Strain-Promoted Azide-Alkyne Cycloaddition-Based PSMA-Targeting Ligands For Multimodal Intraoperative Tumor Detection of Prostate Cancer

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Abstract

Purpose: Strain-promoted azide-alkyne cycloaddition (SPAAC) is a straightforward and multipurpose conjugation strategy. Use of SPAAC to link different functional elements to prostate specific membrane antigen (PSMA) ligands would facilitate the development of a modular platform for PSMA-targeted imaging and therapy of prostate cancer (PCa). As a first proof-of-concept for the SPAAC chemistry platform we synthesized and characterized four dual-labeled PSMA ligands for intraoperative radiodetection and fluorescence imaging of PCa.

Methods: Ligands were synthesized using solid phase chemistry and contained a chelator for ^{111}In or $^{99\text{m}}\text{Tc}$ labeling. The fluorophore IRDye800CW was conjugated using SPAAC chemistry or conventional *N*-hydroxysuccinimide (NHS)-ester coupling. LogD values were measured and PSMA-specificity of these ligands was determined in LS174T-PSMA cells. Tumor targeting was evaluated in BALB/c nude mice with subcutaneous LS174T-PSMA and LS174T wildtype tumors using $\mu\text{SPECT/CT}$ imaging, fluorescence imaging, and biodistribution studies.

Results: SPAAC chemistry increased lipophilicity of the ligands (range LogD: -2.4 to -4.4). *In vivo*, SPAAC chemistry ligands showed high and specific accumulation in s.c. LS174T-PSMA tumors up to 24 hours after injection, enabling clear visualization using $\mu\text{SPECT/CT}$ and fluorescence imaging. Overall, no significant differences between the SPAAC chemistry ligands and their NHS-based counterparts were found (2 h p.i., $p > 0.05$), while ^{111}In -labeled ligands outperformed the $^{99\text{m}}\text{Tc}$ ligands.

Conclusion: Here we demonstrate that our newly developed SPAAC-based PSMA ligands show high PSMA-specific tumor targeting. Use of click-chemistry in PSMA ligand development opens up the opportunity for fast, efficient and versatile conjugations of multiple imaging moieties and/or drugs.

Introduction

Prostate cancer (PCa) is the second most common cancer in men worldwide, leading to a substantial morbidity and mortality [1]. About 90% of PCa patients have a localized tumor at initial screening and are candidates for surgery [2, 3]. The prostate is located between a number of critical structures, and as a consequence the surgeon has to perform very narrow tumor resections [4, 5]. In approximately 15–65% of PCa patients, dependent on the disease stage, tumor resection is incomplete caused by positive surgical tumor margins upon removal of the primary tumor and/or incomplete removal of tumor positive lymph nodes in the pelvis [6–8]. Innovative approaches to improve intraoperative tumor visualization could increase the chance of complete surgical resection of all tumor tissue. Here we focused on fluorescence imaging combined with radionuclide detection of PCa using dual-labeled PSMA-targeting ligands [9].

In recent years many PSMA-targeting ligands have been developed, each suitable for different applications (e.g. radionuclide imaging, radionuclide therapy, fluorescent imaging) [10–13]. To develop a versatile platform that enables easy synthesis of PSMA ligands for different purposes, strain-promoted

azide-alkyne cycloaddition (SPAAC, N₃-DBCO) can be used, a well-known form of click chemistry [14, 15]. Click chemistry reactions proceed with ease under mild nontoxic conditions (*i.e.* at room temperature in water) tolerating the presence of a wide range of functional groups. Hence, strain-promoted cycloadditions provide fast and highly efficient chemistry to link PSMA ligands to different functional elements [14, 16]. Nonetheless, there is still uncertainty about the influence of the hydrophobic functional group dibenzocyclooctene that is introduced with SPAAC on the pharmacokinetics and non-specific uptake of the ligands [14, 17, 18]. Therefore, the aim of the current study is to develop small molecule PSMA ligands that can be conjugated to different functional components using SPAAC chemistry (N₃-DBCO) and to evaluate the effects of the hydrophobic dibenzocyclooctene group on binding affinity, pharmacokinetics and biodistribution of the ligand.

As a first proof-of-concept for the SPAAC chemistry-based PSMA-targeting platform, we synthesized dual-labeled PSMA ligands that were conjugated with the near-infrared (NIR) fluorophore IRDye800CW using SPAAC chemistry. The NIR fluorescent dye can be used for fluorescence imaging to allow direct visualization of tumor tissue during surgery. This enables a more precise removal of the primary tumor with less positive surgical margins as a result [19]. Moreover, a chelator for either technetium-99m (^{99m}Tc) or indium-111 (¹¹¹In) labeling was added to the ligand. The addition of a gamma-emitting radionuclide label in the dual-labeled PSMA ligand allows for intraoperative detection of metastatic lymph nodes [20]. Here, we compared the SPAAC-based PSMA-targeting ligands with similar ligands where conventional *N*-hydroxysuccinimide (NHS)-ester coupling of IRDye800CW was used. With this, we could determine the effect of conjugation strategy (SPAAC vs NHS) on the PSMA-binding affinity, biodistribution and pharmacokinetics of these novel dual-labeled ligands. As a secondary aim, we evaluated the differences in affinity and biodistribution of the ¹¹¹In-labeled ligands and their ^{99m}Tc-labeled equivalents.

