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Prostate-Specific Membrane Antigen Targeted Imaging and Therapy of Prostate Cancer Using a PSMA Inhibitor as a Homing Ligand

Sumith A. Kularatne,[†] Kevin Wang,[‡] Hari-Krishna R. Santhapuram,[‡] and Philip S. Low*[,†]

Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, Indiana 47907, and Endocyte, Inc., 3000 Kent Avenue, West Lafayette, Indiana 47906

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Abstract: Prostate cancer (PCa) is a major cause of mortality and morbidity in Western society today. Current methods for detecting PCa are limited, leaving most early malignancies undiagnosed and sites of metastasis in advanced disease undetected. Major deficiencies also exist in the treatment of PCa, especially metastatic disease. In an effort to improve both detection and therapy of PCa, we have developed a PSMA-targeted ligand that delivers attached imaging and therapeutic agents selectively to PCa cells without targeting normal cells. The PSMA-targeted radioimaging agent (DUPA−^{99m}Tc) was found to bind PSMA-positive human PCa cells (LNCaP cell line) with nanomolar affinity (K_D = 14 nM). Imaging and biodistribution studies revealed that DUPA−^{99m}Tc localizes primarily to LNCaP cell tumor xenografts in nu/nu mice (% injected dose/gram = 11.3 at 4 h postinjection; tumor-to-muscle ratio = 75:1). Two PSMA-targeted optical imaging agents (DUPA−FITC and DUPA−rhodamine B) were also shown to efficiently label PCa cells and to internalize and traffic to intracellular endosomes. A PSMA-targeted chemotherapeutic agent (DUPA−TubH) was demonstrated to kill PSMA-positive LNCaP cells in culture (IC_{50} = 3 nM) and to eliminate established tumor xenografts in nu/nu mice with no detectable weight loss. Blockade of tumor targeting upon administration of excess PSMA inhibitor (PMPA) and the absence of targeting to PSMA-negative tumors confirmed the specificity of each of the above targeted reagents for PSMA. Tandem use of the imaging and therapeutic agents targeted to the same receptor could allow detection, staging, monitoring, and treatment of PCa with improved accuracy and efficacy.

Keywords: Prostate-specific membrane antigen; PSMA-targeted imaging and therapy; radioimaging and optical imaging of prostate cancer; tubulysin prodrug; diagnosis of prostate cancer; chemotherapy for prostate cancer

Introduction

Prostate cancer (PCa) is the most common male malignancy in western society, amounting to ~230,000 new cases/year in the US.[1] More males die from PCa (>30,000/year) than any other malignancy except lung cancer,[1] and the cumulative cost of treating PCa patients has been estimated at $8−10 billion/year in the US.[2] Advanced stages of PCa can also significantly impact quality of life due to bone disintegration, pain, obstruction of urination, and erectile dysfunction among other disorders.[3]

Although neoplastic transformation begins in the prostate gland, malignant cells can eventually metastasize to other parts of the body, including bones, rectum, and bladder. Because metastatic PCa is difficult to treat, early detection

* Corresponding author. Mailing address: Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, IN 47907. Phone: 765-494-5273. Fax: 765-494-5272. E-mail: plow@purdue.edu.
[†] Purdue University.
[‡] Endocyte, Inc.

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constitutes the most effective strategy for minimizing disease-related morbidity and mortality. Early diagnosis of PCa is most commonly achieved by digital rectal exam, blood test for prostate specific antigen (PSA), or a prostate biopsy.[4] However, only more advanced stages of disease can be detected by a digital rectal exam[5] and prostate biopsies can be expensive and painful.[6] Further, the accuracy of the PSA test has been criticized[7] due to its elevation during benign prostatic hypertrophy (BPH) or prostatitis and due to its decline during treatment for BPH or baldness.[8] Although transrectal ultrasound together with magnetic resonance imaging (MRI) and computerized tomography (CT) can effectively reveal the extent of prostate enlargement and growth asymmetry, these exams are too expensive for routine screening and may not distinguish malignant disease from BPH.[9] Clearly, better methods for assessing onset and spread of PCa could greatly reduce the frequency of advanced stage disease.

