as an anticancer strategy, and PCa cells may be sensitive to agents that induce oxidative stress [29,30]. In the present study, we demonstrate that TPEN is highly cytotoxic to PCa cells by causing oxidative stress and have developed a targeted liposomal delivery system to harness the potential of Zn²⁺-chelation therapy for selective PCa treatment

Figure 7. Liposomal loading results in increased retention and reduced hepatotoxicity. (A) *Ex vivo* imaging of liver where FD is mice treated with free dye and Lipo is mice treated with aptamer-targeted liposomal dye. (B) Spleen and kidneys from mice dosed with either free dye or targeted dye-loaded liposomes. (C) C4-2 tumors from the same mice as in A and B. (D) *Ex vivo* fluorescence imaging of tumors derived from C4-2 (top) and PC3 (bottom) cells. Mice were treated only with aptamer-targeted liposomes in this experiment. (E) Hematoxylin and Eosin stain of liver sections from mice treated with 4 mg/kg of either free TPEN or TPEN-loaded liposomes. TPEN: N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine.

Figure 8. Aptamer-targeted liposomes specifically deliver contents to and reduce tumor growth of PSMA⁺ tumors. (A) Growth of C4-2-derived tumors, normalized to starting volume. Error bars represent standard error. **(B)** Growth of PC3-derived tumors, normalized to starting volume. Error bars represent standard error.

while minimizing systemic toxicities. Our Ap-Lips have potential to localize to PSMA⁺ tumor cells *in vivo* by both passive targeting, through the enhanced permeability and retention effect, and by active targeting with the SZTI aptamer.

We, and others, have reported TPEN cytotoxicity in PCa and colon cancer cells requires a threshold dose, with virtually no cytotoxicity below 4 μ M and essentially complete cytotoxicity with 5 μ M treatment, but these effects were highly time-dependent [8,31]. To develop appropriate dosing regimens, it is important to identify at what point after treatment TPEN-mediated effects become irreversible. Here, we established that exogenous TPEN is cytotoxic within 3 h of treatment and that Cu²⁺/Zn²⁺ is ineffective at rescuing from the cytotoxic effects of TPEN after 8 h. This new information suggests that TPEN rapidly causes cells to commit

to apoptosis. The effects of TPEN have largely been attributed to Zn²⁺ chelation, yet TPEN has higher affinity for Cu²⁺ than Zn²⁺ [32]. Our results show that Cu²⁺ is as effective as Zn²⁺ for rescue, consistent with enzyme(s) that require both Cu²⁺ and Zn²⁺ cofactors being targets for TPEN treatment. One such candidate is SOD1, an ROS-detoxifying enzyme that requires both Cu²⁺ and Zn²⁺ cofactors. Chelating Cu²⁺ and Zn²⁺ may reduce SOD1 activity, causing oxidative stress, inducing apoptosis, and partially explaining why cells treated with TPEN can be rescued with Cu²⁺, even hours after TPEN treatment (Figure 2A & B). Consistent with this interpretation, TPEN cytotoxicity was rescued by the antioxidant NAC (Figure 2C), or by the SOD1 mimetic MnTBAP (Figure 2D). Rescue was only partial, however, consistent with other processes also contributing to TPEN cytotoxicity. This is not surprising, given the ubiquitous use of Cu²⁺ and Zn²⁺ as cofactors for transcription factors and other enzymes. Zn²⁺ may also be directly involved in inhibiting apoptosis, since Zn²⁺ depletion results in degradation of the antiapoptotic protein XIAP [6,7].

Our studies with oxidation-sensitive fluorescent dyes Bodipy-C11 (Figure 1A) and DCFH (Figure 1B) established that oxidative stress is induced by TPEN, while studies with MitoTracker Green (Figure 1) demonstrated that TPEN altered mitochondria morphology. When treated with TPEN and Bodipy, PCa cells displayed much more green fluorescence than controls, indicating high levels of oxidative stress (Figure 1A). We confirmed these results with an additional ROS-sensitive dye, DCFH (Figure 1B). Using MitoTracker Green, we also observed morphological changes in mitochondria and granular bodies in cells undergoing oxidative stress due to TPEN treatment (especially in PC3 cells; Figure 1C). These morphological changes may reflect mitochondrial damage linked to cytochrome C release and apoptosis [33]. Although the cause of these morphological changes are not clearly understood, these data suggest mitochondria are stressed when cells are treated with TPEN, and this may be an important aspect of cytotoxicity due to Zn²⁺ chelation. These results demonstrate that TPEN induces both oxidative stress and alterations to mitochondrial morphology consistent with apoptosis. Importantly, the IC₅₀ of TPEN is two to threefold lower in cancer cells than in noncancerous cells, indicating that there is a therapeutic window for selective cancer therapy [31]. These data collectively implicate ROS and oxidative stress as the primary mechanisms of TPEN cytotoxicity.