Materials And Methods

Synthesis of ligands

We synthesized KuE-based PSMA ligands with the chelator mercaptoacetylglycylglycylglycine (MAG3) for ^{99m}Tc labeling, or the chelator 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) for ¹¹¹In labeling using standard Fmoc solid phase chemistry. After cleavage from the resin and intermediate preparative HPLC purification, fluorophore IRDye800CW was conjugated to the ligands via either SPAAC or NHS-ester coupling in solution. Full synthetic procedures can be found in the supplementals (Page 1 – 6, Fig. S1-3). The ligands are further referred to as: PSMA-N048 (KuE-linker-DOTA-SPAAC-IRDye800CW), PSMA-N049 (KuE-linker-MAG3-SPAAC-IRDye800CW), PSMA-N050 (KuE-linker-DOTA-NHS-IRDye800CW) and PSMA-N051 (KuE-linker-MAG3-NHS-IRDye800CW) (Fig. 1, Fig. 2A).

Cell culture

The LS174T colon carcinoma cell line was purchased from the American Type Culture Collection. LS174T-PSMA cells were created by stable transfection with human PSMA using the plasmid pcDNA3.1-hPSMA [6]. Wildtype LS174T colon carcinoma cells were used as a control. All cells were cultured in RPMI 1640 medium supplemented with 10% FCS and 2 mM glutamine (5% CO₂, 37°C). Additionally, LS174T-PSMA cells were cultured in the presence of 0.3 mg/ml G418 geneticin.

Radiolabeling

Indium-111: Ligands (1–20 µg,) were radiolabeled under metal-free conditions with ¹¹¹InCl₃ (Curium) in 0.5 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 5.5, twice volume of ¹¹¹InCl₃). Labeling was performed at 90°C for 30 minutes [21]. Ethylenediamine-tetraacetic acid (EDTA, 50 mM) was added to a final concentration of 5 mM after the incubation. Specific activity after labeling ranged from 1–10 MBq/µg. Ligands were purified by a Sep-Pak C18 light cartridge (Waters) and eluted from the cartridge with 50% ethanol in water.

Technetium-99m: Ligands (1–20 µg,) were radiolabeled in 45 µl ammonium acetate (NH₄Ac, 0.25M, pH 8) and 15 µl freshly prepared disodium tartate buffer (50 mg/ml in 0.25M NH₄Ac), under metal-free conditions. Ascorbic acid buffer was prepared just before labeling (3 mg/ml in 10 mM HCl). Next, 5 µl of freshly prepared stannous chloride dihydrate (SnCl₂) buffer (4 µg/ml in ascorbic acid buffer) was added simultaneously with ^{99m}TcO₄⁻ in saline, followed by incubation for 30 min at 90°C. Specific activity after labeling ranged from 1–30 MBq/µg. Ligands were purified by a Sep-Pak C18 light cartridge (Waters) and eluted from the cartridge with 50% ethanol in water.

ITLC/HPLC: Radiochemical yield (RCY) was determined by instant thin-layer chromatography (ITLC) using silica gel-coated paper (Agilent Technologies) and 0.1 M ammonium acetate containing 0.1 M EDTA pH 5.5 (¹¹¹In) or 0.1 M Sodium Citrate pH 6.0 (^{99m}Tc), as the mobile phase. In addition, RCY was measured using reverse-phase high performance liquid chromatography (RP-HPLC) on an Agilent 1200 system (Agilent Technologies) with an in-line radiodetector (Elysia-Raytest). A C18 column (5 µm, 4.6 x 250 mm; HiChrom) was used at a flow rate of 1 ml/min. We used the following buffer system: buffer A, triethylammonium acetate (TEAA, 10mM, pH 7); buffer B, 100% methanol; and a gradient of 97–0% buffer A (35 min).

Binding, internalization and IC50 assay

Internalization assay: Binding and internalization characteristics of all ligands were compared using LS174T-PSMA and wildtype LS174T cells, cultured to confluency in 6-wells plates. Cells were incubated with 50,000 counts per minute (cpm) ¹¹¹In- or ^{99m}Tc-labeled PSMA ligand (0.1–0.25 pmol/well) in 1 ml binding buffer (RPMI/0.5% BSA) for 2 h at 37°C. Nonspecific binding was determined by co-incubation with 2-(phosphonomethyl)pentane-1,5-dioic acid (2-PMPA, 21.57 µM). Cells were washed with PBS and incubated with acid buffer (0.1 M acetic acid, 154 mM NaCl, pH 2.6) for 10 minutes at 0°C to retrieve the membrane-bound fraction. After this, the membrane-bound fraction was collected, cells were washed,

lysed with 1.5 ml 0.1 M NaOH and cell lysis (intracellular activity) was collected. Membrane-bound activity and intracellular activity fractions were measured in a gamma-counter (2480 WIZARD² Automatic Gamma Counter, PerkinElmer) [6, 22].