Treatment for PCa most commonly involves surgery, radiation therapy, hormone administration, and/or chemotherapy. Unfortunately, none of these therapies is highly effective against metastatic disease, and each has sufficient disadvantages that patients often decline their use. While localized PCa can be treated by removal of malignant tissue,[10] radical prostatectomy may result in loss of urinary control and impotence.[11] Radiation therapy can also cause impotence, rectal bleeding,[3] and increased risk of colon and bladder cancer,[12] and treatment of invasive or metastatic PCa is often limited to palliative hormonal therapy and/or chemotherapy. While hormonal treatment induces remission of hormonally responsive cancer, the longevity of tumor remission is limited and it is not without significant toxicity, including liver damage, cardiovascular disease, weight gain, and osteoporosis.[13] And although chemotherapy (e.g., mitoxantrone) may also extend lifespan,[14] side effects of such antimitotic drugs often outweigh their benefits. Therefore, safer and more potent methods of treating PCa are widely needed.

In an effort to improve both detection and treatment of PCa, we initiated a search for low molecular weight ligands that would selectively target attached drugs to PCa cells without promoting their uptake by healthy cells. Prostate-specific membrane antigen (PSMA, folate hydrolase I, glutamate carboxypeptidase II), a plasma membrane-associated protein,[15] is overexpressed on the vast majority of PCa.[16] While the physiological function of PSMA remains controversial, its expression is largely limited to PCa cells,[16,17] where malignant transformation leads not only to its up-regulation but also to its translocation from internal organelles to the cell surface. For unknown reasons, PSMA is also expressed in the neovasculature of most other solid tumors (but not in the vasculature of healthy tissues),[16,18] and in the kidneys, albeit at significantly lower levels in human kidneys[16,17] than murine kidneys.[19] For drug targeting applications, perhaps the most important characteristic of PSMA is that it undergoes internalization through clathrin-coated pits and rapidly recycles to the cell surface for

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additional rounds of internalization.[20] Collectively, these unique features render PSMA an excellent candidate for use in tumor-targeted drug delivery.

For the above reasons, a variety of efforts have been made to develop PSMA-targeted imaging agents for use in the diagnosis and monitoring of PCa.[21−27] Indeed, a variety of low molecular weight inhibitors of PSMA have been radiolabeled and used to image human PCa xenografts (LNCaP and PC-3 cell lines) in athymic nude mice.[23,28−30] In this report, we describe the design and use of a PSMA-specific ligand for the selective delivery of both imaging and therapeutic agents to a human PCa xenograft (LNCaP cells) in nu/nu mice. We also demonstrate that the PSMA-specific ligand can deliver sufficient tubulysin, a microtubule inhibitor, to established solid LNCaP tumors in athymic mice to induce their long-term remission without causing measurable toxicity to healthy tissues.

Experimental Section

Materials. Sodium pertechnetate was purchased from Cardinal Health (Indianapolis, IN). [^{3}H]-Thymidine was obtained from Moravek Biochemicals (Brea, CA), and 2-(phosphonomethyl)-pentanedioic acid (PMPA) was from Axxora Platform (San Diego, CA). Tubulysin B was provided by Endocyte Inc. (W. Lafayette, IN). HC Matrigel was obtained from BD Bioscience (San Jose, CA). All other chemicals were purchased from major suppliers.

Synthesis of DUPA Conjugates. DUPA−^{99m}Tc was synthesized as described in the companion paper (DOI 10.1021/mp9000712) to this report.[31] The synthesis of DUPA−TubH will be described in detail elsewhere (manuscript in preparation). DUPA−FITC was synthesized by solid phase methodology as follows (see Scheme 1). Universal NovaTag resin (50 mg, 0.53 mM) was swollenwith dichloromethane (DCM) (3 mL) followed by dimethylformamide (DMF, 3 mL). A solution of 20% piperidine in DMF (3 × 3 mL) was added to the resin, and argon was bubbled for 5 min. The resin was washed with DMF (3 × 3 mL) and isopropyl alcohol (*i*-PrOH, 3 × 3 mL). After swelling the resin in DMF, a solution of DUPA(O^{t}Bu)-OH (1.5 equiv), HATU (2.5 equiv) and DIPEA (4.0 equiv) in DMF was added. Argon was bubbled for 2 h, and resin was washed with DMF (3 × 3 mL) and *i*-PrOH (3 × 3 mL). After swelling the resin in DCM, a solution of 1 M HOBt in DCM/trifluoroethane (TFE) (1:1) (2 × 3 mL) was added. Argon was bubbled for 1 h, the solvent was removed and resin was washed with DMF (3 × 3 mL) and *i*-PrOH (3 × 3 mL). After swelling the resin in DMF, a solution of Fmoc-Phe-OH (2.5 equiv), HATU (2.5 equiv) and DIPEA (4.0 equiv) in DMF was added. Argon was bubbled for 2 h, and resin was washed with DMF (3 × 3 mL) and *i*-PrOH (3 × 3 mL). The above sequence was repeated for 2 more coupling steps for addition of 8-aminooctanoic acid and fluorescein isothiocyanate or rhodamine B isothiocyanate. Final compound was cleaved from the resin using a trifluoroacetic acid (TFA): H_2O:triisopropylsilane:cocktail (95:2.5:2.5) and concentrated under vacuum. The concentrated product was precipitated in diethyl ether and dried under vacuum. The crude product was purified using preparative RP-HPLC [λ = 488 nm; solvent gradient: 1% B to 80% B in 25 min, 80% B wash 30 min run; A = 10 mM NH_4OAc, pH = 7; B = acetonitrile (ACN)]. ACN was removed under vacuum, and pure fractions were freeze-dried to yield DUPA−FITC as a brownish-orange solid. RP-HPLC: t_R = 8.0 min (A = 10 mM NH_4OAc, pH = 7.0; B = ACN, solvent gradient: 1%

