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PSMA-specific theranostic nanoplex for combination of TRAIL gene and 5-FC prodrug therapy of prostate cancer

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ABSTRACT

Metastatic prostate cancer causes significant morbidity and mortality and there is a critical unmet need for effective treatments. We have developed a theranostic nanoplex platform for combined imaging and therapy of prostate cancer. Our prostate-specific membrane antigen (PSMA) targeted nanoplex is designed to deliver plasmid DNA encoding tumor necrosis factor related apoptosis-inducing ligand (TRAIL), together with bacterial cytosine deaminase (bCD) as a prodrug enzyme. Nanoplex specificity was tested using two variants of human PC3 prostate cancer cells in culture and in tumor xenografts, one with high PSMA expression and the other with negligible expression levels. The expression of EGFP-TRAIL was demonstrated by fluorescence optical imaging and real-time PCR. Noninvasive ^{19}F MR spectroscopy detected the conversion of the nontoxic prodrug 5-fluorocytosine (5-FC) to cytotoxic 5-fluorouracil (5-FU) by bCD. The combination strategy of TRAIL gene and 5-FC/bCD therapy showed significant inhibition of the growth of prostate cancer cells and tumors. These data demonstrate that the PSMA-specific theranostic nanoplex can deliver gene therapy and prodrug enzyme therapy concurrently for precision medicine in metastatic prostate cancer.

1. Introduction

Prostate cancer remains the second most frequently diagnosed cancer and the second leading cause of cancer-related death among men in the United States [1,2]. In particular, there is a critical, unmet need to find effective treatments for castration-resistant metastatic prostate cancer. Advances in theranostics, where detection is combined with therapy, are paving the way for precision medicine in cancer [3,4]. Theranostic nanoparticles are being developed in these strategies as novel platforms to target cancer cells specifically [5,6] with real-time visualization using noninvasive imaging [7–9]. These theranostic nanoparticles have been developed to deliver a variety of agents [10,11], including chemotherapy and nucleic acids such as plasmid DNA (pDNA) [10–12], and small interfering RNA (siRNA) [13,14].

Our purpose here was to develop a theranostic nanoplex to deliver a prodrug enzyme and pDNA expressing a gene of therapeutic interest with the ultimate goal of targeting advanced, metastatic prostate cancer (Fig. 1). Prostate-specific membrane antigen (PSMA) provided an attractive target to achieve specificity to castration-resistant prostate cancer [15,16]. PSMA is a type II integral membrane protein that has abundant expression on the surface of prostate cancer, particularly in castration-resistant, advanced, and metastatic disease. Urea-based small molecule inhibitors of PSMA have been applied as targeting moieties for diagnostic imaging probes and drugs for prostate cancer [17–19]. Here we used a urea-based small molecular PSMA inhibitor, (2-(3-[1-carboxy-5-[7-(2,5-dioxo-pyrrolidin-1-yloxycarbonyl)-heptanoylamino]-pentyl]-ureido)-pentanedioic acid (MW 572.56) as the targeting moiety linked to the nanoplex through a polyethyl glycol (PEG) chain for targeting PSMA.

Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) has attracted attention in cancer gene therapy [20,21]. The TRAIL protein belongs to the TNF cytokine superfamily. After binding with death receptors (DR4 and DR5) and decoy receptors

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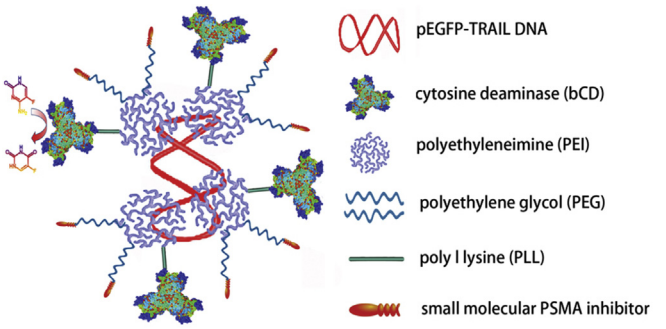

Fig. 1. Structure of PSMA-targeted nanoplex **1**.