IC₅₀: The 50% inhibitory concentration (IC₅₀) of the ligands was determined using LS174T-PSMA cells in a competitive binding assay. The LS174T-PSMA cells were cultured to confluency in 6-wells plates, followed by incubation on ice for 2 h in 1 mL of binding buffer (RPMI/0.5% BSA) with 50,000 cpm of ¹¹¹In-labeled PSMA-617 and a series of increasing concentrations (0.01–300 nM) of unlabeled PSMA ligands. After incubation, cells were washed with 2 ml PBS and lysed with 1.5 ml 0.1 M NaOH. Cell lysis was collected from the plate and the cell-associated activity was measured in a gamma-counter and IC₅₀ values were calculated using GraphPad Prism software version 5.03.

Lipophilicity

LogD values of all radiolabeled ligands were determined by adding 300,000 cpm (0.6–1.5 pmol) to a mixture of 3 ml PBS (pH 7.4) and 3 ml n-octanol. Tubes were vortexed vigorously for 1 min and centrifuged for 5 min at 201×g. The concentration of radioactivity in a defined volume of each layer was measured in a well-type gamma-counter.

Subcutaneous tumor model

Animal experiments were performed in 8–10 weeks old male BALB/c nude mice (Janvier). Animals were housed under non-sterile conditions in individually ventilated cages (Blue line IVC, 4–5 mice per cage) with cage enrichment present and free access to water and chlorophyll-free animal chow (Sniff GmbH). Mice were subcutaneously inoculated with 3.0 x 10⁶ LS174T-PSMA cells in the right flank and 1.5 x 10⁶ LS174T wildtype cells in the left flank, diluted in 200 µL of complete RPMI 1640 medium. When xenografts were approximately 0.1 cm³ (10–14 days after tumor inoculation), tracers were injected intravenously in the tail vein. The biotechnicians performing the s.c. and i.v. injections were blinded for the experimental groups and tumor-bearing mice were block-randomized into groups based on tumor size. All experiments were conducted in accordance to the guidelines of the Revised Dutch Act on Animal Experimentation and approved by the institutional Animal Welfare Committee of the Radboud university medical center.

Biodistribution, fluorescence imaging and µSPECT/CT imaging

Mice were injected intravenously with 10 MBq ¹¹¹In-labeled PSMA-N048, -N050 or PSMA-617 as control (0.3 nmol, molar activity 33.3 MBq/nmol) or 15 MBq ^{99m}Tc-labeled PSMA-N049 or -N050 (0.3 nmol, molar activity 50 MBq/nmol) in PBS/0.5% BSA. Two hours post injection (p.i.), mice were euthanized by CO₂/O₂ asphyxiation and images were acquired with the IVIS fluorescence imaging system (Xenogen VivoVision IVIS Lumina II, PerkinElmer), using an acquisition time of 30 s. Subsequently, µSPECT/CT images were acquired (U-SPECT II, MILabs) with a 1.0 mm diameter pinhole mouse collimator tube [23]. Mice were

scanned for 30 min followed by a CT scan (spatial resolution 160 μm , 65 kV, 615 μA) for anatomical reference. $\mu\text{SPECT/CT}$ scans were reconstructed with MILabs reconstruction software, using an ordered-subset expectation maximization algorithm, energy windows 154–188 keV and 220–270 keV for ^{111}In , and 126–154 keV for $^{99\text{m}}\text{Tc}$, 1 iteration, 16 subsets, voxel size of 0.4 mm. $\mu\text{SPECT/CT}$ scans were analyzed and maximum intensity projections (MIPs) were created using the Inveon Research Workplace software version 4.1 (Siemens Preclinical Solutions). NIRF images were analyzed using Living Image software version 4.2 (Caliper Life Sciences). Tumors, blood, and relevant organs and tissues were dissected, weighed, and radioactivity in each sample was quantified using a well-type gamma-counter. The results were expressed as percentage of injected dose per gram of tissue (%ID/g).

Pharmacokinetics

To determine the pharmacokinetics of the ligands, nine groups of five mice received an intravenous injection of 0.3 nmol ^{111}In -labeled PSMA-N048 or PSMA-N050 (10 MBq/mouse, molar activity 33.3 MBq/nmol) or $^{99\text{m}}\text{Tc}$ -labeled PSMA-N049 (5 MBq/mouse, molar activity 16.7 MBq/nmol) in PBS/0.5% BSA. At 2, 4, and 24 h p.i. mice were euthanized followed by dissection. Tissues of interest were dissected, weighed and measured for radioactivity in a gamma-counter as described above. For each ligand, two mice from the 24 h groups underwent repeated $\mu\text{SPECT/CT}$ and NIRF imaging (2, 4 and 24 h p.i.). During imaging, mice were anesthetized with 2.5% isoflurane inhalation anesthesia and kept warm with a heating pad. Images were acquired and analyzed as described above.

