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A Low Molecular Weight PSMA-Based Fluorescent Imaging Agent for Cancer

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Abstract

We synthesized YC-27 **3** to provide a fluorescent imaging probe for the prostate-specific membrane antigen (PSMA), a marker for hormone-independent prostate cancer and tumor neovasculature, with suitable pharmacokinetics for use *in vivo*. Immediate precursor trifluoroacetate salt of 2-(3-{5-[7-(5-amino-1-carboxy-pentylcarbamoyl)-heptanoylamino]-1-carboxy-pentyl}-ureido)-pentanedioic acid **2** was conjugated with a commercially available near-infrared light emitting dye (IRDye 800CW) to provide **3** in 72% yield. YC-27 **3** demonstrated a PSMA inhibitory activity of 0.37 nM and was capable of generating target-to-nontarget ratios of at least 10 in PSMA-expressing PC3-PIP vs. PSMA-negative PC3-flu tumors *in vivo*. YC-27 **3** may be useful for study of PSMA-expressing tissue in preclinical models or for intraoperative guidance.

Keywords

Prostate-specific membrane antigen; NAALADase; fluorescence; urea; molecular imaging

Introduction

The prostate-specific membrane antigen (PSMA), while expressed in prostate tumor epithelium, has a curious property in that it is expressed in the neovasculature of many solid tumors but not in that of prostate cancer [1,2,3,4,5,6,7]. Because of that property, an ¹¹¹In-labeled monoclonal antibody to an extracellular epitope of PSMA, ¹¹¹In-J591, was capable of identifying renal, bladder, lung, breast, colorectal and pancreatic tumors in a Phase I clinical imaging study [7]. That study validated ¹¹¹In-J591 as a vascular targeting agent in human subjects. Since then other reports have further studied PSMA expression in certain tumor types. Baccala et al. noted that clear cell renal cell carcinoma expresses significantly more PSMA in its neovasculature than does the papillary variety [5]. Furthermore, angiomyolipoma, a benign renal lesion, did not express PSMA. As an enzyme with an extracellular active site PSMA

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represents an excellent target for imaging and therapy directed toward solid tumor neovasculature in addition to prostate cancer itself.

Complete detection and eradication of primary tumor and metastatic foci are required to effect a cure in patients with cancer, however, current preoperative assessment often misses small metastatic deposits. More sensitive imaging techniques than computed tomography, magnetic resonance imaging and even positron emission tomography (PET), which can be used easily in the operating suite, are required. An old technique, recently revisited because of improved optics and fluorescent dye chemistry, is intraoperative photodiagnosis (PDD) [8]. Fluorescein dyes have been used intraoperatively to identify brain tumors and verify the clarity of tumor margins since 1948 [8]. A recent report describes its utility in identifying brain metastases [9]. A long history of the use of 5-aminolevulinic acid (5-ALA) for brain tumor resection is also evident, and its use has been associated with improvement in progression-free survival [10]. PDD can be performed easily during surgery due to the lack of a need for complex imaging equipment. All that is needed is a light-emitting diode to excite the fluorophore, which can be administered systemically or “painted” on the tissue directly. More recent incarnations of PDD have used quantum dots [11], and more advanced dyes, such as indocyanine green (ICG) [12], which emit in the near-infrared (NIR) region of the spectrum, enabling reasonable tissue penetration of emitted (and detected) light. Applications have included nontargeted approaches, such as preoperative evaluation of the vascular integrity of surgical flaps or identification of nodules of hepatocellular carcinoma [13]. Targeted approaches are also emerging, such as use of a fluorophore-conjugated anti-CEA antibody to identify colon or pancreatic cancer [14], or the use of NIR activatable probes that emit light only when cleaved by a tumor-associated protease [15]. Here we describe a PSMA-binding NIR probe that demonstrates pharmacokinetics suitable for targeting PSMA *in vivo*.

