Theranostic Agents for Photodynamic Therapy of Prostate Cancer by Targeting Prostate-Specific Membrane Antigen

Xinning Wang1, Brian Tsui2, Gopolakrishnan Ramamarthy1, Ping Zhang3, Joseph Meyers1, Malcolm E. Kenney3, Jonathan Kiechle4, Lee Ponsky4, and James P. Basilion1,5

Abstract

Prostatectomy has been the mainstay treatment for men with localized prostate cancer. Surgery, however, often can result in major side effects, which are caused from damage and removal of nerves and muscles surrounding the prostate. A technology that can help surgeons more precisely identify and remove prostate cancer resulting in a more complete prostatectomy is needed. Prostate-specific membrane antigen (PSMA), a type II membrane antigen highly expressed in prostate cancer, has been an attractive target for imaging and therapy. The objective of this study is to develop low molecular weight PSMA-targeted photodynamic therapy (PDT) agents, which would provide image guidance for prostate tumor resection and allow for subsequent PDT to eliminate unresectable or remaining cancer cells. On the basis of our highly negatively charged, urea-based PSMA ligand PSMA-1, we synthesized two PSMA-targeting PDT conjugates named PSMA-1-Pc413 and PSMA-1-IR700. In in vitro cellular uptake experiments and in vivo animal imaging experiments, the two conjugates demonstrated selective and specific uptake in PSMA-positive PC3pip cells/tumors, but not in PSMA-negative PC3Ilu cells/tumors. Further in vivo photodynamic treatment proved that the two PSMA-1-PDT conjugates can effectively inhibit PC3pip tumor progression. The two PSMA-1-PDT conjugates reported here may have the potential to aid in the detection and resection of prostate cancers. It may also allow for the identification of unresectable cancer tissue and PDT ablation of such tissue after surgical resection with potentially less damage to surrounding tissues.

Mol Cancer Ther; 15(8); 1–11. ©2016 AACR.

Introduction

PSA testing has allowed significantly more men to be diagnosed and treated for prostate cancer. Approximately 220,800 new diagnoses and 27,540 deaths from the disease are projected in 2015 in the United States (1). Over 90% of men have localized tumors at initial screenings and are candidates for radical prostatectomies (2). However, at surgery, cancer has been shown to extend outside the prostate (pathologic stage C) in 20% to 42% of patients (3), surgery fails to halt the disease in approximately 20% of the patients who undergo radical prostatectomy, and recurrence rate of this disease is more than 60% (4–6).

During radical prostatectomy, surgeons have difficulty accessing prostate cancer invasion; therefore, many malignant nodules escape detection, leading to disease recurrence. A retrospective multivariate analysis by Wright and colleagues of incomplete resection of prostate cancer in more than 65,000 patients who underwent radical prostatectomies (7) found that positive surgical margins were associated with a 2.6-fold increased unadjusted risk of prostate cancer–specific mortality and are an independent predictor of mortality. This study also underscored the need for surgeons to optimize surgical techniques to achieve negative surgical margins to increase sound oncological outcomes. However, surgical achievement of this without side effects is challenging because the prostate is surrounded by many nerves and muscle fibers controlling different excretory and erectile functions that are difficult but necessary to avoid. In 1983, Walsh defined nerve locations around the prostate and inspired a number of “nerve-sparing” surgical approaches, including robotic-assisted laparoscopic prostatectomy (8). Unfortunately, the success of these approaches to mitigate side effects is mixed, (9, 10) and surgical approaches are still associated with significant morbidity, for example, incontinence (3%–74%) and impotence (30%–90%; refs. 11–16). There remains an unmet clinical need to improve surgical techniques for identifying and removing cancerous tissue without damaging surrounding tissues during prostatectomy. Recently, Neuman and colleagues showed that the near-infrared (NIR) fluorescence probe YC-27 can improve the surgical treatment of prostate cancer and reduce the rate of positive surgical margins in real-time laparoscopic extirpative surgery (17).

Photodynamic therapy (PDT) is a minimally invasive therapy used clinically in the treatment of cancers and other diseases (18–20). PDT uses photosensitizers which are pharmacologically inactive until exposed to light in the presence of oxygen. The active drug forms reactive oxygen species such as singlet oxygen to kill...
Materials and Methods

General
PSMA targeting peptide Glu-CO-Glu’-Amar-Ahx-Glu-Glu-Glu-Lys-NH₂ (PSMA-1; Supplementary Fig. S1) for PSMA-targeted imaging of prostate cancer (37, 38). In this study, we have developed two PSMA-1-based PDT conjugates (PSMA-1-PDT). PSMA-1-Pc413 (Fig. 1A) and PSMA-1-IR700 (Fig. 1C). Pc413 is an analogue of the second-generation phthalocyanine PDT drug Pc4 which is currently in clinical trials (39). IR700 is a commercially available NIR phthalocyanine dye which has PDT activity (40–42). The two conjugates reported here were found to be effective as theranostic conjugates, allowing both targeted-bioimaging and targeted-PDT of prostate cancer.