Intraperitoneal tumor model

LS174T-PSMA cells (1.0×10^6) in 200 μl of complete RPMI 1640 medium were injected intraperitoneally and grew for 28 d after inoculation. Six male BALB/c nude mice with intraperitoneally growing LS174T-PSMA tumors were intravenously injected with ^{111}In -labeled PSMA-N050 (10 MBq and 0.3 nmol/mouse). Two hours p.i., $\mu\text{SPECT/CT}$ imaging was performed preoperatively (30 min), followed by NIRF imaging of the mice in supine position after surgical removal of skin, abdominal muscle layers, and peritoneum. After *in vivo* image acquisition, the visualized tumors were resected, followed by NIRF imaging to control whether residual tumor tissue was *in situ*.

Statistics

Statistical analyses were performed with Graphpad Prism, version 5.03. Results are presented as mean $\pm$ standard deviation (SD). Differences in *in vitro* affinity and tumor/organ uptake *in vivo* were tested for significance using a one-way ANOVA with a Tukey multiple comparison posttest. Differences were considered significant at $P < 0.05$, two-sided.

Results

Design and synthesis

In order to synthesize dual-labeled ligands that fit the active site as well as the entrance funnel of PSMA, we replaced the more C-terminal glutamic acid residue of PSMA-1007 with a lysine [11, 24, 25].

The side chain of this lysine was designed to be oriented towards the exterior of PSMA in the same manner as the side chain of the original glutamic acid is (Fig. S4), providing space for conjugation of multiple functional moieties. To the side chain of this lysine we added another lysine or azidolysine to enable two groups that could be further functionalized with both a metal chelator and a fluorophore. Most of the synthesis was performed on the solid phase, including the addition of a DOTA or MAG3 chelator. After cleavage from the resin we conjugated all ligands in solution with IRDye800CW via either SPAAC (N_3 -DBCO) or NHS-ester based chemistry.

Radiolabeling of the ligands

RCY of ^{111}In -labeled ligands (PSMA-N048 and PSMA-N050) exceeded 93% in all experiments (specific activity 10 MBq/ μg). RCY of $^{99\text{m}}\text{Tc}$ labelled ligands ranged between 15% and 69% (specific activity 30 MBq/ μg). All ligands were purified, resulting in a radiochemical purity > 95% as determined by ITLC and HPLC. Labelling of all four ligands was stable in human serum at 37°C up to 2 hours as determined by RP-HPLC (Fig. S5).

In vitro characterization of the ligands

To characterize the four ligands we first measured the distribution coefficient (LogD at pH 7.4), to determine the effect of the dibenzocyclooctene group on the lipophilicity of the PSMA ligand (Fig. 2A). LogD of $^{99\text{m}}\text{Tc}$ -PSMA-N049 was - 2.4 compared with - 3.5 for its NHS-based equivalent $^{99\text{m}}\text{Tc}$ -PSMA-N051 ($p < 0.01$). The LogD of ^{111}In -PSMA-N048 (SPAAC) was - 3.2 as compared with - 4.4 for ^{111}In -PSMA-N50 (NHS) ($p < 0.001$). Hence, as expected the ligands with the dibenzocyclooctene group for SPAAC chemistry were more lipophilic. Use of MAG3 compared with DOTA as a chelator increased lipophilicity as well ($p < 0.01$). In addition to the lipophilicity, we determined the IC_{50} values of all ligands, which ranged between 184 nM and 475 nM ($p = 0.11$) (Fig. 2A, Fig. S6).

Next, we verified the PSMA-binding potential of our ligands in an *in vitro* binding and internalization assay using PSMA-positive and negative cells, in which all four ligands showed PSMA-specific binding (Fig. 2B). Use of SPAAC chemistry led to a significantly lower total binding of the ligand; $3.9\% \pm 0.5\%$ for $^{99\text{m}}\text{Tc}$ -PSMA-N049 and $8.9\% \pm 0.6\%$ for ^{111}In -PSMA-N048, compared with NHS-conjugated variants; $22.9\% \pm 1.6\%$ for $^{99\text{m}}\text{Tc}$ -PSMA-N051 and $17.2\% \pm 3.5\%$ for ^{111}In -PSMA-N050 (Fig. 2B, $p < 0.001$). As a reference, we determined the total binding of ^{111}In -PSMA-617 in our assay, which was $13.4\% \pm 3.5\%$. For all ligands, the internalized fraction ranged between 61.0% and 80.6% of the total cell-associated activity, and did not differ significantly (Fig. 2A). Minimal binding and internalization were observed in PSMA-negative cells or PSMA-positive cells incubated with an excess of 2-PMPA, demonstrating that, despite their higher lipophilicity, the SPAAC chemistry-based ligands do not suffer from non-specific binding and uptake (Fig. 2B).