Materials and Methods

General

All reagents and solvents were purchased from either Sigma-Aldrich (Milwaukee, WI) or Fisher Scientific (Pittsburgh, PA). 2-{3-[5-[7-(2,5-Dioxo-pyrrolidin-1-yloxy-carbonyl)-heptanoylamino]-1-(4-methoxy-benzyloxycarbonyl)-pentyl]-ureido}-pentanedioic acid bis-(4-methoxy-benzyl) ester **1** was prepared according to [16]. H-Lys(Boc)-OBu-HCl was purchased from Chem-Impex International (Wood Dale, IL). The *N*-hydroxysuccinimide (NHS) ester of IRDye 800CW was purchased from LI-COR Biosciences (Lincoln, NE). ¹H NMR spectra were obtained on a Bruker Avance 400 MHz Spectrometer. ESI mass spectra were obtained on a Bruker Esquire 3000 plus system. Purification by high-performance liquid chromatography (HPLC) was performed on a Varian Prostar System (Varian Medical Systems, Palo Alto, CA).

Chemistry

Trifluoroacetate salt of 2-(3-{5-[7-(5-amino-1-carboxy-pentylcarbamoyl)-heptanoylamino]-1-carboxy-pentyl}-ureido)-pentanedioic acid (2**)**—To a solution of **1** (0.065 g, 0.020 mmol, [16]) in CH₂Cl₂ (2 mL) was added triethylamine (0.040 mL, 0.285 mmol), followed by H-Lys(Boc)-OBu-HCl (0.028 g, 0.083 mmol). After stirring for 2 h at room temperature, the solvent was evaporated on a rotary evaporator. A solution of TFA/CH₂Cl₂ 1:1 (2 mL) was then added to the residue and stirred for 1 h at room temperature. The crude material was purified by HPLC (column, Econosphere C18, 10μ, 250 × 10 mm; retention time, 15 min; mobile phase, A = 0.1% TFA in H₂O, B = 0.1% TFA in CH₃CN; gradient, 0 min = 5% B, 25 min = 25% B; flow rate, 4 mL/min) to afford 0.032 g (66%) of **2**. ¹H NMR (400 MHz, D₂O) δ 4.24–4.28 (m, 1H), 4.17–4.20 (m, 1H), 4.08–4.12 (m, 1H), 3.08–3.12 (m, 2H), 2.88–2.92 (m, 2H), 2.41–2.44 (m, 2H), 2.19–2.21 (m, 2H), 2.05–2.16 (m, 3H), 1.57–1.93 (m,

7H), 1.21–1.50 (m, 10H), 1.21 (m, 4H). ESI-Mass calcd for $C_{26}H_{46}N_5O_{11} [M]^+$ 604.3, found 604.0.

YC-27 (3)—To a solution of **2** (0.3 mg, 0.43 μ mol) in DMSO (0.1 mL) was added *N,N*-diisopropylethylamine (0.002 mL, 11.4 μ mol), followed by the NHS ester of IRDye 800CW (0.3 mg, 0.26 μ mol). After stirring for 2 h at room temperature, the reaction mixture was purified by HPLC (column, Econosphere C18 5 μ , 150 $\times$ 4.6 mm; retention time, 22 min, mobile phase, A = 0.1% TFA in H_2O , B = 0.1% TFA in CH_3CN ; gradient, 0 min = 0% B, 5 min = 0% B, 45 min = 100% B; flow rate, 1 mL/min) to afford 0.3 mg (72%) of **3**. ESI-Mass calcd for $C_{72}H_{97}N_7O_{25}S_4 [M]^+$ 1587.5, found 794.3 $[M+H]^{2+}$, 1587.6 $[M]^+$.

Fluorescence Properties—Fluorescence spectra were recorded using a Varian Cary Eclipse fluorescence spectrophotometer (Varian Medical Systems) with excitation from a Xenon arc lamp. YC-27 **3** was dissolved in water. All of the fluorescence measurements were performed in aqueous solution under ambient conditions. The fluorescence quantum yield of **3** was measured using an aqueous solution of ICG ($\Phi = 0.016$ [17], excitation wavelength at 775 nm) as the standard. The fluorescence intensity data were collected in the spectral region 780 – 900 nm over which quantum yield was integrated. Time-resolved intensity decays were recorded using a PicoQuant Fluotime 100 time-correlated single-photon counting (TCSPC) fluorescence lifetime spectrometer (PicoQuant, Berlin, DE). The excitation was obtained using a pulsed laser diode (PicoQuant PDL800-B) with a 20 MHz repetition rate. The fluorescence intensity decay of **3** was analyzed in terms of the single-exponential decay using the PicoQuant Fluofit 4.1 software with deconvolution of the instrument response function and nonlinear least squares fitting. The goodness-of-fit was determined by the χ^2 value.