Synthesis of PSMA-1-SMCC
Coupling of PSMA-1 to sulfosuccinimidyl-4-(N-maleimido-pentanedioic acid (Cys-CO-Glu) was custom-made by Bachem Bioscience Inc. pc413 was synthesized by Fmoc chemistry as reported previously (38). S)-2-(3-(S)-5-amino-1-carboxyphenyl)ureido)pentanedioic acid (Cys-CO-Glu; Supplementary Fig. S1) was custom-made by Bachem Bioscience Inc. pc413 was provided by M.E. Kenney’s laboratory. All the other chemicals were purchased from Sigma-Aldrich Inc.

Synthesis of PSMA-1-Pc413
Pc413 was first dissolved in 1 mL of DMF, then PSMA-1-SMCC in 100 μL of 100 mmol/L phosphate buffer, pH 8.0 was added (Supplementary Fig. S3A). The reaction mixture was stirred at room temperature for 2 hours and purified by preparative HPLC using gradient B (Supplementary Methods) to get purified PSMA-1-Pc413. Yield: 63%. Retention time: 15.4 minutes. MALDI-MS: C₁₀₂H₁₃₄N₂₀₂O₁₅S₅I₃, 1306.7 (found); 1306.4 (calculated; Supplementary Fig. S2B and S2C).

Synthesis of PSMA-1-IR700
Coupling of PSMA-1 to IRDye700DX NHS ester (LI-COR Biosciences) was performed in 100 mmol/L phosphate buffer, pH 7.0. PSMA-1 (1 mg) in 200 μL of phosphate buffer was added to 0.5 mg of IRDye700DX NHS ester in 200 μL of phosphate buffer (Supplementary Fig. S4A). The reaction was performed at room temperature overnight. The crude product was purified by preparative HPLC using gradient C (Supplementary Methods). Yield: 43%. Retention time: 28.7 minutes. MALDI-MS: C₁₁₆H₁₃₂N₂₅Na₄O₄₃S₆I₃, 1840.9 (found, M-2C₄H₁₅0₃NaN₃O₁₀S₅S; 1841.0 (calculated; Supplementary Fig. S4B and S4C).

Cell culture
Retrovirally transfected PSMA-positive PC3pip cells and transfection control PC3flu cells were obtained from Dr. Michel Sadelain in 2000 (Laboratory of Gene Transfer and Gene Expression, Gene Transfer and Somatic Cell Engineering Facility, Memorial-Sloan Kettering Cancer Center, New York, NY). The cells were last checked by Western blot analysis in 2014; no genetic authentication was performed. Cells were maintained in RPMI1640 medium (Invitrogen Life Technology) with 2 mmol/L L-glutamine and 10% FBS at 37°C and 5% CO₂ under a humidified atmosphere.

Competitive binding assay
The assay was carried out as reported previously (38) by incubation of PSMA-Pc413 or PSMA-1-IR700 in the presence of 12 nmol/L N-[N-[(S)-1,3-dicarboxypropyl]carbamoyl]-S-[4′H]-methyl-L-cysteine (H-ZJ24; Supplementary Fig. S1; GE Healthcare Life Sciences). Radioactivity of cell pellet was counted by scintillation counter. The concentration required to inhibit 50% of binding was determined (IC₅₀) by GraphPad Prism 3.0.

In vitro cellular uptake studies
PC3pip and PC3flu cells were plated on coverslips at about 70% confluency. Twenty-four hours later, cells were incubated with 1 μmol/L of PSMA-1-Pc413 or PSMA-1-IR700 for various times. After incubation, cells were washed three times with PBS, fixed with 4% paraformaldehyde, counterstained with 2-(4-aminophenyl)-6-indolecarbamidine dihydrochloride (DAPI), mounted with Fluor-Mount aqueous mounting solution, and observed under Leica DM4000B fluorescence microscopy (Leica Microsystems Inc.). Competition experiments were performed by coincubation of PC3pip and PC3flu cells with 1 μmol/L of PSMA-1-Pc413 or PSMA-1-IR700 and 10 μmol/L of Cys-CO-Glu (Supplementary Fig. S1; ref. 38) for 4 hours.
In vitro phototoxicity assay

Cell viability in the dark and under light exposure was evaluated by CellTitre 96 Aqueous Cell Proliferation Assay (Promega Corporation). Cells (3,000/well) were seeded in 96-well culture plates the day before treatment. On treatment day, cells were incubated with 1 μmol/L of PSMA-1-Pc413 or PSMA-1-IR700 for 1 or 4 hours. After incubation, cells were washed three times with 200 μL of cold RPMI media and irradiated under light (>500 nm; Apollo Horizon Projector, Acco Brands; Supplementary Fig. S5A for the spectrum of the light and Supplementary Method) with irradiance of 8.3 mW/cm² and radiant exposure of 0.5 J/cm². Twenty-four hours later, CellTitre 96 Aqueous reagent was added to each well. After a 3-hour incubation at 37°C, the absorbance at 490 nm was measured.

Singlet oxygen detection

Photo-induced singlet oxygen generation was detected by the chemical method using 1,3-diphenylisobenzofuran (DPBF) as the chemical quencher (43, 44). The decay of DPBF was monitored by the absorption at 411 nm. Briefly, DPBF (50 μmol/L) in ethanol was added to PSMA-1-Pc413 and PSMA-1-IR700. The irradiation wavelength used to excite PSMA-1-Pc413 and PSMA-1-IR700 was 672 nm and 690 nm, respectively. The absorbance at 411 nm was measured on an Infinite M200 Plate Reader (Tecan Group Ltd.).