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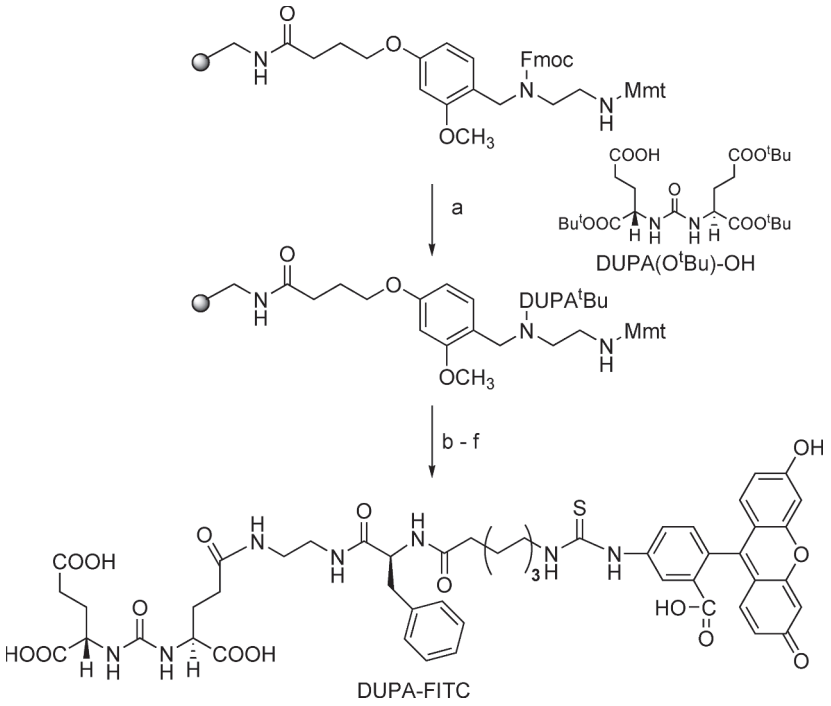
Scheme 1[a]



[a] Reagents and conditions: (a) (i) 20% piperidine/DMF, rt, 10 min; (ii) DUPA(O^{t}Bu)-OH, HATU, DIPEA, 2 h; (b) 1 M HOBt in DCM/TFE (1:1), 1 h; (c) (i) 20% piperidine/DMF, rt, 10 min; (ii) Fmoc-Phe-OH, HATU, DIPEA, 2 h; (d) (i) 20% piperidine/DMF, rt, 10 min; (ii) Fmoc-8-aminooctanoic (EAO) acid, HATU, DIPEA, 2 h; (e) (i) 20% piperidine/DMF, rt, 10 min; (ii) fluorescein isothiocyanate (FITC) or rhodamine B isothiocyanate, DIPEA/DMF; (f) TFA/H_2O/TIPS (95:2.5:2.5), 30 min.

B to 50% B in 10 min, 80% B wash 15 min run). ^{1}H NMR (DMSO-d_6/D_2O): δ 0.98−1.27 (ms, 9H); 1.45 (b, 3H); 1.68−1.85 (ms, 11H); 2.03 (m, 8H); 2.6−3.44 (ms, 12H); 3.82 (b, 2H); 4.35 (m, 1H); 6.53 (d, J = 8.1 Hz, 2H), 6.61 (dd, J = 5.3, 3.5 Hz, 2H); 6.64 (s, 2H); 7.05 (d, J = 8.2 Hz, 2H), 7.19 (m, 5H); 7.76 (d, J = 8.0 Hz, 1H); 8.38 (s, 1H). HRMS (ESI) (m/z): $(M + H)^+$ calcd for $C_{51}H_{59}N_7O_{15}S$, 1040.3712, found, 1040.3702. UV/vis: λ_{max} = 491 nm.

