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

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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 un-resectable or remaining cancer cells. Based on 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 \textit{in vitro} cellular uptake experiments and \textit{in vivo} animal imaging experiments the two conjugates demonstrated selective and specific uptake in PSMA-positive PC3pip cells/tumors, but not in PSMA-negative PC3flu cells/tumors. Further \textit{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.
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 (pathological stage C) in 20-42% 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 over 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 et al. of incomplete resection of prostate cancer in over 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, e.g. incontinence (3-74%) and impotence (30-90%) (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 et al. 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 cells. Various agents, including porphyrins and phthalocyanines, have been examined as photosensitizers (21-23). Most photosensitizers are fluorescent and some can emit near-infrared (NIR) light that can be used for in vivo imaging to identify the location of cancer cells and provide image-guided PDT treatment, potentially leading to improved therapeutic accuracy and outcome (21). The main challenge for PDT treatment is off-target tissue accumulation and activation of photosensitizer leading to cell death in normal tissue (24). Development of a highly selective delivery method for photosensitizers will minimize side effects and generate better therapeutic outcomes.

Prostate specific membrane antigen (PSMA) is a unique membrane bound glycoprotein originally discovered in the androgen-dependent LNCaP human prostate adenocarcinoma cell line (25). PSMA is overexpressed in prostate cancer. Expression of PSMA correlates with the stage of disease and Gleason score (26). PSMA expression is also higher in prostate cancer cells from hormone-refractory patients, (26, 27) and increased PSMA expression has been shown to be an independent marker of disease recurrence (27-29). In addition to being overexpressed in prostate cancer, PSMA is also expressed in the neovasculature of many solid tumors (30), making PSMA a promising target for both imaging (31-33) and treatment of prostate cancer (34-
The objective of this study is to develop PSMA-targeted PDT agents that could be used for surgical guidance and allow for subsequent PDT to eliminate unresectable or “missed” cancer cells. Previously, we have developed a peptide-based, highly negatively charged PSMA ligand (PSMA-1) (Figure S1) for PSMA-targeted imaging of prostate cancer (37, 38). In this study, we have designed two PSMA-1 based PDT conjugates (PSMA-1-PDT), PSMA-1-Pc413 (Figure 1A) and PSMA-1-IR700 (Figure 1C). Pc413 is an analogue of the second generation phthalocyanine PDT drug Pc4 which is currently in clinical trials (39), and IR700 is a commercially available near-infrared 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.

Methods and Materials

General. PSMA targeting peptide Glu-CO-Glu'-Amc-Ahx-Glu-Glu-Glu-Lys-NH₂ (PSMA-1) was synthesized by Fmoc chemistry as previously reported (38). (S)-2-(3-((S)-5-amino-1-carboxypentyl)ureido)pentanedioic acid (Cys-CO-Glu, Figure S1) was custom made by Bachem Bioscience Inc. PC413 was provided by Dr. Malcolm E. Kenney’s laboratory. All the other chemicals were purchased from Sigma-Aldrich Inc.

Synthesis of PSMA-1-SMCC. Coupling of PSMA-1 to sulfo-succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) (Thermo Scientific) was performed in 100mM phosphate buffer, pH 7.0. PSMA-1 (200nmol) was dissolved in 100uL of phosphate buffer; then 400nmol of sulfo-SMCC in 500µL of phosphate buffer was added (Figure S2A). The reaction mixture was left at room temperature overnight. The crude product was purified by preparative HPLC using gradient A (supplementary methods). Yield: 78%. Retention time: 24.8min. MALDI-MS: C₅₈H₈₇N₁₁O₂₃, 1306.7 (found); 1306.4 (calculated) (Figures S2B-C)
Synthesis of PSMA-1-Pc413. Pc413 was first dissolved in 1mL of DMF, then PSMA-1-SMCC in 100µL of 100mM phosphate buffer, pH 8.0 was added (Figure 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: 15.4min. MALDI-MS: C_{102}H_{134}N_{20}O_{25}S_{3}, 1996.9 (found, M-C_{7}H_{19}NOSi); 1996.3 (calculated) (Figures S3B-C).