In vivo NIR imaging studies

Animal experiments were performed according to guidelines of the animal care and use committee at Case Western Reserve University. The animal care and use committee at Case Western Reserve University approved the protocol for animal experiments. In vivo NIR imaging studies were performed using an In Vivo Imaging System (IVIS) (PerkinElmer) for detecting the NIR fluorescence of PSMA-1-Pc413 and PSMA-1-IR700 in nude mice. Nude mice were subcutaneously implanted with PC3 pip cells and PC3 flu cells on their right and left flanks, respectively. The absorbance at 411 nm was measured on an Infinite M200 Plate Reader (Tecan Group Ltd.).

In vitro cellular uptake results of PSMA-1-Pc413 and PSMA-1-IR700. B, in vitro cellular uptake results of PSMA-1-Pc413. PSMA-positive PC3pip cells and PSMA-negative PC3flu cells on coverslips were incubated with no probe (0 minute) or 1 μmol/L of PSMA-1-Pc413 for 15 minutes, 30 minutes, 1 hour, and 4 hours. Nuclei were stained using DAPI (false color blue) and uptake of PSMA-1-Pc413 was assessed by fluorescence microscopy (false color red). Specificity of PSMA-1-Pc413 for PSMA binding was evaluated by incubation of PC3pip and PC3flu cells with 1 μmol/L of PSMA-1-Pc413 and 10 μmol/L of Cys-Co-Glu, last column. Signal in PC3pip cells was significantly competed by Cys-Co-Glu, suggesting the binding of PSMA-1-Pc413 to PSMA is specific. Images are taken at 40×. Representative images are shown from three independent experiments. C, chemical structure of PSMA-1-IR700. D, in vitro cellular uptake results of PSMA-1-IR700. PSMA-positive PC3pip cells and PSMA-negative PC3flu cells on coverslips were incubated with no probe (0 minute) or 1 μmol/L of PSMA-1-IR700 for 15 minutes, 30 minutes, 1 hour, and 4 hours. The nuclei were stained by DAPI (false color blue) and uptake of PSMA-1-IR700 was assessed by fluorescence microscopy (false color red). Specificity of PSMA-1-IR700 to PSMA was evaluated by incubation of PC3pip and PC3flu cells with 1 μmol/L of PSMA-1-IR700 and 10 μmol/L of Cys-Co-Glu, last column. Signal in PC3pip cells was significantly competed by Cys-Co-Glu, suggesting the binding of PSMA-1-IR700 to PSMA is specific. Images are taken at 40×. Representative images are shown from three independent experiments.
University (IACUC #120024). Six- to eight-week-old male athymic nude mice were implanted subcutaneously with \(1 \times 10^6\) of PSMA-negative PC3flu and PSMA-positive PC3pip on the left and right dorsum, respectively. Animals were used when tumor lengths reached 10 mm. PSMA-1-PDT was injected intravenously via the tail vein. Fluorescence imaging was performed using the Maestro In Vivo Imaging System (Perkin-Elmer). During imaging, mice were anesthetized with isoflurane. After imaging, the mice were euthanized, and tissues were harvested for ex vivo imaging.

For in vivo competition experiments, mice were co-injected with 1 nmol of PSMA-1-Pc413 and 1,000 nmol of ZL-MCC-Ahx-YYYG (Supplementary Fig. S1), an analogue of PSMA-1 with similar binding affinity (37), or 1 nmol of PSMA-1-IR700 conjugate and 1,000 nmol of PSMA-1. Different competitors were used for PSMA-1-Pc413 and PSMA-1-IR700 due to their different pharmacokinetic behaviors. Multispectral images were unmixed into their component spectra (PSMA probes, autofluorescence, and background). Component images were used to quantify the average fluorescence intensity associated with the tumors by creating regions of interest around the tumors.

In vivo PDT treatment of subcutaneous PC3pip tumors

Six- to eight-week-old male athymic nude mice were implanted subcutaneously with \(1 \times 10^6\) PC3pip cells. Tumor volume was measured in vivo using calipers and calculated by the following formula: Tumor volume = \(length \times width \times depth\) (45). Tumors reaching approximately 50 mm\(^3\) in volume were selected for the study. To test the PDT activity of PSMA-1-Pc413, selected mice were divided into 4 groups with each group having 5 mice. Groups: (i) no treatment; (ii) mice were injected at a dosage of 0.1 mg/kg; (iii) mice were injected at a dosage of 0.25 mg/kg; or (iv) mice were injected at a dosage of 0.5 mg/kg. The mice were imaged before injection, immediately following injection, and 24 hours postinjection. At 24 hours postinjection, the tumors were subjected to PDT treatment using 672-nm light from a diode laser (Applied Optonics Corp; see Supplementary Fig. S5B for the spectrum of the laser light). The 672-nm laser diode was chosen as it was previously used with success in prior studies (46) due to its proximity to the peak of Pc413 absorbance (676 nm) and because of the availability of laser diodes. Light was delivered through a GRIN-lens terminate multimode fiber (OZ Optics) and tumors were illuminated with irradiance of 33.3 mW/cm\(^2\), radiant exposure of 150 J/cm\(^2\) at 672 nm (Supplementary Methods). The mice were imaged posttreatment and allowed to recover.