Characterization of DUPA−Rhodamine B. Purple solid, analytical HPLC: t_R = 8.4 min (A = 10 mM NH_4OAc, pH = 7.0; B = CAN, solvent gradient: 1% B to 70% B in 10 min, 80% B wash 15 min run); 1.08−1.53 (21H); 1.65 (b, 4H); 1.84−2.20 (ms, 6H); 2.83 (m, 1H); 2.97 (b, 3H); 3.01 (b, 3H); 3.36 (b, 9H); 3.90 (b, 2H); 4.62 (s, 1H); 6.52 (m, 5H); 7.18 (m, 5H); 7.82 (m, 2H); 8.20 (m, 1H); 8.35 (s, 1H). LRMS (ESI) (m/z): $(M)^+$ calcd for $C_{59}H_{76}N_9O_{13}S$, 1151.35, found, 1151.19. UV/vis: λ_{max} = 555 nm.

Cell Culture and Animals. LNCaP, KB, and A549 cells were obtained from American Type Culture Collection. Cells were grown as a monolayer using 1640 RPMI medium containing 10% heat-inactivated fetal bovine serum, sodium pyruvate (100 mM) and 1% penicillin streptomycin in a 5% CO_2:95% air-humidified atmosphere at 37 °C.

Male *nu/nu* mice were purchased from NCI Charles River Laboratories and maintained on normal rodent diet during the study.

Preparation of DUPA−^{99m}Tc Radioimaging Agent. A solution of sodium pertechnetate (1.0 mL, 15 mCi) was added conjugate (0.14 mg), sodium α-D-glucoheptonate dihydrate (80 mg), stannous chloride dihydrate (0.80 mg), and sufficient NaOH to achieve a pH of 7.2 upon rehydration with water. The vial was heated in a boiling water bath for 18 min and then cooled to rt before use.

Binding Affinity and Specificity of DUPA−^{99m}Tc. LNCaP cells (150,000 cells/well in 500 μL) were seeded into 24-well Falcon plates and allowed cells to form monolayers over 48 h. Spent medium in each well was replaced with fresh medium (0.5 mL) containing increasing concentrations of DUPA−^{99m}Tc in the presence or absence of 100-fold excess (ex) PMPA. After incubating for 1 h at 37 °C, cells were rinsed with medium (2× 1.0 mL) and tris buffer (1× 1.0 mL). After dissolving cells in 0.25 M $NaOH_{(aq)}$ (0.5 mL), cells were transferred into individual γ-counter tubes and radioactivity was counted using a γ-counter (Packard, Packard Instrument Company). K_D was calculated by plotting bound radioactivity versus the concentration of radiotracer using GraphPad Prism 4.

Flow Cytometry. LNCaP cells were seeded into a T75 flask and allowed to form a monolayer over 48 h. After trypsin digestion, release cells were transferred into centrifuge tubes (1×10^6 cells/tube) and centrifuged. The medium was replaced with fresh medium containing DUPA−FITC (100 nM) in the presence or absence of 100-fold excess PMPA and incubated for 1 h at 37 °C. After rinsing with fresh medium (2× 1.0 mL) and tris buffer (1× 1.0 mL), cells were resuspended in PBS (1.0 mL) and cell bound fluorescence

(Cytomics F500, Beckman Coulter). Untreated LNCaP cells in PBS served as a negative control.

Confocal Microscopy. LNCaP cells (100,000 cells/well in 1 mL) were seeded into poly-D-lysine microwell Petri dishes and allowed cells to form monolayers over 24 h. Spent medium was replaced with fresh medium containing DUPA−FITC (100 nM) or DUPA−rhodamine (100 nM) in the presence or absence of 100-fold excess PMPA and cells were incubated for 1 h at 37 °C. After rinsing with fresh medium (2× 1.0 mL) and PBS (1× 1.0 mL), confocal images were acquired using a confocal microscopy (FV 1000, Olympus).

Tumor Models, Imaging, and Biodistribution Studies. Five-week-old male nu/nu mice were inoculated subcutaneously with LNCaP cells (5.0×10^6/mouse in 50% HC Matrigel), KB cells, or A549 (1.0×10^6/mouse in RPMI medium) on their shoulders. Growth of the tumors was measured in two perpendicular directions every 2 days using a caliper (body weights were monitored on the same schedule), and the volumes of the tumors were calculated as $0.5 \times L \times W^2$ (L = longest axis and W = axis perpendicular to L in millimeters). Once tumors reached between 400 and 500 mm^3 in volume, animals were treated with DUPA−^{99m}Tc (67 nmol, 150 μCi) in saline (100 μL). After 4 h, animals were sacrificed by CO_2 asphyxiation. Images were acquired by a Kodak Imaging Station (In-Vivo FX, Eastman Kodak Company) in combination with CCD camera and Kodak molecular imaging software (version 4.0). Radioimages: illumination source = radio isotope, acquisition time = 3 min, *f*-stop = 4, focal plane = 5, FOV = 160, binning = 4. White light images: illumination source = white light transillumination, acquisition time = 0.05 s, *f*-stop = 16, focal plane = 5, FOV = 160 with no binning.