Synthesis of PSMA-1-IR700. Coupling of PSMA-1 to IRDye700DX NHS ester (Li-Cor Biosciences) was performed in 100mM phosphate buffer, pH 7.0. PSMA-1 (1mg) in 200µL of phosphate buffer was added to 0.5mg of IRDye700DX NHS ester in 200µL of phosphate buffer (Figure 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: 28.7min. MALDI-MS: C_{116}H_{125}N_{21}Na_{4}O_{44}S_{6}Si_{3}, 1840.9 (found, M-2C_{14}H_{30}NNa_{2}O_{10}S_{3}Si); 1841.0 (calculated) (Figures S4B-C).

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 in 2014; no genetic authentication was performed. Cells were maintained in RPMI1640 medium (Invitrogen Life Technology) with 2mM L-glutamine and 10% Fetal Bovine Serum at 37°C and 5% CO₂ under a humidified atmosphere.

Competitive binding assay. The assay was carried as previously reported (38) by incubation PC3pip cells with different concentrations of PSMA-1-PDT in the presence of 12nM N-[N-[(S)-1,3-dicarboxypropyl]carbamoyl]-S-[³H]-methyl-L-cysteine (³H-ZJ24) (Figure S1) (GE
Healthcare Life Sciences). Radioactivity of cell pellet was counted by scintillation counter. The concentration required to inhibit 50% of binding was determined (IC$_{50}$) 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 1µM 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-amidinophenyl)-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 co-incubation of PC3pip and PC3flu cells with 1µM of PSMA-1-Pc413 or PSMA-1-IR700 and 10µM of Cys-CO-Glu (Figure S1) (38) for 4 hours.

**In vitro phototoxicity assay.** Cell viability in the dark and under light exposure was evaluated by CellTiter 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 1µM of PSMA-1-Pc413 or PSMA-1-IR700 for 1 or 4 hours. After incubation, cells were washed three times with 200µL of cold RPMI media and irradiated under light (>500nm) (Apollo Horizon Projector, Acco Brands) (Figure S5A for the spectrum of the light and supplementary method) with irradiance at 8.3mW/cm$^2$ and radiant exposure at 0.5J/cm$^2$. Twenty-four hours later, CellTiter 96 Aqueous reagent was added to each well. After a 3-hour incubation at 37°C, the absorbance at 490nm 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 411nm. Briefly, DPBF (50µM) in
ethanol was added to PSMA-1-PDT conjugate solutions in PBS or in RPMI1640 media. The irradiation wavelength used to excite PSMA-1-Pc413 and PSMA-1-IR700 was 672nm and 690nm, respectively. The absorbance at 411nm 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 (IACUC#120024). Six to eight weeks 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 10mm. 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 anaesthetized 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 1nmol of PSMA-1-Pc413 and 1000nmol of ZJ-MCC-Ahx-YYYG (Figure S1), an analogue of PSMA-1 with similar binding affinity (37), or 1nmol of PSMA-1-IR700 conjugate and 1000nmol 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 quantitate the average fluorescence intensity associated with the tumors by creating regions of interest (ROIs) 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: (1) no treatment; (2) mice were injected at a dosage of 0.1 mg/kg; (3) mice were injected at a dosage of 0.25 mg/kg; or (4) mice were injected at a dosage of 0.5 mg/kg. The mice were imaged before injection, immediately following injection, and 24-hour post-injection. At 24-hour post-injection, the tumors were subjected to PDT treatment using 672 nm light from a diode laser (Applied Optronics Corp) (see Figure 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 method). The mice were imaged post-treatment and allowed to recover. For mice receiving PSMA-1-IR700, selected mice were divided into three groups with each group having 5 mice. Groups: (1) no treatment; (2) mice were injected at a dosage of 0.25 mg/kg on days 0, 4 and 8 and treated with 50 J/cm$^2$ of laser light (supplementary methods) at 1-hour post-injection on these three days; (3) mice were injected at a dosage of 0.5 mg/kg on day 0, 4 and 8 and treated with light at 1-hour post-injection on these three days. A diode LED light source (L690-66-60, Marubeni America Co.) (see Figure S5C for the spectrum of the diode LED) was used to irradiate PSMA-1-IR700 at 690 nm due to its proximity to the peak of IR700 absorbance (690 nm) as reported by others [41-43]. Mice were treated with irradiance of 31.8 mW/cm$^2$ and radiant exposure of 50 J/cm$^2$. No radiant exposure higher than 50 J/cm$^2$ was tried, otherwise the LED light unit would be too hot. Mice were monitored daily, and tumor size was measured every other day until animals were euthanized.
**Histology studies with H&E staining.** PC3pip tumors were harvested 1 day after single PDT treatment with 0.5mg/kg of PSMA-1-PDT conjugates. Tumors were fixed in 4% paraformaldehyde and embedded into paraffin blocks. Serial 10µm slice sections were fixed on slide glasses and H&E stained. Sections were photographed using the 10X objective. Control tumors were harvested from mice received same dose of drugs, but without light illumination.