Biological Studies

NAALADase Assay—PSMA inhibitory activity of **3** was determined using a fluorescence-based assay according to a previously reported procedure [18]. Briefly, lysates of LNCaP cell extracts (25 μ L) were incubated with the inhibitor (12.5 μ L) in the presence of 4 μ M *N*-acetylaspartylglutamate (NAAG) (12.5 μ L) for 120 min. The amount of glutamate released by NAAG hydrolysis was measured by incubation with a working solution (50 μ L) of the Amplex Red Glutamic Acid Kit (Molecular Probes Inc., Eugene, OR) for 60 min. Fluorescence was measured with a VICTOR³V multilabel plate reader (Perkin Elmer Inc., Waltham, MA) with excitation at 530 nm and emission at 560 nm. Inhibition curves were determined using semi-log plots, and IC_{50} values were determined at the concentration at which enzyme activity was inhibited by 50%. Assays were performed in triplicate. Enzyme inhibitory constants (K_i values) were generated using the Cheng-Prusoff conversion [19]. Data analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California).

Cell Culture and Animal Models—Both PSMA-expressing (PC3-PIP) and non-expressing (PC3-flu) prostate cancer cell lines [1] were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Invitrogen) and 1% Pen-Strep (Biofluids, Camarillo, CA). All cell cultures were maintained in 5% carbon dioxide (CO_2), at 37.0°C in a humidified incubator. Animal studies were undertaken in compliance with the regulations of the Johns Hopkins Animal Care and Use Committee. Six- to eight-week-old male, non-obese diabetic (NOD)/severe-combined immunodeficient (SCID) mice (Charles River Laboratories, Wilmington, MA) were implanted subcutaneously (s.c.) with PC3-PIP and PC3-flu cells (2×10^6 in 100 μ L of Matrigel) at the forward left and right flanks, respectively. Mice were imaged or used in *ex vivo* biodistribution assays when the xenografts reached 5 to 7 mm in diameter.

In vivo Imaging and Ex vivo Biodistribution—Mouse #1 was injected with 10 nmol and mouse #2 with 1 nmol of **3** in 200 μ L of PBS intravenously (i.v.) *via* the lateral tail vein. Mouse #3 was injected with 1 nmol of **3** and also co-injected with 1 μ mol of the known PSMA inhibitor 2-{3-[1-carboxy-5-(4-iodo-benzoylamino)-pentyl]-ureido}-pentanedioic acid (DCIBzL) [20, 21] in 200 μ L of PBS i.v. to assess for PSMA binding specificity. Images were acquired at an array of post-injection (PI) time points starting at 10 min PI using a dedicated small animal optical imaging instrument, the Pearl Imager (LI-COR Biosciences). The Pearl Imager uses diffusive lasers optimized for IRDye 800CW. The instrument employs a CCD camera with a field-of-view of 11.2 cm $\times$ 8.4 cm at the surface of the imaging bed. The scan time was less than 30 sec to complete white light, 700 nm channel and 800 nm channel image acquisition. Images are displayed using a pseudocolor output with corresponding scale. All images were acquired at the same parameter settings and are scaled to the same maximum values. Imaging bed temperature was adjusted to 37°C. Animals received inhalational anesthesia (isoflurane) through a nose cone attached to the imaging bed. Animals were sacrificed by cervical dislocation for *ex vivo* imaging studies at the end of acquisition of the *in vivo* images. *Ex vivo* images were acquired first by midline surgical laparotomy and then again by harvesting liver, spleen, stomach, small intestine, kidneys, urinary bladder, PC3-PIP and PC3-flu tumors and displaying them individually on plastic Petri dishes. Estimates of signal output were provided by drawing three circular regions of interest within each tumor and determining the average signal (arbitrary units)/area using the manufacturer's software.

Results and Discussion

We synthesized low molecular weight PSMA-based NIR imaging agent YC-27 **3** in ~ 47% overall yield in two steps from the NHS ester of a PSMA-binding urea that we recently reported (Scheme 1) [16]. The reactions were readily completed at room temperature and involved extension of the linker moiety with an amine function that could then be reacted with the NHS ester of a commercially available NIR dye (IRDye 800CW, LI-COR Biosciences). The electronic spectrum of **3** exhibited an absorbance maximum at 774 nm with an extinction coefficient of 158,900 M⁻¹. Upon excitation, **3** provided intense fluorescence with an emission maximum at 792 nm and a fluorescence lifetime of 443 psec in aqueous solution. Using an excitation wavelength of 775 nm, **3** demonstrated a fluorescence quantum yield of 0.053 in aqueous solution relative to ICG, which demonstrated a quantum yield of 0.016 [17], attesting to the efficiency of this IRDye 800CW-based compound. That is significant because ICG has been used previously for intraoperative tumor mapping [12].