Following imaging, animals were dissected and selected tissues were collected to preweighed γ-counter tubes. Radioactivity of preweighed tissues and DUPA−^{99m}Tc (67 nmol, 150 μCi) in saline (100 μL) was counted in a γ-counter. CPM values were decay corrected, and results were calculated as % ID/gram of wet tissue and tumor-to-tissue ratios.

In Vitro Potency of DUPA−TubH. LNCaP cells were seeded into 24-well (50,000 cells/well in 500 μL) Falcon plates and allowed cells to form monolayers over 48 h. Spent medium was replaced with fresh medium (0.5 mL) containing increasing concentrations of DUPA−TubH in the presence or absence of 100-fold excess PMPA, and cells incubated for an additional 2 h at 37 °C. Cells were washed 3× with fresh medium and incubated in fresh medium (0.5 mL) for 66 h at 37 °C. Spent medium in each well was replaced with fresh medium (0.5 mL) containing [^{3}H]-thymidine (1 μCi/mL), and cells were incubated for 4 h at 37 °C to allow [^{3}H]-thymidine incorporation. Cells were then rinsed with medium (3× 0.5 mL) and treated with 5% trichloroacetic acid (0.5 mL) for 10 min at rt. Cells were dissolved in 0.25 M NaOH (0.5 mL), transferred into individual vials containing Ecolume scintillation cocktail (3.0 mL), and counted in pany). IC_{50} was calculated by plotting % ^{3}H-thymidine incorporation versus log concentration of DUPA−TubH using GraphPad Prism 4.

In Vivo Potency of DUPA−TubH. Healthy male nu/nu mice were administered with multiple doses of freshly prepared DUPA−TubH dissolved in saline (200 μL) via lateral tail injection on days zero, 2, 4, 6, 8, and 10. Body weights and clinical observations were monitored prior to dosing and daily thereafter from day zero to 12. Chronic maximum tolerance dose was determined by plotting % weight change versus days on therapy, and any animals with a body weight loss of 20% or more over two consecutive days were euthanized.

Male nude mice bearing LNCaP xenograft tumors were then treated with 1.5 μmol/kg DUPA−TubH (for tumors of 90−130 mm^3) or 2.0 μmol/kg DUPA−TubH (for tumors of 320−360 mm^3) dissolved in 200 μL of saline via lateral tail vein injection. Treatments were conducted 3× per week for two weeks. Tumor volumes and body weights were measured on the same schedule. In vivo efficacy was evaluated by plotting tumor volume versus days on therapy.

Results

Design and Labeling of a PSMA-Targeted Radiotracer. In an effort to identify a high affinity targeting ligand for delivery of attached imaging and therapeutic agents to PSMA-expressing PCa cells, we conducted in silico docking studies on a series of PSMA inhibitors using a high resolution crystal structure of GCP-II in complex with the PSMA inhibitor termed GPI-18431 (PDB ID code 2C6C).[35] Due to its favorable binding mode (in silico), ease of synthesis, high experimental affinity for purified PSMA (K_i = 8 nM),[36] and availability of a free carboxylic acid not required for PSMA binding, 2-[3-(1,3-dicarboxypropyl)-ureido]pentanedioic acid (DUPA, Figure 1a) was selected as a possible targeting ligand.

Initial analysis of the tumor targeting specificity of DUPA-linked drugs required synthesis of a radiolabeled conjugate that would allow quantitation of its distribution in live animals. Because technetium (^{99m}Tc) is the major radioimaging nuclide used clinically, a common chelator of ^{99m}Tc, diaminopropionic acid-Asp-Cys,[32] was selected for attachment to DUPA. Thus, the PSMA crystal structure revealed a gradually narrowing access funnel 20 Å deep that extends from the protein surface to a binuclear zinc atom in the catalytic site.[35] By modeling the peptide spacer to fit the contours of this funnel, we were able to generate a targeting moiety that did not compromise DUPA's affinity for PSMA. Details and images of the fit of the peptide spacer to the 20

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