**Statistics.** Statistical analyses were carried out using 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 Figures S2-S4. PSMA-1 and Pc413 were coupled through the heterobifunctional linker sulfo-SMCC (Figures S2A and S3A). PSMA-1-IR700 was synthesized by reaction of PSMA-1 with commercially available IRDye700DX NHS ester in PBS (Figure S4A). PSMA-1-Pc413 has maximum absorbance (λ<sub>max</sub>) at 676nm and maximum emission (λ<sub>Em</sub>) at 678nm (Figure S6A) and PSMA-1-IR700 has λ<sub>max</sub> at 690nm and λ<sub>Em</sub> at 698nm (Figure S6B), both concurring those of Pc4 (47) and IR700 (40). Conjugation of PSMA-1 and Pc413 resulted in a molecule with better solubility characteristics than the parent Pc413, likely due to the presence of 3 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±0.22nM) and PSMA-1-IR700 (IC$_{50}$=2.2±0.15nM) had binding affinities greater than 4.6-fold compared to the related ligand Cys-CO-Glu (Figure S1) (38) (IC$_{50}$=10.2±0.31nM) (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 vitro* 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 detectable amount of fluorescence uptake was observed in PSMA-negative PC3flu cells for either PSMA-1 conjugates (Figure 1). In contrast, for both targeted PDT agents, fluorescence intensity in PSMA-positive PC3pip cells increased with prolonged incubation time (Figures 1B and 1D). 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 0.5J/cm$^2$. At 1μM of PSMA-1-Pc413, 65.0±1.8% of PC3pip cells were killed, while only 22.5±3.0% PC3flu cells were killed at the same condition (P=0.0012) (Figure 2A). In the presence of 10μM of Cys-CO-Glu, the PDT activity of PSMA-1-Pc413 (1μM) to PC3pip cells was reduced approximately 6-fold (Figure 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 phototoxocity was observed even with prolonged incubation time (4 hours) and higher radiant exposure (up to 2.0J/cm², Figure 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 1,3-diphenylisobenzofuran (DPBF) to quantify the production of singlet oxygen by each of our PDT agents by following the changes in the absorbance at 411nm (43, 44). As shown in Figure 2C, the absorbance at 411nm 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 ¹O₂. When the test was performed in RPMI1640 media containing 10% FBS (Figure 2D), the decay of DPBF resembled that in PBS for PSMA-1-Pc413; however, the decrease of absorbance at 411nm 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 vivo imaging**