In order to deliver TPEN to cells in a manner that would reduce the risk of toxicity, we encapsulated the chelator in pegylated liposomes targeted with SZTI01 aptamer. In a neutral or basic environment, TPEN is highly hydrophobic and is insoluble in water. We

utilized a pH 5.5 citrate buffer, which effectively solubilized the drug, up to 100 mM. This allowed for TPEN to be delivered via the interior of the liposome; however, concentrations much higher than 1 mM prevented liposome formation, indicating that even protonated TPEN may still interact with the lipid membrane at high concentrations. Once liposomes were formed, we assessed them for size distribution (Figure 3A–C). NTA, DLS and TEM all confirmed that liposomes were almost equal to 100 nm in diameter with few large or small outliers. The zeta potential of the liposomes was found to be slightly negative, which is to be expected as the particles are coated with DNA aptamers, which have a negative charge from the phosphodiester backbone. After confirming the formation of liposomes, in order to ascertain the leakage rate, we dialyzed liposomes containing TPEN and found dialysis against PBS at 37°C showed that approximately 50% of the contents had leaked after 8 h (Supplementary Figure 2B). These results demonstrate that our liposomes are stable enough to carry their contents, even lipophilic small molecules such as TPEN, for an extended period of time, and deliver them to targeted sites. We also measured the change in diameter of liposomes over time and found that while there was relatively little change to the average diameter, the distribution did change slightly (Supplementary Figure 4B) to favor slightly smaller particles. This likely is a result of larger particles precipitating out of solution. There is no evidence of liposome fusion. These results indicate that the liposomes are stable for up to 48 h at 37°C, making them a good candidate for *in vivo* use.

Zn²⁺ is a vital micronutrient required for many biochemical processes; its depletion can lead to severe side effects. Administration of TPEN via tail vein injection in mice resulted in ataxia, convulsions and death within 20 min [12]. If Zn²⁺ chelation were to be used clinically, it must be targeted to PCa cells to limit these side effects. Our Ap-Lips seek to address this need. Histological studies of mice treated with TPEN or TPEN-loaded liposomes revealed possible hepatic toxicity after a single dose of TPEN, which was ameliorated when TPEN was delivered via liposomes (Figure 7E). Mice treated with IR DYE 800CW-loaded liposomes showed more dye delivery to the liver, kidneys, spleen and C-42 tumors when compared with mice that received free dye (Figure 7A–C) after 48 h. These results are unsurprising, as altered accumulation and enhanced delivery is well-documented for nanosized particles, however, this can result regardless of targeting. Our imaging study was expanded to examine if aptamer-targeted liposomes preferentially bound to PSMA⁺ cells over PSMA⁻ cells, as we observed *in vitro*. To test the specificity of our targeted liposomes we used a model in which two tumors

were grown on opposite flanks, one with targeted cells and one with untargeted cells. Doing so allowed us to study if targeted liposomes had increased delivery to the targeted tumor over the nontargeted tumor, and removed the variability that can occur between mice. We found that, qualitatively, tumors established from C4-2 cells had higher fluorescence than those established from PC3 cells (Figure 7D). This establishes that SZTI01-targeted liposomes increased delivery of small molecules and preferentially delivered their cargo to tumors established from PSMA⁺ expressing cells both *in vitro* and *in vivo*, as demonstrated by our imaging studies, and reduced liver toxicity. Additionally, delivery of TPEN via liposomes resulted in a significant reduction in tumor size when compared with nontreated controls in both targeted and nontargeted prostate cancer cell lines (Figure 8). We believe a combination of enhanced permeability and retention and decreased renal clearance results in prolonged circulation time and increased liposomal uptake in both tumors. PSMA⁺ tumors may attract targeted liposomes that actively bind to the tumor and are internalized. Although in these studies tumors from C4-2 cells still increased in size despite treatment, our study was limited to a single dose, which caused no toxicity as measured by weight loss. A dose-escalation trial may reveal that higher dosages are safe, and more effective at reducing tumor size.