We determined the *in vitro* PSMA inhibitory activity of **3** using a fluorescence-based NAALADase assay [18]. This assay is free from the interference of IRDye 800CW because the excitation/emission maxima of IRDye 800CW are remote from those of resorufin (λ_{ex} = 563 nm, λ_{em} = 587 nm), which provides the fluorescent readout in the assay. The K_i value of **3** was 0.37 nM with 95% confidence intervals from 0.18 nM to 0.79 nM. Under the same experimental conditions, the K_i value of the known PSMA inhibitor ZJ-43 [22] was 2.1 nM, indicating the high inhibitory capacity of **3**. The inhibition curve of **3**, which is expressed with respect to the amount of glutamate released from hydrolysis of NAAG, is shown in Figure 1.

Figure 2 (mouse #1) depicts the pharmacokinetic behavior of **3** *in vivo*. In this experiment 10 nmol of **3** was administered i.v. and the animal was imaged repeatedly over a three day period. Although difficult to quantify as these are planar images, one can see clearly increased uptake in the PSMA+ PC3-PIP tumor relative to the control (PSMA-negative) PC3-flu tumor at 18.5 h PI through 70.5 h PI (Figure 2C through 2M). Using quantitative real time polymerase chain reaction (qRT-PCR) we measured the relative amounts of PSMA mRNA expression in extracts of the tumors in mice #1–3, and confirmed that PC3-PIP tumors (left flank) expressed PSMA mRNA at levels several million times higher than PC3-flu tumors (right flank) (data not

shown). Panels L and M show emission from the intact, living, unshaven animal, while panels N and O are postmortem studies with organs exposed. Note that in L one can barely discern the kidneys, a known target site for PSMA [23,24,25], while the kidneys are clearly visible in O when exposed. A portion of that renal light emission may be due to clearance of this relatively hydrophilic compound. The estimated target-to-nontarget ratio (PC-3 PIP vs. PC-3 flu light output) was 10 when comparing the tumors from panel M (70.5 h PI).

The experiment in Figure 3 was performed with 10-fold less **3** administered than in the previous experiment. Despite reducing the concentration of **3**, PSMA+ PC3-PIP tumor could be seen clearly at one day PI (Figure 3, mouse #2, *Left Panels*). DCIBzL, a known, high-affinity PSMA inhibitor, was co-administered with **3** as a test of binding specificity (Figure 3, mouse #3, *Right Panels*). Nearly all of the light emission from target tumor, as well as kidneys, was blocked, demonstrating the specificity of this compound for PSMA *in vivo*. The estimated target-to-nontarget ratio (PC-3 PIP vs. PC-3 flu light output) was 26 when comparing the tumors from panel F (20.5 h PI). By administering 1 nmol to this ~ 25 g mouse, we have realized the high sensitivity of *in vivo* optical imaging, rivaling that of the radiopharmaceutical-based techniques. For example, 1 nmol converts to 1.6 μg injected. If we synthesized a similar compound labeled with ^{18}F or other radionuclide at 1,000 mCi/ μmol (37 GBq/ μmol), and administered a standard dose of 200 μCi (7.4 MBq) to a mouse, we would be injecting 0.3 μg .

Interestingly, in mouse #1, which received 10 nmol of **3**, we observed a small degree of non-specific uptake at the 23 h time point, manifested as uptake within PSMA-negative PC3-flu tumors. That finding could be due to enhanced permeability and retention of **3**. No non-specific uptake/retention was observed at a similar, 20.5 h, time point in mouse #2, which received a 10-fold lower dose. That finding suggests the need for further optimization of dose and timing for *in vivo* applications.