Animals bearing both PSMA-positive PC3pip and PSMA-negative PC3flu tumors were used to demonstrate non-invasive imaging and examine the bio-distribution of PSMA-1-PDT conjugates in vivo. Selective uptake was observed in PSMA-positive PC3pip tumors. As shown in Figure 3A, the fluorescence intensity of PSMA-1-Pc413 in PC3pip tumors increased gradually,
peaking at 24 hours post-injection, 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 post-injection (P=0.029). High fluorescence was observed in the upper back of the animals at early time points, but was cleared after 24 hours post-injection; this is likely due to non-specific accumulation in the fatty region of the neck (46). To confirm that binding of PSMA-1-Pc413 is specific to PSMA, \textit{in vivo} competition experiments were performed. Previous \textit{in vivo} competition experiments have shown that ZJ-MCC-Ahx-YYYG (Figure S1), an analogue of PSMA-1 with similar binding affinity (37), can effectively compete with PSMA-1-IR800 and PSMA-1-Cy5.5 \textit{in vivo} (38); therefore, we used it again to compete with PSMA-1-Pc413 \textit{in vivo}. When the mice were co-injected with 1nmol of PSMA-1-Pc413 and 1000nmol of ZJ-MCC-Ahx-YYYG, the fluorescent intensity in PC3pip tumors decreased (Figures 3C-D). At 24-hour post-injection, fluorescence in PC3pip tumors decreased about 30% (P=0.0067), while 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 post-injection, mice were euthanized and tissues such as skin, liver, stomach, heart, lung, spleen, kidneys, PC3pip tumor, PC3flu tumor and bladder were taken for \textit{ex vivo} imaging. PC3pip tumor showed bright fluorescent signal, while other tissues had minimal amount of fluorescence signal (Figure 3B).

PSMA-1-IR700 showed different pharmacokinetics compared to PSMA-1-Pc413. PSMA-1-IR700 reached highest accumulation in PC3pip tumor within 30 minutes post-injection, then dropped rapidly (Figure 4A). Compared to PSMA-1-Pc413, PSMA-1-IR700 demonstrated better selectivity. At 4-hour post-injection, the signal in PC3pip tumor (23.3±3.8counts/s) was more than 3.6-fold higher than in PC3flu tumor (6.4±3.3counts/s) (P=0.0018). For \textit{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 (Figure S1) (38). When mice were co-injected with excess amount of PSMA-1, the signals in PC3pip tumor reduced significantly (Figure 4C). At 4-hour post co-injection of 1nmol of PSMA-1-IR700 and 1000nmol of PSMA-1, the fluorescent signal in PC3pip tumor was 86% (P=0.0002) lower compared to animals received PSMA-1-IR700 only (Figure 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 (Figure 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 150J/cm² of light at 672nm at 24-hour post-injection to take advantage of the maximum peak of PSMA-1-Pc413 tumor accumulation. Ex vivo images of tissues at 24-hour post-injection of 0.5mg/kg of PSMA-1-Pc413 showed that PSMA-1-Pc413 was mainly located inside PC3pip tumor (Figures S8A-B), 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 since 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 (Figure 5A). Treated tumors showed immediate loss of fluorescence indicating photobleaching from the activation of Pc413 (Figure 5B). The loss of fluorescence
was also observed when mice were treated with 50J/cm² of light. Swelling around the treated site was observed within hours after treatment. Tumor volume was significantly reduced starting on day 5 post-injection for mice receiving 0.25mg/kg (P=0.001) or 0.50mg/kg (P=0.0004) of PSMA-1-Pc413 when compared to untreated controls (Figure 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 dose of 0.25mg/kg and 0.5mg/kg, tissue damage was observed in some animals due to tumor damage/necrosis; no other adverse effects were observed.