Pharmacokinetics of SPAAC-based ligands

To establish the pharmacokinetics of the SPAAC-based PSMA ligands, the *ex vivo* biodistribution of ^{99m}Tc -PSMA-N049 and ^{111}In -PSMA-N048 was determined 2, 4 and 24 h p.i. in mice bearing LS174T PSMA-positive and negative tumors. ^{99m}Tc -PSMA-N049 showed PSMA-specific tumor uptake $> 8\% \text{ID/g}$, which was stable up to 24 hours after injection (Fig. 3A, Supplementary table 1). Tumor uptake of ^{111}In -PSMA-N048 was $23.8 \pm 3.9\% \text{ID/g}$ and $22.7 \pm 1.5\% \text{ID/g}$ 2 and 4 hours p.i., respectively. Yet, after 24 hours p.i. tumor uptake of ^{111}In -PSMA-N048 significantly decreased to $17.6 \pm 1.8\% \text{ID/g}$ ($p < 0.05$). Tumor uptake of the ^{111}In -labeled ligand PSMA-N048 was significantly higher compared with the ^{99m}Tc -labeled ligand PSMA-N049 at all three timepoints measured ($p < 0.05$ for all timepoints).

For both ligands, the amount of tracer present in the blood over time decreased from 2–3 $\% \text{ID/g}$ (2 h p.i.) to $< 0.3\% \text{ID/g}$ (24 h p.i.). Furthermore, accumulation of the two ligands in other organs including the PSMA-negative tumor, spleen, liver, prostate and salivary glands was low ($< 5\% \text{ID/g}$) at the 2 h p.i. timepoint and further decreased over the course of 24 h, leading to high tumor-to-background ratios at all timepoints measured (Supplementary table 1). $\mu\text{SPECT/CT}$ and fluorescence imaging of the mice revealed that both ligands clearly visualized the subcutaneous LS174T-PSMA tumors (left flank) up to 4 hours p.i. (Fig. 3B).

Biodistribution comparison of SPAAC- and NHS-based ligands

To determine the effect of conjugation chemistry (SPAAC vs. NHS) on the *in vivo* performance of the PSMA ligands, their tumor and normal tissue uptake was determined in mice bearing both a PSMA-positive and negative LS174T tumor two hours after injection. Despite the high tumor uptake of all four ligands tested, the NHS-based ligands showed higher tumor accumulation as compared to their SPAAC-based counterparts (not significant, $p > 0.05$). Tumor uptake of PSMA-N048 (SPAAC) and PSMA-N050 (NHS) labeled with ^{111}In were $21.2 \pm 1.2\% \text{ID/g}$ and $25.3 \pm 2.0\% \text{ID/g}$, respectively. Lowest uptake in the PSMA-expressing tumors was measured for the ^{99m}Tc -labeled ligands; $12.0 \pm 1.4\% \text{ID/g}$ and $17.7 \pm 3.7\% \text{ID/g}$ for PSMA-N049 (SPAAC) and PSMA-N051 (NHS), respectively. In our LS174T s.c. tumor model, uptake of ^{111}In -PSMA-N050 was significantly higher than both ^{99m}Tc -labeled ligands ($p < 0.01$) and control ligand ^{111}In -PSMA-617 ($18.5 \pm 1.9\% \text{ID/g}$, $p < 0.05$). All four ligands showed a significantly higher uptake in the PSMA-positive tumor as compared to the PSMA-negative tumor ($p < 0.001$) (Fig. 4A, Supplementary table 2).

All ligands showed rapid blood clearance and minimal uptake in muscle, bone, salivary glands and prostate at 2 hours after injection, leading to high tumor-to-organ ratios (Fig. 4B, Supplementary table 2). The SPAAC chemistry-based ligands showed a higher liver uptake as compared to their NHS-based equivalents; $4.3 \pm 0.3\% \text{ID/g}$ (SPAAC) vs. $1.7 \pm 0.3\% \text{ID/g}$ (NHS) for ^{99m}Tc and $3.4 \pm 0.3\% \text{ID/g}$ (SPAAC) vs. $1.2 \pm 0.1\% \text{ID/g}$ (NHS) for ^{111}In ($p < 0.05$). Ligand accumulation in the excretory organ, the kidneys, was

similar for both conjugation strategies, but lower for the ^{99m}Tc -labeled ligands (range 73.9–81.6 %ID/g), compared with the ^{111}In -labeled ligands (> 120 %ID/g, $p < 0.001$).

Multimodal imaging of PSMA-positive tumors

To compare the multimodal imaging potential of our SPAAC chemistry-based ligands to that of their NHS-based equivalents, we scanned mice using both a $\mu\text{SPECT/CT}$ and a fluorescence scanner. Figure 5 shows representative back- and side-view $\mu\text{SPECT/CT}$ images (Fig. 5A) and the corresponding fluorescence images (Fig. 5B) of all four ligands two hours after injection. No large differences in imaging potential were observed between the SPAAC and NHS-based ligands. All ligands could clearly visualize the subcutaneous LS174T-PSMA tumors (right flank), without showing clearly visible uptake in the PSMA-negative LS174T tumors (left flank). In addition, multimodal images visualized high renal tracer accumulation in all mice.