Histology studies with H&E staining

PC3pip tumors were harvested 1 day after single PDT treatment with 0.5 mg/kg of PSMA-1-PDT conjugates. Tumors were fixed in 4% paraformaldehyde and embedded into paraffin blocks. Serial 10-mm slice sections were fixed on slide glasses and hematoxylin and eosin (H&E) stained. Sections were photographed using the 10× objective. Control tumors were harvested from mice received same dose of drugs, but without light illumination.

Statistical analysis

Statistical analyses were carried out using Microsoft Excel. Student t test was used to compare the treatment effects with controls. \(P < 0.05\) was used to determine statistical significance.

Results

Synthesis

The synthesis and characterization of PSMA-1-Pc413 and PSMA-1-IR700 is shown in Supplementary Figs. S2–S4. PSMA-1 and Pc413 were coupled through the heterobifunctional linker sulfo-SMCC (Supplementary Figs. S2A and S3A). PSMA-1-IR700 was synthesized by reaction of PSMA-1 with commercially available IRDye700DX NHS ester in PBS (Supplementary Fig. S4A). PSMA-1-Pc413 has maximum absorbance at 667 nm and maximum emission at 678 nm (Supplementary Fig. S6A) and PSMA-1-IR700 has \(\lambda_{\text{max}}\) at 690 nm and \(\lambda_{\text{Em}}\) at 698 nm (Supplementary Fig. S6B), both concurring those of Pc413 and IR700. Conjugation of PSMA-1 and Pc413 resulted in a molecule with better solubility characteristics than the parent Pc413, likely due to the presence of three negatively charged glutamate residues in PSMA-1. As a result, PSMA-1-Pc413 (logP value = –1.35 ± 0.14) is water soluble. The conjugate PSMA-1-IR700, logP = –2.46 ± 0.22, also has good water solubility. The water solubility of our conjugates eliminates the need for a complicated drug formulation for systemic delivery.

In vitro competition binding results

Competition binding assay showed that both PSMA-1-Pc413 (IC\(_{50}\) = 2.1 ± 0.22 nmol/L) and PSMA-1-IR700 (IC\(_{50}\) = 2.2 ± 0.15 nmol/L) had binding affinities greater than 4.6-fold compared with the related ligand Cys-CO-Glu (Supplementary Fig. S1; ref. 38; IC\(_{50}\) = 10.2 ± 0.31 nmol/L; Table 1) and an affinity similar to that measured in previous studies using PSMA-1-Cy5.5 or PSMA-1-IR800 (38).

In vitro uptake results

To examine the uptake of PSMA-targeted PDT conjugates, in vivo cellular uptake of PSMA-1-Pc413 and PSMA-1-IR700 in PSMA-positive PC3pip cells and PSMA-negative PC3-flu cells were performed and visualized by fluorescence microscopy. No significant uptake was observed in PSMA-negative cells. In PSMA-positive cells, fluorescence intensity was observed only in the regions of the cell where also PSMA localization was detected. These results indicate that the PSMA-targeted conjugates were efficiently internalized by the cells.

Table 1. In vitro competition binding results of PSMA-1-Pc413 and PSMA-1-IR700

<table>
<thead>
<tr>
<th>Cys-CO-Glu</th>
<th>PSMA-1-Pc413</th>
<th>PSMA-1-IR700</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC(_{50}) (nmol/L)</td>
<td>10.2 ± 0.31</td>
<td>10.2 ± 0.31</td>
</tr>
<tr>
<td>IC(_{50}) (nmol/L)</td>
<td>2.1 ± 0.22</td>
<td>2.1 ± 0.22</td>
</tr>
<tr>
<td>IC(_{50}) (nmol/L)</td>
<td>2.2 ± 0.15</td>
<td>2.2 ± 0.15</td>
</tr>
</tbody>
</table>

Note: The assay was carried out by incubating PC3pip cells (5 × 10\(^4\)) with different concentrations of PSMA-1-PDT conjugates in the presence of 12 nmol/L N-[N-(Cys-L-3-dicarboxypropyl)carbamoyl]-S-[\(^{3}H\)]-methyl-L-cysteine (\(^{3}H\)-ZJ24). The concentration required to inhibit 50% of binding is determined (IC\(_{50}\)) by GraphPad Prism 3.0. Values represent mean ± SD of three independent experiments. Both PSMA-1-Pc413 and PSMA-1-IR700 showed better binding affinity than the related ligand Cys-CO-Glu.
detectable amount of fluorescence uptake was observed in PSMA-negative PC3flu cells for either PSMA-1 conjugates (Fig. 1). In contrast, for both targeted PDT agents, fluorescence intensity in PSMA-positive PC3pip cells increased with prolonged incubation time (Fig. 1B and D). When an excess amount of Cys-CO-Glu was included in the incubation, no fluorescence signal was observed, confirming that cellular uptake of fluorescence was attributed to the specific binding of PSMA-1–PDT conjugates to PSMA. Once internalized into PC3pip cells, the PSMA-1–PDT conjugates were located in the perinuclear position, concurring with our previous results with PSMA-NIR conjugates (38).