(DcR1 and DcR2), the extrinsic apoptotic pathway is triggered by TRAIL through recruitment of the Fas-associated death domain protein to the death domain. The increased expression of death receptors in most cancer cells results in TRAIL inducing apoptosis, with minimal toxicity to normal cells [22,23]. Because the killing activity of TRAIL is cancer-specific and has little effect on most normal cells and tissues, TRAIL is being actively investigated as a promising anticancer protein against a broad range of cancer cells and tissues [24–26]. However, rapid clearance *in vivo* has proven to be a major impediment in achieving effective therapy [27,28]. In contrast to TRAIL protein therapy, TRAIL gene therapy, where TRAIL DNA is delivered into tumor cells through a suitably designed plasmid, provides an attractive alternative through continuous production of TRAIL *in situ*. We used pEGFP (enhanced green fluorescent protein)-TRAIL pDNA in our theranostic nanoplex. The plasmid is expressed as an EGFP-TRAIL fusion protein. The TRAIL component is the therapeutic while EGFP is used as an imaging reporter in cells and *ex vivo* to monitor the expression and metabolism of EGFP-TRAIL. Polyethylenimine (PEI) was used as the pDNA vector. In addition to pEGFP-TRAIL, the nanoplex delivered the prodrug enzyme bacterial cytosine deaminase (bCD), which converts 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU). 5-FU is a classical chemotherapeutic agent [29], and the conversion of 5-FC to 5-FU is detectable by ^{19}F magnetic resonance spectroscopy (MRS). Although TRAIL [30,31] and bCD-mediated 5-FU therapy [32,33] have been applied separately for cancer treatment, non-specific delivery, especially in the case of 5-FU, may create significant collateral damage and normal tissue toxicities. By incorporating bCD in the nanoplex, 5-FU is formed in the immediate proximity of the cancer cells. In addition, TRAIL as a monotherapy may not provide sufficient tumor control [34,35]. A combination of both strategies was therefore incorporated for effective cancer-selective therapy, to minimize damage to normal tissue, and to reduce drug resistance [36,37]. Although yeast CD (yCD) demonstrates higher activity in converting convert 5-FC to 5-fluorouracil (5-FU), the higher stability of bCD [38] made it an attractive choice for nanoplex synthesis. We used Cy5.5 as the fluorescent moiety of the nanoplex. Due to its emission in the near-infrared (NIR) region (680–900 nm) of the electromagnetic spectrum, Cy5.5 enables *in vivo* optical imaging because of limited autofluorescence at these wavelengths. Noninvasive, *in vivo*, real-time imaging of Cy5.5 was used to detect the temporal and spatial distribution of the nanoplex.

2. Materials and methods

2.1. Materials

All organic chemicals and solvents of analytical grade were from Aldrich (St. Louis, MO) and Sigma (Milwaukee, WI) unless otherwise specified. N-succinimidyl-S-acetylthiopropionate (SATP), N-[e-maleimidocaproyloxy)-succinimide ester (EMCS), succinimidyl 4-formylbenzoate (SFB) and succinimidyl 6-hydrazinonicotinamide acetone hydrazine (SANH) were obtained from Pierce (Rockford, IL). Maleimide-PEG-NH_2 (3.4 kDa) was procured from Nanocs (New York, NY). pEGFP-TRAIL and pEGFP-U6 plasmids were purchased from Addgene (Cambridge, MA).

2.2. Determination of size distribution and zeta potential of PSMA-targeted nanoplex 1

The hydrodynamic radius and size distribution of PSMA-targeted nanoplex **1** were determined by dynamic light scattering (DLS, 10 mW He–Ne laser, 633 nm wavelength). PSMA-targeted Nanoplex **1** was prepared at varying N/P ratios of 5, 10, 30, and 50 by adding a PBS buffer solution (20 mM, pH 7.4) of compound **8** (600 μL, varying concentrations) to a distilled water solution of pDNA (400 μL, 400 μg/mL), followed by vortexing for 5 s and incubating for 10 min at room temperature. The DLS measurements were performed in triplicate. The average zeta potential of bCD alone, compound **8** (carrier alone) and PSMA-targeted nanoplex **1** (N/P = 50) in deionized water were measured with a Zetasizer Nano ZS instrument (Malvern) equipped with a clear standard zeta capillary electrophoresis cell cuvette from 20 acquisitions with a concentration of approximately 1 mg/mL. The measurements were performed in triplicate.

2.3. Cell culture

Human prostate cancer PC3 cells transfected to overexpress PSMA (PC3-PIP) or transfected with the plasmid alone (PC3-Flu) were obtained from Dr. Warren Heston (Cleveland Clinic, Cleveland, OH). Fetal bovine serum, penicillin, and streptomycin were from Invitrogen (Carlsbad, CA). Cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum in a humidified incubator at 37°C/5% CO_2.

2.4. In vitro cell culture studies

In vitro therapeutic efficacy of PSMA-targeted nanoplex **1** was evaluated in PC3-PIP and PC3-Flu cells by an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma, Milwaukee, WI). PC3-PIP and PC3-Flu cells (8×10^3 cells/well) in 96-well plates were incubated for 24 h in RPMI 1640 in a humidified environment with 5% CO_2 at 37 °C prior to treatment. To evaluate the efficacy of TRAIL gene therapy, cells were treated with PSMA-targeted nanoplex **1** (concentration of pDNA: 2 μg/ml, N/P = 50). To test the therapeutic efficacy of the 5-FC/bCD strategy, cells were treated with 5-FC (3 mM) and PSMA-targeted nanoplex **1** in which the pEGFP-TRAIL pDNA was replaced with pEGFP-U6 pDNA (concentration of pDNA: 2 μg/ml, N/P = 50). To evaluate the combined therapeutic efficacy of TRAIL gene therapy and 5-FC/bCD strategy, cells were treated with PSMA-targeted nanoplex **1** (concentration of pDNA: 2 μg/ml, N/P = 50) with the addition of 5-FC (3 mM). The cells were further incubated for 24 and 48 h at 37 °C. The MTT reagent (in 20 μL PBS, 5 mg/mL) was then added to each well. The cells were further incubated for 5 h at 37 °C. After incubation, 100 μL sodium dodecyl sulfate (SDS) solution (10 mg/mL) was added in each well, and the plates were kept in dark overnight. The absorbance (A) at 490 nm was recorded by a microplate reader (Bio-rad, USA). The cell viability (y) was calculated by $y=(A_{treated}/A_{control}) \times 100\%$, where $A_{treated}$ and $A_{control}$ are the absorbance of the cells cultured with treatment and fresh culture medium, respectively.