A wide variety of low molecular weight PSMA-based imaging agents have been synthesized by us and others, including those using the urea scaffold [16,18,22,24,26,27,28,29,30,31,32, 33]. Those compounds have primarily been radiopharmaceuticals, but optical agents exist. In two separate studies Humblet et al. reported the synthesis of mono- and polyvalent NIR fluorescent phosphonate derivatives for imaging PSMA, but little accumulation in PSMA-expressing tumors was evident in the former study [27] while no *in vivo* results were reported in the latter [31]. Liu et al have also synthesized fluorescent phosphonate derivatives and have demonstrated their PSMA-binding specificity and intracellular localization *in vitro* [30]. Recently Kularatne et al. have synthesized fluorescent (fluorescein and rhodamine) urea derivatives that demonstrate PSMA migration to endosomes [32]. We arrived at YC-27 **3** based on structure-activity relationships developed for PSMA-binding ureas, which were focused on improving pharmacokinetics for use *in vivo* by optimization of the linker-chelate complex [16]. Calculated hydrophobicity values [34] suggest that **3** should be considerably more hydrophobic (ALogD = 5.96) than radiopharmaceuticals such as [^{125}I]DCIBzL (ALogD = 1.19), perhaps accounting for its long tumor retention, which is desirable for an optical imaging agent intended for intraoperative use. We confirmed greater hydrophobicity of **3** relative to DCIBzL through reverse-phase HPLC (data not shown).

Conclusions

We synthesized NIR imaging agent YC-27 **3** in reasonable yield using mild, well established conditions. Because of its salutary pharmacokinetics *in vivo*, YC-27 **3** is promising for clinical investigation of PSMA-expressing tissue, such as prostate tumors or solid tumor neovasculature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

PSMA, prostate-specific membrane antigen; YC-27, 2-((*E*)-2-((*E*)-3-((*E*)-2-(3,3-dimethyl-5-sulfo-1-(4-sulfobutyl)indolin-2-ylidene)ethylidene)-2-(4-sulfophenoxy)cyclohex-1-enyl vinyl)-3,3-dimethyl-1-((3*S*,7*S*)-1,3,7,22-tetracarboxy-5,13,20,28-tetraoxo-4,6,12,21,27-pentaazatritriacontan-33-yl)-3*H*-indolium-5-sulfonate; NAAG, *N*-acetylaspartylglutamate; DCIBzL, 2-{3-[1-carboxy-5-(4-iodo-benzoylamino)-pentyl]-ureido}-pentanedioic acid; ZJ-43, 2-[3-(1-carboxy-3-methyl-butyl)-ureido]-pentanedioic acid; NAALADase, *N*-acetylated- α -linked-acidic-dipeptidase; PBS, phosphate-buffered saline..

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Figure 1.
IC₅₀ curve of compound **3** using a fluorescence-based NAALADase assay

Figure 2.

In vivo imaging of a NOD/SCID mouse (mouse #1), bearing PC3-PIP (forward left flank) and PC3-flu (forward right flank) tumors. Mouse #1 received 10 nmol of **3** and dorsal (animal prone) and ventral (animal supine) views were obtained. Dorsal and ventral views at 40 min PI (A, B, respectively); 18.5 h (C, D); 23 h (E, F); 42.5 h (G, H); 68 h (I, J). Dorsal view of pre-injection image (K). Dorsal and ventral views 70.5 h PI (L, M). Images after midline laparotomy (N) and individually harvested organs (O) on a Petri dish at 70.5 h PI. Images were scaled to the same maximum (arbitrary units).

Figure 3.

Left Panels: *In vivo* imaging of a NOD/SCID mouse (mouse #2), bearing PC3-PIP (forward left flank) and PC3-flu (forward right flank) tumors. Mouse #2 received 1 nmol of **3** and dorsal (animal prone) and ventral (animal supine) views were obtained. Dorsal and ventral views of the pre-injection image (A, B, respectively); 10 min PI (C, D); 20.5 h (E, F); 24 h (G, H). Images after midline laparotomy (I) and individually harvested organs (J) on a Petri dish at 24 h PI. *Right Panels:* Mouse #3 in same orientation as mouse #2. Mouse #3 received 1 nmol of **3** co-injected with 1 μ mol of DCIBzL, which served as a blocking agent to test binding specificity. Images were scaled to the same maximum (arbitrary units).

Scheme 1.

(a) H-Lys(Boc)-OBu-HCl, Et_3N , CH_2Cl_2 ; (b) TFA: CH_2Cl_2 = 1:1; (c) IRDye800CW-NHS, DIPEA, DMSO