In vitro cytotoxicity of PDT

To determine the in vitro phototoxicity of PSMA-1-Pc413, PC3pip, and PC3flu cells were incubated with PSMA-1-Pc413 at 37°C for 1 hour and then exposed to light with radiant exposure of 0.5 J/cm². At 1 µmol/L of PSMA-1-Pc413, 65.0 ± 1.8% of PC3pip cells were killed, whereas only 22.5 ± 3.0% PC3flu cells were killed at the same condition (P = 0.0012; Fig. 2A). In the presence of 10 µmol/L of Cys-CO-Glu, the PDT activity of PSMA-1-Pc413 (1 µmol/L) to PC3pip cells was reduced approximately 6-fold (Supplementary Fig. S7), suggesting dependence on PSMA expression. In contrast, no changes were observed in its activity to PC3flu tumors. The presence of excess amount of Cys-CO-Glu also had no effect on the PDT activity of unconjugated PC413. These results indicated that PSMA-1-Pc413 can preferentially kill PSMA-positive PC3pip cells.

When cells were treated with PSMA-1-IR700, however, no phototoxicity was observed even with prolonged incubation time (4 hours) and higher radiant exposure (up to 2.0 J/cm²; Fig. 2B). We also utilized a LED diode as the light source and obtained similar results. To begin to understand the difference in PDT efficacy between the agents, we measured singlet oxygen generation of the conjugates.

Singlet oxygen (1O₂) is believed to play a key role in the efficacy of PDT. We used DPBF to quantify the production of singlet oxygen by each of our PDT agents by following the changes in the absorbance at 411 nm (43, 44). As shown in Fig. 2C, the absorbance at 411 nm decreased quickly after PSMA-1-Pc413 or PSMA-1-IR700 in PBS was added to DPBF solution.
and light irradiation was applied, suggesting high efficacy in generation of reactive $^1$O$_2$. When the test was performed in RPMI1640 media containing 10% FBS (Fig. 2D), the decay of DPBF resembled that in PBS for PSMA-1-Pc413; however, the decrease of absorbance at 411 nm was significantly less in RPMI media than in PBS for PSMA-1-IR700, indicating that PSMA-1-IR700 was not effective in generating singlet oxygen in RPMI media, potentially explaining the poorer $in$ $vitro$ cytotoxicity of PSMA-1-IR700.

In $vitro$ imaging

Animals bearing both PSMA-positive PC3pip and PSMA-negative PC3flu tumors were used to demonstrate noninvasive imaging and examine the biodistribution of PSMA-1-$Pi$D$T$ conjugates $in$ $vitro$. Selective uptake was observed in PSMA-positive PC3pip tumors. As shown in Fig. 3A, the fluorescence intensity of PSMA-1-Pc413 in PC3pip tumors increased gradually, peaking at 24 hours postinjection, and then slowly declined. The fluorescence was also observed in PC3flu tumors, but was 1.5-fold less than that in PC3pip tumors after 2 hours postinjection ($P = 0.029$). High fluorescence was observed in the upper back of the animals at early time points, but was cleared after 24 hours postinjection; this is likely due to nonspecific accumulation in the fatty region of the neck (46). To confirm that binding of PSMA-1-Pc413 is specific to PSMA, $in$ $vitro$ competition experiments were performed. Previous $in$ $vitro$ competition experiments have shown that ZJ-MCC-Ahx-YYYG (Supplementary Fig. S1), an analogue of PSMA-1 with similar binding affinity (37), can effectively compete with PSMA-1-IR800 and PSMA-1-Cy5.5 $in$ $vitro$ (38); therefore, we used it again to compete with PSMA-1-Pc413 $in$ $vitro$. When the mice were coinjected with 1 nmol of PSMA-1-Pc413 and 1,000 nmol of ZJ-MCC-Ahx-YYYG, the fluorescence intensity in PC3pip tumors decreased (Fig. 3C and D). At 24 hours postinjection, fluorescence in PC3pip tumors decreased about 30% ($P = 0.0067$), whereas no change in fluorescence intensity was observed in PC3flu tumors ($P = 0.345$). In all cases, inclusion of the unlabeled competitor ligand reduced binding in PSMA-positive PC3pip tumors to uptake levels measured in the receptor-negative PC3flu tumors. Five days postinjection, mice were euthanized and tissues such as skin, liver, stomach, heart, lung, spleen, kidneys, PC3pip tumor, PC3flu tumor, and bladder were taken for ex $vitro$ imaging. PC3pip tumor showed bright fluorescent signal, whereas other tissues had minimal amount of fluorescence signal (Fig. 3B).

PSMA-1-IR700 showed different pharmacokinetics compared with PSMA-1-Pc413. PSMA-1-IR700 reached highest

Figure 3.