For mice treated with PSMA-1-IR700, we first tried to treat the animals at 2-hour and 4-hour post-injection to allow the conjugate to distribute in the tumor and clear from the body; however, no PDT effect was observed. We then moved the treatment time to 1-hour post-injection to maximize tumor associated drug at the time of irradiation. *Ex vivo* tissue images taken at 1-hour post-injection of 0.5mg/kg of PSMA-1-IR700 showed fluorescent signal was mainly observed in the kidneys, followed by the PC3pip tumor, but not in other organs (Figures S8C-D), indicating that 1-hour post-injection can offer acceptable selectivity. In order to improve the treatment outcome, mice received PSMA-1-IR700 on days 0, 4 and 8 and were treated with light at 1-hour post-injection. Similar to PSMA-1-Pc413, fluorescence signal in PC3pip tumors was dose-dependent (Figure 5D). Fluorescence signal in PC3pip tumors disappeared after irradiation to light (Figure 5E). The loss of fluorescence was also observed at a lower dose of 30J/cm² light. Significant tumor growth inhibition was observed starting on day 7 post PDT treatment. Compared to untreated control mice, tumor sizes in mice exposed to PDT treatments were significantly reduced. The PDT effect of PSMA-1-IR700 was dose-dependent (Figure 5F).
Treatment did not affect physical appearance and activity of the mice; no overt toxicity was observed.

**Histological studies**

For histological analysis, tumors were extracted 24 hours after single PDT treatment with PSMA-1-PDT. Tissues were processed using hematoxylin and eosin staining. Pathological analysis showed dramatic differences between the treated and untreated tumors (Figure 6). Nuclei in cancer cells treated with PSMA-1-PDT conjugates were much smaller compared to untreated tumors, indicating that the cells were damaged; in contrast the untreated tumor was not damaged and the cells were intact (49).

**Discussion**

Effective PSMA-targeted photodynamic immunotherapy has been demonstrated by Watanabe *et al.* (43) both *in vitro* and *in vivo*. Compared to 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-a 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 paper we report the synthesis and *in vitro* and *in vivo* studies of two PSMA-1-PDT conjugates, PSMA-1-Pc413 and PSMA-1-IR700. Both conjugates had improved binding affinity compared to the related ligand Cys-CO-Glu (38), and their binding affinities were similar to the unconjugated PSMA-1 (38) (Table 1). *In vitro* cellular uptake experiments demonstrated that the two PSMA-1-PDT conjugates are selectively and specifically taken up by PSMA-positive PC3pip cells but not by PSMA-negative PC3flu cells (Figure 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 (Figures 3-4).
measurable non-specific uptake of PSMA-1-Pc413 observed in PSMA-negative PC3flu tumors may be related to the *in vivo* stability of the molecule. The non-specific binding to tumors was also observed when unconjugated Pc413 was administered to mice. Since 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 tetraazatetrabenzoporphyrin 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 (Figures 3-4), 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-PDT 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 non-targeted tissues. Further, 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 (Figure 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 (Figure 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 (Figure 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 confluence of the absorption of the PDT agents and the emission wavelengths along with measurement of similar absorption by each agent (Figures S5-6) 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 media with 10% FBS (Figure 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 to 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 (Figure 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 (Figure 5F). This lower efficacy of PSMA-1-IR700 may be due to lower radiant exposure (50J/cm²) compared to PSMA-1-Pc413 treatment (150J/cm²).
treatment toxicity was not observed for either agent based on physical appearance and activity of the mice, consistent with the \textit{ex vivo} organ images.

There is an unmet 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 pharmacological effect. This has been demonstrated using antibody targeted IR700 in mixed culture studies to demonstrate PDT killing of cells over expressing 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, i.e. 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 neovasculature 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 anti-cancer 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.

Acknowledgements

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References


Table 1: *In vitro* competition binding results of PSMA-1-Pc413 and PSMA-1-IR700. The assay was carried out by incubating PC3pip cells ($5 \times 10^5$) with different concentrations of PSMA-1-PDT conjugates in the presence of 12nM N-[(S)-1,3-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.