Image-guided surgery with dual-labeled ligands

To evaluate the applicability of the dual-labeled PSMA ligands for image-guided resections in a more clinically relevant setting, we injected six mice with intraperitoneal LS174T PSMA-positive tumors with our ligand ^{111}In -PSMA-N050. Two hours after injection of the ligand, a preoperative $\mu\text{SPECT/CT}$ scan was acquired, on which multiple i.p. tumors could be visualized (Fig. 6C). Next, we carried out image-guided resection of the i.p. tumors, of which Fig. 6 shows one illustrative example. NIR fluorescence imaging immediately revealed a tumor at the injection site, also visible on $\mu\text{SPECT/CT}$ (Fig. 6A1-2, Fig. 6C red arrow). After removal of the tumor at the injection site, fluorescence imaging revealed a large tumor in the abdomen (Fig. 6A3-4) and multiple tumors close to the spleen and pancreas (Fig. 6A3-5), all visible on $\mu\text{SPECT/CT}$ as well (Fig. 6C green and pink arrows). Last, small nodules beneath the liver were found (Fig. 6A5, Fig. 6C yellow arrow). All resected tumors showed fluorescent uptake (Fig. 6B). Nevertheless, the kidneys were highly fluorescent as well, which hampered detection of tumor lesions in close proximity to these excretory organs in mice.

Discussion

In this study we used strain-promoted azide-alkyne cycloaddition (SPAAC) chemistry to synthesize dual-labeled PSMA-targeting ligands that could aid in the intraoperative detection and resection of PCa. The SPAAC-chemistry based ligands (PSMA-N048 and PSMA-N049) showed high tumor uptake and fast clearance from other organs. Importantly, performance of the SPAAC-based ligands was similar to their NHS-based equivalents. Together, this suggests the feasibility of SPAAC chemistry as a versatile conjugation strategy in high-affinity PSMA ligand design. Furthermore, the ^{111}In -labeled ligands showed a significantly higher tumor uptake compared with the ^{99m}Tc -labeled counterparts.

Click chemistry has been used in many research fields because of its beneficial characteristics, including high yield, high specificity, and simplicity [26]. More specifically, it enables reasonably fast kinetics under aqueous conditions in the presence of a wide range of functional groups and no high temperatures are required [27]. The recent development of less cytotoxic and copper-free click chemistry reactions such as

SPAAC has allowed its application in the field of medicine. Hence, the SPAAC-based conjugation strategy presented in this study provides a versatile platform in which PSMA ligands can easily be coupled to different chelators, fluorophores or anti-cancer drugs. For example, it offers the opportunity to click various imaging moieties (*e.g.* fluorophores, radionuclides or MRI contrast agents) to the ligand for preclinical microscopy, as well as clinical diagnostic, pre- and intraoperative imaging of PCa. Moreover, theranostic tracers could be synthesized that include therapeutic elements such as α - or β -emitting radionuclides for radioligand therapy, photosensitizers for PSMA-targeted photodynamic therapy or anti-cancer drugs including chemotherapeutics and immunomodulatory agents. To elucidate the influence of the dibenzocyclooctene group on ligand characteristics, we compared this fast and efficient way of conjugation with conventional NHS-ester chemistry. Withal, the SPAAC-based PSMA ligands showed similar high affinity towards PSMA *in vitro*, and specific uptake and retention in PSMA-positive tumors *in vivo*, compared to NHS-based ligands.

Literature has indicated that the dibenzocyclooctene group, present in the ligand after SPAAC chemistry, is rather hydrophobic and could potentially negatively impact the affinity and non-specific binding of small PSMA-targeting ligands *in vitro* [28, 29]. On the contrary, Wirtz *et al.* found that higher lipophilicity of their PSMA-I&T based ligands is beneficial in terms of affinity and internalization, possibly because of ligand interaction with a lipophilic binding pocket of PSMA [30]. LogD determination in our study showed that SPAAC chemistry conjugation of IRDye800CW indeed leads to more lipophilic ligands. We determined the IC_{50} values of all four ligands to be in the same order of magnitude (184–475 nM), which was lower than that of the high-affinity PSMA-617 ligand (IC_{50} : 8.5 nM). Wirtz *et al.* found that increasing the lipophilicity of their PSMA-I&T based ligands is beneficial for ligand internalization in LNCaP cells [30]. In contrast to this, no differences in internalization rate were observed between the SPAAC ligands and their NHS-based counterparts in our study. We, however, observed significantly higher overall binding (membrane bound + internalized fraction) of the NHS-based ligands compared with the SPAAC-variants in LS174T PSMA-positive cells *in vitro*.

Previously, our group performed a dose and time optimization study with dual-labeled ligands similar to the ones described here and showed that 2 hours p.i. was the optimal time point for imaging [9]. However, higher lipophilicity was found to increase plasma protein binding and alter the pharmacokinetic profile of these PSMA ligands [30, 31]. Furthermore, recent literature has shown that other changes in the ligand such as variations in linker and chelator can also cause significant changes in binding affinities, pharmacokinetics and biodistribution [32]. Therefore, we evaluated the pharmacokinetics and optimal imaging time-point of the two more hydrophobic SPAAC-chemistry based ligands (PSMA-N048 and PSMA-N049). Both ligands showed high and specific tumor uptake in PSMA-positive xenografts, with high tumor to background ratios at all timepoints (2, 4 and 24 h). This resulted in a clear visualization of the tumors using both fluorescence imaging and μ SPECT/CT imaging.