Imaging of PSMA-1-Pc413 in mice bearing flank PC3pip and PC3flu tumors. A, $in$ $vitro$ Maestro imaging of a typical mouse treated with PSMA-1-Pc413. Mice received 1 nmol of PSMA-1-Pc413 via tail vein injection and then were imaged at the designated times. Representative images are shown of $n = 5$. B, $ex$ $vitro$ imaging of mice organs at 5 days postinjection of PSMA-1-Pc413. The fluorescent signal in PC3pip tumor was significantly higher than in other organs. C, $in$ $vitro$ Maestro imaging of mice injected with 1 nmol of PSMA-1-Pc413 and 1,000 nmol of a selective PSMA receptor binding molecule, ZJ-MCC-Ahx-YYYG. Images are on the same scale as in Fig. 3A. Blockade of fluorescent uptake in PC3pip tumors was observed. D, quantification of fluorescent signal intensity in PC3pip and PC3flu tumors from the mice used in Fig. 3A and C. Values represent mean ± SD of 5 animals.
accumulation in PC3pip tumor within 30 minutes postinjection, then dropped rapidly (Fig. 4A). Compared with PSMA-1-Pc413, PSMA-1-IR700 demonstrated better selectivity. At 4 hours postinjection, the signal in PC3pip tumor (23.3 ± 3.8 counts/s) was more than 3.6-fold higher than in PC3flu tumor (6.4 ± 3.3 counts/s; P = 0.0018). For in vivo competition experiments, we first tried to use excess amount of ZJ-MCC-Ahx-YYYG to compete with PSMA-1-IR700 as we did with PSMA-1-Pc413; however, ZJ-MCC-Ahx-YYYG failed to block the binding of PSMA-1-IR700. PSMA-1-IR700 had different pharmacokinetics from PSMA-1-Pc413, clearing very quickly from the body. To better compete for binding, we tested unconjugated PSMA-1 (Supplementary Fig. S1; ref. 38). When mice were coinjected with excess amount of PSMA-1, the signals in PC3pip tumor reduced significantly (Fig. 4C). At 4 hours after coinjection of 1 nmol of PSMA-1-IR700 and 1,000 nmol of PSMA-1, the fluorescent signal in PC3pip tumor was 86% (P = 0.0002) lower compared with animals received PSMA-1-IR700 only (Fig. 4D). In contrast, no significant change was observed in the signals in PC3flu tumors (P = 0.065). Ex vivo tissue images showed that PSMA-1-IR700 was mainly accumulated in PC3pip tumors (Fig. 4B). Some signal was observed in the kidneys but was weaker than that in PC3pip tumors.

In vivo photodynamic treatment of PC3pip tumors

To test the photodynamic efficacy of PSMA-1-Pc413, mice-bearing PC3pip tumors were irradiated with 150 J/cm² of light at 672 nm at 24 hours postinjection to take advantage of the maximum peak of PSMA-1-Pc413 tumor accumulation. Ex vivo images of tissues at 24 hours postinjection of 0.5 mg/kg of PSMA-1-Pc413 showed that PSMA-1-Pc413 was mainly located inside PC3pip tumor (Supplementary Fig. S8A and S8B), showing good selectivity of the conjugate. Mice receiving no drug and no light were used as controls. Control groups in which mice receive light only or drug only were not included in this study as light alone or drug alone results in no PDT effect (42, 48). Maestro images showed that fluorescence signal in PC3pip tumor increased when more drug was administered (Fig. 5A). Treated tumors showed immediate loss of fluorescence indicating photobleaching from the activation of Pc413 (Fig. 5B). The loss of fluorescence was also observed when mice were treated with 50 J/cm² of light. Swelling around the treated site was observed within hours after treatment. Tumor volume was significantly reduced starting on day 5 postinjection for mice receiving 0.25 mg/kg (P = 0.001) or 0.50 mg/kg (P = 0.0004) of PSMA-1-Pc413 when compared with untreated controls (Fig. 5C). As the drug dose increased, improved treatment efficacy was observed. The increased drug accumulation in the tumor, therefore, likely led to better treatment results. At the
In vivo photodynamic treatment of PSMA-positive PC3pip tumors. Values represent mean ± SD of 5 animals. A, quantification of PSMA-1-Pc413 fluorescent signal in PC3pip tumors. Signal in PC3pip tumors increased when mice received increased dose of PSMA-1-Pc413. B, loss of fluorescent signal in PC3pip tumors after PSMA-1-Pc413 PDT treatment. Mice received 0.5 mg/kg PSMA-1-Pc413 and were treated by laser light (672 nm) with radiant exposure of 150 J/cm² at 24 hours postinjection. Tumor is indicated by the red circle. This loss of fluorescence after PDT indicated the activation of PSMA-1-Pc413 by light. C, tumor growth inhibition by PSMA-1-Pc413 PDT treatment in PC3pip tumors. Tumors were irradiated with 150 J/cm² light (672 nm) at 24 hours postinjection (red arrow). Significant tumor regression was observed and the response was dose dependent. Values represent mean ± SD of 5 tumors. P values are obtained by comparison with control group (*, P < 0.05). D, quantification of PSMA-1-IR700 fluorescent signal in PC3pip tumors. Signal in PC3pip tumors increased when mice received increased dose of PSMA-1-IR700. E, loss of fluorescent signal in PC3pip tumors after PSMA-1-IR700 PDT treatment. Mice received 0.5 mg/kg PSMA-1-IR700 and were treated by light (690 nm) with radiant exposure at 50 J/cm² at 1 hour postinjection. Tumor is indicated by the red circle. This loss of fluorescence after PDT indicated the activation of PSMA-1-IR700 by light. F, tumor growth inhibition by PSMA-1-IR700 PDT treatment in PC3pip tumors. Mice received PSMA-1-IR700 on days 0, 4, and 8 (red arrows). PDT treatment was performed at 1 hour postinjection. Significant tumor regression was observed and the response was dose dependent. Values represent mean ± SD of 5 tumors. P values are obtained by comparison with control group (*, P < 0.05).