<table>
<thead>
<tr>
<th></th>
<th>Cys-CO-Glu</th>
<th>PSMA-1-Pc413</th>
<th>PSMA-1-IR700</th>
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<td>IC$_{50}$ (nM)</td>
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<td>2.1±0.22</td>
<td>2.2±0.15</td>
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FIGURE LEGENDS

Figure 1: (A) Chemical structure of PSMA-1-Pc413. (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 1µM 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 1µM of PSMA-1-Pc413 and 10µM 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 40X. 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 1µM 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-Pc413 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 1µM of PSMA-1-IR700 and 10µM 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 40X. Representative images are shown from three independent experiments.

Figure 2: (A) Phototoxicity of PSMA-1-Pc413. PC3pip and PC3flu cells were incubated with different concentrations of PSMA-1-Pc413 or Pc413 at 37°C for 1 hour in RPMI medium, cells were then treated by light with radiant exposure at 0.5J/cm². PSMA-1-Pc413 showed selective PDT activity in PSMA-positive PC3pip cells (*: P<0.05). Values are mean ± SD of 8 replicates.
(B) Phototoxicity of PSMA-1-IR700. PC3pip and PC3flu cells were incubated with 1µM of PSMA-1-IR700 or IR700 at 37°C for 4 hours in RPMI medium, the cells were then kept in dark or exposed to light with different radiant exposure. However, no phototoxicity was observed. Values are mean ± SD of 8 replicates. (C) Generation of singlet oxygen by PSMA-1-Pc413 and PSMA-1-IR700 in PBS. Generation of singlet oxygen was detected by the indirect DPBF method. The absorbance at 411nm decreased significantly when PSMA-1-Pc413 or PSMA-1-IR700 in PBS was added to DPBF solution and light irradiation was applied, suggesting a high efficacy in generation of reactive \(^1\text{O}_2\). Values are mean ± SD of 3 replicates. (D) Generation of singlet oxygen by PSMA-1-Pc413 and PSMA-1-IR700 in RPMI1640 media. PSMA-1-Pc413 can effectively generate singlet oxygen in RPMI media as shown by the reduced absorbance at 411nm, however, PSMA-1-IR700 was not as good in generating single oxygen in RPMI1640 media as in PBS. Values are mean ± SD of 3 replicates.

Figure 3: Imaging of PSMA-1-Pc413 in mice bearing flank PC3pip and PC3flu tumors. (A) In vivo Maestro imaging of a typical mouse treated with PSMA-1-Pc413. Mice received 1nmol 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 vivo imaging of mice organs at 5 days post-injection of PSMA-1-Pc413. The fluorescent signal in PC3pip tumor was significantly higher than in other organs. (C) In vivo Maestro imaging of mice injected with 1nmol of PSMA-1-Pc413 and 1000nmol of a selective PSMA receptor binding molecule, ZJ-MCC-Ahx-YYYG. Images are on the same scale as in Figure 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 Figure 3A and 3C. Values represent mean ± SD of 5 animals.
Figure 4: Imaging of PSMA-1-IR700 in mice bearing flank PC3pip and PC3flu tumors. (A) *In vivo* Maestro imaging of mice treated with PSMA-1-IR700. Mice received 1nmol of PSMA-1-IR700 through tail vein injection and then were imaged at the indicated time points. Representative images of N=5 mice are shown. Selective uptake in PC3pip tumors was observed. (B) *Ex vivo* imaging of mice organs at 48-hour post-injection of PSMA-1-IR700. The fluorescent signal in PC3pip tumor was significantly higher than in other organs. (C) *In vivo* Maestro imaging of mice injected with 1nmol of PSMA-1-IR700 and 1000nmol of PSMA-1. Images are on the same scale as in Figure 4A. Blockade of fluorescent uptake in PC3pip tumors was observed. (D) Fluorescent signal quantification of PSMA-1-IR700 in PC3pip and PC3flu tumors from mice used in Figure 4A and 4C. Values represent mean ± SD of 5 animals.