In vivo, higher lipophilicity was reported to increase, but also decrease tumor uptake of various click-chemistry based tracers [26, 28–30, 33]. In the case of PSMA ligands, higher ligand lipophilicity of PSMA-I&T-based ligands led to an increase in tumor uptake in LNCaP xenografts [30]. In addition, Böhmer *et al.*

developed and characterized a copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) based PSMA ligand for PET imaging called [^{18}F]PSMA-MIC01 [34]. This CuAAC-based ligand showed specific tumor uptake in LNCaP xenografts (11.7 ± 4.2 %ID/g, 1 h p.i.) with only minor non-specific uptake in other organs. In this LNCaP tumor model, uptake of the click-chemistry based [^{18}F]PSMA-MIC01 was even higher compared with [^{68}Ga]Ga-PSMA-11 (6.8 ± 6.3 %ID/g), conventionally used for PET imaging of PCa patients. In our study, the SPAAC chemistry PSMA ligands showed similar or even higher tumor uptake; 9 %ID/g for $^{99\text{m}}\text{Tc}$ -PSMA-N049 and 21 %ID/g for ^{111}In -PSMA-N048 (2 h p.i., LS174T-PSMA xenograft model).

Our ligands, including the SPAAC-based ones, showed low non-specific accumulation in other organs (*e.g.* PSMA-negative tumor, prostate, salivary glands). Yet, as might be expected, a more than twofold higher liver uptake was measured for the SPAAC variants. These data are in line with the statement of Notni *et al.* that a lipophilic character of the ligand, induced by large hydrophobic groups (*i.e.* aromatics such as in dibenzocyclooctene), is prone to increase the fraction of slow hepatobiliary clearance [29]. In addition, the study of Zhang *et al.* showed that addition of lipophilic quinoline groups in PSMA ligand [^{18}F]DCFPyl led to a large increase in liver accumulation in mice, primates and PCa patients [33].

For radioguided surgery, $^{99\text{m}}\text{Tc}$ and ^{111}In are used because they emit γ -photons detectable with a handheld gamma probe. In the present study, as a secondary aim, we evaluated differences between DOTA-based ligands suitable for ^{111}In -labeling (PSMA-N048, PSMA-N050) and MAG3-based ligands for labelling with $^{99\text{m}}\text{Tc}$ (PSMA-N049, PSMA-N051). Chelation of ^{111}In in DOTA leads to a neutral charge whereas chelation of $^{99\text{m}}\text{Tc}$ in MAG3 leads to a net charge of -1, which might be advantageous since the introduction of negative charges to increase PSMA affinity and ligand uptake in PSMA-positive tumors was reported in multiple studies [9, 35–37]. However, use of MAG3 compared with DOTA as a chelator increased lipophilicity, which could also influence tumor uptake and affinity of the PSMA ligand. Our results show that the ^{111}In -DOTA ligands have a significantly higher uptake in the s.c. LS174T PSMA-positive tumors compared with the $^{99\text{m}}\text{Tc}$ -MAG3 ligands. Besides favorable *in vitro* and *in vivo* properties of the ^{111}In -labeled ligands, labeling of $^{99\text{m}}\text{Tc}$ in the MAG3 chelator resulted in low RCY (15%-69%), which was highly variable between each labeling. In comparison, labeling with ^{111}In always resulted in high RCY ($\geq 93\%$). Furthermore, use of MAG3 as a chelator led to more difficulties with the synthesis and overall stability of the PSMA-N051 ligand (data not shown). Consequently, DOTA-based ligands might be preferred over MAG3-based variants.

As shown in the i.p. model presented in this study, tumors located deeper inside the surgical cavity may not be visualized with NIR fluorescence imaging alone due to the absorption of the emitted fluorescent light in overlying tissues (2–3 mm) [38]. This further emphasizes the importance of dual-labeled NIR fluorescence and radionuclide targeting agents that, in addition to preoperative tumor localization, allow intraoperative mapping of more deeply situated tumor lesions with a gamma probe. The findings of the current study encourage clinical studies with PSMA-targeted dual-labeled ligands to enable image-guided

complete resection of all PCa lesions during radical prostatectomy, preventing cancer-recurrences and improving the chances for curative PCa surgeries.

In conclusion, we developed four dual-labeled ligands which all showed high PSMA affinity and excellent PSMA-specific tumor uptake. We compared NHS and SPAAC chemistry-based approach to attach the fluorophore IRDye800CW. Overall, no significant differences between the SPAAC chemistry ligands and their NHS-based counterparts were found, while ^{111}In -labeled ligands outperformed the $^{99\text{m}}\text{Tc}$ ligands. These results inspire the use of click chemistry conjugations in PSMA ligand development to enable fast, efficient and easy coupling of various chelators, dyes or even anti-cancer drugs.