Histologic studies

For histologic analysis, tumors were extracted 24 hours after single PDT treatment with PSMA-1-PDT. Tissues were processed using H&E staining. Pathologic analysis showed dramatic differences between the treated and untreated tumors (Fig. 6). Nuclei in cancer cells treated with PSMA-1-PDT conjugates were much smaller compared with untreated tumors, indicating that the cells were damaged; in contrast the untreated tumor was not damaged and the cells were intact (49).
Tumors were taken out at 24 hours after PDT treatment. was observed in PSMA-positive LNCaP cells, but no pyropheophorbide, as reported in 2009 (50). Selective cell killing are easy to be synthesized and more cost-effective. To date, the treated by light with radiant exposure at 50 J/cm² light at 1 hour postinjection. postinjection without light irradiation.

Received 0.5 mg/kg of PSMA-1-IR700 and tumors were taken out at 24 hours 150 J/cm² light at 24 hours postinjection. Tumors were taken out at 24 hours 0.5 mg/kg of PSMA-1-PC413, irradiated with light with radiant exposure at 150 J/cm² light at 1 hour postinjection.

Discussion

Effective PSMA-targeted photodynamic therapy has been demonstrated by Watanabe and colleagues (43) both in vitro and in vivo. Compared with antibody, low molecular weight molecules are easy to be synthesized and more cost-effective. To date, the only PSMA-targeted PDT agent is a PSMA inhibitor conjugate of pyropheophorbide, as reported in 2009 (50). Selective cell killing was observed in PSMA-positive LNCaP cells, but no in vivo results have been reported since then. In this article, we report the synthesis and in vitro and in vivo studies of two PSMA-1–PTD conjugates, PSMA-1-PC413 and PSMA-1-IR700. Both conjugates had improved binding affinity compared with the related ligand Cys-CO-Glu (38), and their binding affinities were similar to the unconjugated PSMA-1 (ref. 38; Table 1). In vitro cellular uptake experiments demonstrated that the two PSMA-1–PTD conjugates are selectively and specifically taken up by PSMA-positive PC3pip cells but not by PSMA-negative PC3flu cells (Fig. 1). In vivo imaging studies showed that both PSMA-1-PC413 and PSMA-1-IR700 selectively accumulate in PSMA-positive PC3pip tumor, although PSMA-1-IR700 had better selectivity than PSMA-1-PC413 (Figs. 3 and 4). The measurable nonspecific uptake of PSMA-1-PC413 observed in PSMA-negative PC3flu tumors may be related to the in vivo stability of the molecule. The nonspecific binding to tumors was also observed when unconjugated PC413 was administered to mice. As it is similar in level to free PC413, there may be a stability issue with the agent and we are working to investigate this potential issue. If this is the case, proposed synthesis to correct this is to conjugate PSMA-1 to Pc413 through the tetraazatetrazenoporphyrin group instead of through the axial siloxy group similar to the synthesis of PSMA-1-IR700.

In vivo imaging studies found that PSMA-1-Pc413 and PSMA-1-IR700 showed dramatically different pharmacokinetics (Figs. 3 and 4), with PSMA-1-IR700 accumulation and clearance from the tumor and the body occurring much more rapidly. This might be attributed to differences in the hydrophobicity of the two molecules; the more hydrophobic PSMA-1-PC413 (LogP = −1.35 ± 0.14) may adhere to blood proteins, remaining in the body longer than the less hydrophobic PSMA-1-IR700 (LogP = −2.46 ± 0.22). This agrees with previous evidence showing that fluorophores can affect the pharmacokinetic behavior of the conjugate (38). Further this explains why it is critical to match the pharmacokinetics of competitive ligands to that of the PSMA-1–PTD molecules for effective competition studies. In ex vivo organ imaging, fluorescence was mainly observed in PSMA-positive PC3pip tumors, indicating the potential for a low toxicity to other organs during PDT treatment, due to selective irradiation of the lesion and low levels of PDT agents in nontargeted tissues. Furthermore, the proposed use of these ligands to guide surgery and then selectively irradiate remaining inoperable tumor tissue will further reduce off-target toxicity by specifically localizing light to target tissues.

In vitro phototoxicity experiments (Fig. 2A) showed that PSMA-1-Pc413 was more potent against PSMA-positive PC3pip cells than for PSMA-negative PC3flu cells. This result was consistent with the in vitro cellular uptake results (Fig. 1B), in which selectively higher cellular uptake of PSMA-1-Pc413 was observed for PSMA-positive PC3pip cells than for PSMA-negative PC3flu cells. No phototoxicity was observed for either cell type when cells were treated with PSMA-1-IR700 in RPMI media with 10% FBS (Fig. 2B). Further singlet oxygen generation studies demonstrated that both PSMA-1-Pc413 and PSMA-1-IR700 are able to produce singlet oxygen effectively when activated by light in PBS. The clonality of the absorption of the PDT agents and the emission wavelengths along with measurement of similar absorption by each agent (Figs. S5 and S6) suggests that these are not major contributors to the difference in efficacy. PSMA-1-IR700 was, however, less efficient in generating singlet oxygen than PSMA-1-Pc413 when activated by light in RPMI medium with 10% FBS (Fig. 2D), explaining lack of phototoxicity of PSMA-1-IR700 in vitro. Others have reported photoimmunotherapy in tissue culture cells when IR700 was conjugated to antibody (40, 42); however, this is not the case with our low molecular weight conjugate. At this time, it is unclear what causes this difference in results but it might be related to molecules in RPMI medium with 10% FBS interacting with the "unprotected" IR700 bound to PSMA-1 attenuating free-radical generation, compared with the "protected" IR700 already in close proximity to a large antibody (40, 42), perhaps eliminating medium-dependent inhibition of free-radical generation.