Figure 5: *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 0.5mg/kg PSMA-1-Pc413 and were treated by laser light (672nm) with radiant exposure of 150J/cm² at 24-hour post-injection. 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 150J/cm² light (672nm) at 24-hour post-injection (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 0.5mg/kg PSMA-1-IR700 and were treated by light (690nm) with radiant exposure at 50J/cm² at 1-hour post-injection. 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 post-injection. 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).

Figure 6: Histological analysis of PC3pip tumors. Cell damage was observed in PDT treated PC3pip tumors as compared to non-treatment controls. (A) Control PC3pip tumor receiving PSMA-1-PC413. Mice received 0.5mg/kg of PSMA-1-PC413 and tumors were taken out at 48-hour post-injection without light irradiation. (B) PC3pip tumors receiving PSMA-1-Pc413 and treated with light. Mice received 0.5mg/kg of PSMA-1-PC413, irradiated with light with radiant exposure at 150J/cm² light at 24-hour post-injection. Tumors were taken out at 24hour post PDT treatment. (C) Control PC3pip tumor receiving PSMA-1-IR700. Mice received 0.5mg/kg of PSMA-1-IR700 and tumors were taken out at 24-hour post-injection without light irradiation. (D) PC3pip tumors receiving PSMA-1-IR700 and treated with light. Mice received 0.5mg/kg of PSMA-1-IR700, and treated by light with radiant exposure at 50J/cm² light at 1-hour post-injection. Tumors were taken out at 24-hour post PDT treatment.
Figure 1

(A) PC3pip (PSMA+)

(B) 0 min 15 min 30 min 1 hour 4 hour 4 hour
(Please note: the images show the results over time, with 4 hours marked as +10X Blocker)

PSMA-1-Pc413
Merge with DAPI

(C) PC3flu (PSMA-)

(D) 0 min 15 min 30 min 1 hour 4 hour 4 hour
(Please note: the images show the results over time, with 4 hours marked as +10X Blocker)

PSMA-1-IR700
Merge with DAPI

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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(A) *In vitro* phototoxicity of PSMA-1-Pc413

- PC3pip, PC413
- PC3pip, PSMA-SMCC-PC413
- PC3flu, Pc413
- PC3flu, PSMA-SMCC-Pc413

(C) Generation of singlet oxygen by PSMA-1-PDT conjugates in PBS

(D) Generation of singlet oxygen by PSMA-1-PDT conjugates in RPMI1640 media
(A) PSMA-1-Pc413 *in vivo*

(B) PSMA-1-Pc413, *ex vivo*

Figure 3

(C) PSMA-1-Pc413+ 1000X ZJ-MCC-Ahx-YYYG, *in vivo competition*

(D) Maestro quantification of PSMA-1-Pc413
Figure 4

(A) **PSMA-1-IR700 in vivo**

(B) **PSMA-1-IR700 ex vivo**

(C) **PSMA-1-IR700 + 1000X PSMA-1, in vivo competition**

(D) **Maestro quantification of PSMA-1-IR700**
**Figure 5**

(A) Fluorescence signal–dose response for PSMA-1-Pc413

(B) Loss of signal in PC3pip tumor after PSMA-1-Pc413 PDT treatment

(C) PSMA-1-Pc413 PDT treatment in vivo

(D) Fluorescence signal–dose response for PSMA-1-IR700

(E) Loss of signal in PC3pip tumor after PSMA-1-IR700 PDT treatment

(F) PSMA-1-IR700 PDT treatment in vivo
Figure 6

(A) PC3pip tumor
(0.5mg/kg PSMA-1-Pc413)
(no light irradiation)

(B) PC3pip tumor
(0.5mg/kg PSMA-1-Pc413)
(150J/cm² radiant exposure)

(C) PC3pip tumor
(0.5mg/kg PSMA-1-IR700)
(no light irradiation)

(D) PC3pip tumor
(0.5mg/kg PSMA-1-IR700)
(50J/cm² radiant exposure)
Molecular Cancer Therapeutics

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

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