Our study is particularly timely for men with CRPC. While a few treatments improve survival for men with CRPC, none are curative, and resistance to novel agents inevitably ensues. There is a persistent need for novel strategies to target CRPC cells. Our study provides opportunity to advance a novel strategy of metal chelation to be delivered safely and effectively in men with CRPC. Among various human organs, the prostate gland has the highest and disproportionately high levels of Zn²⁺ concentration. This unique Zn²⁺-rich microenvironment is amenable to metal chelation strategies and our current study is a promising step toward implementing this strategy.

To our knowledge, this is the first report of targeted Zn²⁺ chelation for treatment of PCa. Our *in vitro* studies demonstrated that Ap-Lips were selectively cytotoxic to PSMA⁺ C4-2 cells even in the presence of coculture with PSMA⁻ PC3 cells. We have also demonstrated that aptamer-targeted liposomal delivery of TPEN is an effective anticancer strategy *in vivo*. Targeted liposomal delivery of TPEN significantly reduced tumor volume for both PC3 and C4-2 tumors, consistent with the enhanced permeability and retention effect contributing to efficacy. Targeted C4-2 tumors were, however, even more sensitive to the targeted therapy indicating that active targeting is an independent factor contributing to therapeutic efficacy. These results are significant

given the relatively poor response achieved in clinical trials of metal chelators such as ATN-224 [34]. In light of the poor response of CRPC to conventional therapy, Zn²⁺ chelation offers promise of a new approach to treatment. Our successful demonstration of targeted delivery indicates that this approach may overcome potential toxicities associated with systemic administration of Zn²⁺ chelators and also improve efficacy.

Conclusion & future perspective

There remains an unmet need for the treatment of advanced prostate cancer. A major complicating factor for the treatment of this disease is that recurrent disease is often hormone independent, leaving the primary treatment for prostate cancer ineffective. However, some aspects of advanced PCa are possible avenues for future treatments. As PCa develops, zinc levels in the malignant tissue decrease dramatically compared with the healthy tissue. PCa cells are highly sensitive to changes in zinc concentration, and chelating out the remaining zinc results in PCa cell death. However, this requires a delivery method to ensure that the chelator reaches the site without first chelating other metals from the blood. PCa also expresses high levels of PSMA, and nearly all metastasis to the bone and bladder express this marker as well. The SZTI01 aptamer was designed to recognize this marker and can be used to deliver a variety of molecules to cells expressing this target. Our studies show combining zinc chelation with aptamer-mediated delivery may be a promising and novel treatment for advanced PCa, and may result in improved prognosis for

patients of this disease. We anticipate that further development of zinc chelation therapies for advanced PCa will result in favorable outcomes for these patients, and that Ap-Lips provide a good platform for delivery of not only chelating agents, but also traditional chemotherapy for combination treatments as well.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: <http://www.futuremedicine.com/doi/full/10.2217/nnm-2016-0017>

Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Current status of prostate cancer treatment

- While primary advanced prostate cancer (PCa) has a favorable outcome, with 5-year survival ≈90%, recurrent disease has a much worse prognosis even with new treatments.

Development of novel treatments for advanced prostate cancer

- Recent work has shown that PCa has greatly reduced zinc levels when compared with the normal prostates.
- We investigate the ability of the chelator N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN) to chelate zinc in PCa cells and if this is sufficient to kill PCa cells.
- We develop a targeted delivery vehicle for TPEN, utilizing liposomes and aptamer targeting, to selectively target prostate-specific membrane antigen expressing PCa cells.

Key results of findings

- TPEN acts rapidly when administered to PCa cells *in vitro*, with effects being irreversible within 8 h.
- TPEN elicits its effects via zinc chelation and ROS imbalance, and cells can be rescued from TPEN by supplementing with exogenous zinc or antioxidants.
- Despite high lipophilicity, TPEN can be incorporated into liposomes and targeted to prostate-specific membrane antigen expressing cells using the SZTI01 aptamer.
- SZTI01-targeted liposomes increase the accumulation of dye *in vivo* compared with free dye, and delivery of TPEN in this way results in reduced toxicity at short time points.
- TPEN delivered via aptamer-targeted liposomes results in a significant reduction in PCa tumors in a xenograft model when administered twice weekly.

Implications

- These studies demonstrate that zinc chelation can be used to reduce PCa tumors as a single-agent treatment, and that aptamer-targeted liposomes can be used to safely deliver drugs that are otherwise toxic *in vivo*.

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