Abbreviations

^{111}In : indium-111, $^{99\text{m}}\text{Tc}$: technetium-99m, DBCO: Dibenzylcyclooctyne, NHS: N-Hydroxysuccinimide, NIR: Near infrared, PCa: Prostate cancer, PSMA: Prostate specific membrane antigen, SPAAC: strain promoted azide-alkyne cycloaddition.

Declarations

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Compliance with ethical standards

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Conflict of interest: Y. Derks, D. Löwik, S. Heskamp, P. Laverman and M. Rijpkema are applicants of patent: EP21155853 - PSMA-targeting ligands for multimodal applications.

Ethical approval : All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Figures

Figure 1

Chemical structures of PSMA-N048, PSMA-N049, PSMA-N050 and PSMA-N051. Ligands consisting of KuE (blue), linker (black), SPAAC (red), IRDye800CW (green) and MAG3 or DOTA chelator (purple).

A

Name	Structure	IC ₅₀ (nM)	LogD	Internalization ratio (%)
PSMA-N048	DOTA-SPAAC-IRDye800CW	184.4 (95% CI: 91.0-373.6)	-3.2 ± 0.16	80.6 ± 11.6
PSMA-N049	MAG3-SPAAC-IRDye800CW	358.7 (95% CI: 226.4-568.4)	-2.4 ± 0.39	71.9 ± 13.7
PSMA-N050	DOTA-NHS-IRDye800CW	312.3 (95% CI: 180.8-539.3)	-4.4 ± 0.01	74.1 ± 20.3
PSMA-N051	MAG3-NHS-IRDye800CW	474.9 (95% CI: 271.6-830.5)	-3.5 ± 0.01	61.0 ± 12.8
PSMA-617		8.5 (95% CI: 5.1-14.4)	-4.1 ± 0.01	48.4 ± 12.4

B **Figure 2**

In vitro characterization of PSMA-N048, PSMA-N049, PSMA-N050 and PSMA-N051. (A) IC₅₀ values of ligands as determined in competitive binding assays using LS174T-PSMA cells. Lipophilicity of ligands expressed in logD values. Internalization ratio as determined in LS174T-PSMA cells (B) Membrane binding and internalization kinetics of PSMA-N048, -N049, -N050 and -N051 in LS174T PSMA-positive and negative cells. Non-specific binding was determined by blocking with an excess of 2-PMPA (50 µg). PSMA-617 was added as a positive control.

Figure 3

In vivo pharmacokinetics of ^{99m}Tc -PSMA-N049 and ^{111}In -PSMA-N048. (A) Biodistribution as determined after dissection of ^{99m}Tc -PSMA-N049 (3 MBq/mouse) and ^{111}In -PSMA-N048 (10 MBq/mouse) 2, 4 and 24 hours p.i. (0.3 nmol, $n = 5/\text{group}$). Biodistribution was determined in mice bearing subcutaneous LS174T-PSMA and LS174T wildtype xenografts. Data is expressed as %ID/g $\pm$ SD, ** indicates $p < 0.01$. (B) Representative $\mu\text{SPECT/CT}$ scans and fluorescence images of mice with s.c.

LS174T-PSMA (left) and wildtype LS174T (right) tumors after i.v. injection of 99mTc-PSMA-N049 (3 MBq/mouse) and 111In-PSMA-N048 (10 MBq/mouse) 2, 4 and 24 hours p.i.

Figure 4

In vivo comparison of PSMA-N048, PSMA-N049, PSMA-N050 and PSMA-N051. (A) Biodistribution as determined after dissection and (B) resulting tumor-to-organ ratios of four 111In- (10 MBq/mouse) or 99mTc-labeled (15 MBq/mouse) ligands and positive control PSMA-617 (0.3 nmol, 2 h p.i., n= 5/group). Biodistribution was determined in mice bearing subcutaneous LS174T-PSMA and LS174T wildtype xenografts. Data is expressed as %ID/g ± SD, * indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001.

Figure 5

All ligands clearly visualize PSMA-positive tumors using both $\mu\text{SPECT/CT}$ and fluorescence imaging. Representative $\mu\text{SPECT/CT}$ scans (A) and fluorescence images (B) of mice with s.c. LS174T-PSMA (right) and wildtype LS174T (left) tumors after i.v. injection of ^{111}In - (10 MBq/mouse) or ^{99m}Tc -labeled (15 MBq/mouse) ligands (0.3 nmol, 2 h p.i.).

Figure 6

Multimodal fluorescence and μ SPECT/CT imaging of intraperitoneal PSMA-positive tumors using ^{111}In -PSMA-N050. Mouse with several intraperitoneal LS174T-PSMA tumors located at different depths in the peritoneal cavity. (A) Same scale NIRF images of mouse with several intraperitoneal tumors after i.v. injection of ^{111}In -labeled PSMA-N050 (0.3 nmol, 10 MBq/mouse, 2 h p.i.). (B) NIRF image of removed tumors (C) Corresponding μ SPECT/CT images in supine and left lateral positions.

Supplementary Files

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