In in vivo PDT experiments (Fig. 5C), PSMA-1-Pc413 demonstrated strong inhibition on the growth of PC3pip tumor. After only a single dose, a significant difference in tumor growth was measured between treated and untreated tumors. PSMA-1-IR700 required three doses to inhibit PC3pip tumor growth (Fig. 5F). This lower efficacy of PSMA-1-IR700 may be due to lower radiant exposure (50 J/cm²) compared with PSMA-1-Pc413 treatment (150 J/cm²). Posttreatment toxicity was not observed for either agent based on physical appearance and activity of the mice, consistent with the ex vivo organ images.
There is a net need to visualize and completely resect prostate cancers which have moved outside of the prostate capsule during surgical resection of prostate cancer. We have generated two PSMA-targeting PDT conjugates, PSMA-1-Pc413 and PSMA-1-IR700. Our results demonstrated that the synthesized conjugates have the following advantages as imaging agents: (i) the conjugates bind avidly to cancer cells with quantitative clearance from most healthy tissues, allowing rapid tumor visualization; (ii) the conjugates can enter into cancer cells, providing stable tumor contrast; (iii) the conjugates bind specifically to the tumor marker PSMA, which help create highly defined boundaries of the tumor. These features will allow discrimination between diseased, normal, and neural tissues, help surgeons identify extracapsular disease, which is currently invisible during prostatectomy, and decide the aggressiveness of the surgical approach during the procedure. Furthermore, in addition to tracking tumor location, our conjugates have PDT activity. Selective targeting of PDT agents to cancer cells with PSMA-1 and selective irradiation with light to targeted lesions may result in selective destruction of cancer cells as low expression levels of PSMA in healthy tissues would not lead to enough PSMA-1–PDT agent accumulation for a pharmacologic effect. This has been demonstrated using antibody targeted IR700 in mixed culture studies to demonstrate PDT killing of cells overexpressing the targeted cell surface receptor only (42). It is possible to envision that the described PSMA-1–PDT conjugates can be potentially used to ablate cancer contained within the gland, that is, partial prostatectomy. They could also be used to destroy unresectable tissues and/or missed cancer cells, leading to improved quality and success of radical prostatectomies. The small amount of tissue remaining after the surgery will reduce the burden for elimination of cancer by PDT and improve the therapeutic window for PDT by reducing collateral damage. This approach will reduce the need for subsequent medical treatment. Furthermore, PSMA is also expressed on the neovascularure of many different solid tumors, so this approach could be used for PDT delivery and ablation for these diseases as well.

Conclusion

Our rationally designed dual-functional PSMA-1–PDT conjugates have potential to serve as anticancer agents and to our knowledge represent the first PSMA-targeted PDT agents. Through in vitro and in vivo studies, we have demonstrated that there are differences in the PDT efficacy of each of the two targeted agents that are likely related to the PDT moiety and not the targeting ligand, as the affinity remains unchanged after conjugation to either of the PDT agents. The effectiveness and features of our PSMA-1–Pc413 conjugate suggest that it has potential clinical utility and may represent a next-generation theranostic PDT agent. The dual use of the developed PSMA-1–Pc413 conjugate may offer surgeons photoablation as an adjunct to surgical resection to spare proximate nerves and muscles and eliminate stray cancer tissue and cells, potentially reducing the frequency of unresected tumor and cancer recurrence.

Disclosure of Potential Conflicts of Interest

J.P. Basilion is on the SAB at Akrotome Imaging and Vergent Biosciences, received a commercial research support from Akrotome Imaging, has ownership interest (including patents) in Akrotome, and is a consultant/advisory board member for Akrotome Imaging and Vergent Biosciences. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: X. Wang, J. Kiechle, L. Ponsky, J.P. Basilion
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Wang, B. Tsui, G. Ramamurthy, J. Meyers, J. Kiechle, L. Ponsky, J.P. Basilion
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Wang, B. Tsui, J. Meyers, J. Kiechle, J.P. Basilion
Writing, review, and/or revision of the manuscript: X. Wang, B. Tsui, J. Kiechle, L. Ponsky, J.P. Basilion
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Ramamurthy, P. Zhang, J. Meyers, J.P. Basilion
Study supervision: M.E. Kenney, L. Ponsky, J.P. Basilion

Acknowledgments

The authors acknowledge the laboratory of Dr. John Crabb at the Cole Eye Institute, Cleveland Clinic for assistance with mass spectrometry.

Grant Support

This work was supported by National Foundation of Cancer Research (to J.P. Basilion) and Alpha Omega Alpha Carolyn L. Kuckein Student Research Fellowship (to B. Tsui).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 31, 2015; revised April 18, 2016; accepted May 25, 2016; published OnlineFirst June 13, 2016.

References

Molecular Cancer Therapeutics

Theranostic Agents for Photodynamic Therapy of Prostate Cancer by Targeting Prostate-Specific Membrane Antigen

Xinning Wang, Brian Tsui, Gopolakrishnan Ramamurthy, et al.

*Mol Cancer Ther* Published OnlineFirst June 13, 2016.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-15-0722

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2016/06/11/1535-7163.MCT-15-0722.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.