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PSMA-targeted SPECT agents: Mode of Binding effect on in vitro Performance

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Abstract

BACKGROUND—The enzyme-biomarker prostate-specific membrane antigen (PSMA) is an active target for imaging and therapeutic applications for prostate cancer. The internalization of PSMA has been shown to vary with inhibitors' mode of binding: irreversible, slowly reversible and reversible.

METHODS—In the present study, PSMA-targeted clickable derivatives of an irreversible phosphoramidate inhibitor DBCO-PEG₄-CTT-54 (IC₅₀ = 1.0 nM) and a slowly reversible phosphate inhibitor, DBCO-PEG₄-CTT-54.2 (IC₅₀ = 6.6 nM) were clicked to ^{99m}Tc(CO)₃-DPA-azide to assemble a PSMA-targeted SPECT agent. The selectivity, percent uptake, and internalization of these PSMA-targeted SPECT agents were evaluated in PSMA-positive and PSMA-negative cells.

RESULTS—*In vitro* studies demonstrated that PSMA-targeted SPECT agents exhibited selective cellular uptake in the PSMA-positive LNCaP cells compared to PSMA-negative PC3 cells. More importantly, it was found that ^{99m}Tc(CO)₃-DPA-DBCO-PEG₄-CTT-54 based on an irreversible PSMA inhibitor core, exhibited greater uptake and internalization than ^{99m}Tc(CO)₃-DPA-DBCO-PEG₄-CTT-54.2 constructed from a slowly-reversible PSMA inhibitor core.

CONCLUSIONS—We have demonstrated that a PSMA-targeted SPECT agent can be assembled efficiently using copper-less click chemistry. In addition, we demonstrated that mode of binding has an effect on internalization and percent uptake of PSMA-targeted SPECT agents; with the irreversible targeting agent demonstrating superior uptake and internalization in PSMA+ cells. The approach demonstrated in this work now supports a modular approach for the assembly of PSMA-targeted imaging and therapeutic agents.

Keywords

PSMA; prostate cancer; radioimaging; click chemistry

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INTRODUCTION

Prostate cancer remains second leading cause of cancer death in men and accounts for a third of all new cancer diagnoses in men (1). The cell-surface enzyme prostate-specific membrane antigen (PSMA) is up-regulated and strongly expressed on prostate cancer cells associated with high grade primary, androgen independent, and metastatic tumors (2). PSMA has become an established biomarker for the progression of prostate cancer (3,4) and has attracted significant attention as a target for the delivery of imaging (5–19) and therapeutic agents (20–25). The ability to selectively detect PSMA overexpression in prostate cancer offers the promise for new avenues of diagnosis and earlier therapeutic intervention for patients at risk of an aggressive metastatic stage of the disease.

We have previously demonstrated that phosphoramidate-based peptidomimetic PSMA inhibitors can be chemically outfitted with imaging payloads without diminishing their binding affinity to PSMA (18,19,26). Our leading irreversible phosphoramidate inhibitor, CTT-54 ($IC_{50} = 14$ nM), has been modified to deliver imaging payloads to PSMA positive (PSMA+) cells both *in vitro* and *in vivo*. For example, CTT-54 conjugated to a fluorescent dye was observed to accumulate in PSMA+ cells presumably through the internalization of the PSMA enzyme-inhibitor complex (26). CTT-54 outfitted with ^{18}F was successful in the PET imaging of PSMA+ xenografts in a mouse model (18). More recently, our 1st generation PSMA-targeted SPECT agent was developed by outfitting CTT-54 with ^{99m}Tc through a bifunctional DTPA chelate structure. This phosphoramidate-based targeting agent demonstrated selective uptake in PSMA+ cells both *in vitro* and *in vivo*. Moreover, the PSMA-targeted SPECT agent demonstrated rapid and extensive internalization; greater than 70% within 30 minutes (19).

This present study was focused on two objectives. The first was to develop a clickable PSMA-targeted scaffold for the flexible delivery of imaging or therapeutic payloads to PSMA+ cells. A click-ready radiolabeled chelate structure, $^{99m}Tc(CO)_3$ -DPA-azide [3], was investigated as a SPECT imaging payload for conjugation to a complementary clickable PSMA-targeted scaffolds (DBCO-PEG₄-CTT-54 [5] and DBCO-PEG₄-CTT-54.2 [6]). The *in vitro* performances of the resulting conjugates were evaluated for selectivity, percent uptake, internalization, and cell viability. The second focus was to compare the mode of binding of PSMA inhibitors (irreversible vs. slowly reversible) on the *in vitro* performance of PSMA-targeted SPECT imaging agents. Our lab had previously shown a correlation between the mode of binding and the extent of internalization of the PSMA enzyme-inhibitor complex; irreversible inhibitors induced the greatest extent of internalization (27). Both an irreversible and slowly reversible clickable PSMA-targeted scaffolds were conjugated to **3** and their *in vitro* performances were used to elucidate the effects of mode of binding on internalization of these imaging probes.

EXPERIMENTAL PROCEDURES

Materials

LNCaP and PC-3 cells were obtained from the American Type Culture Collection. Dibenzylcyclooctyne (DBCO)-PEG₄-NHS ester was purchased from Click Chemistry Tools

(Scottsdale, AZ). All other chemicals and cell-culture reagents were purchased from Fisher Scientific (Sommerville, NJ) or Sigma-Aldrich (St. Louis, MO). All solvents used in chemical reactions were anhydrous and obtained as such from commercial sources or distilled prior to use. All other reagents were used as supplied unless otherwise stated. Liquid flash chromatography (silica or C18) was carried out using a Biotage Flash Plus chromatography system. High-resolution mass spectrometry was performed using an ABS 4800 MALDI TOF/TOF Analyzer (Applied Biosystems), at the School of Molecular Biosciences, Washington State University, Pullman, WA. ^1H NMR chemical shifts are relative to tetramethylsilane ($\delta=0.00$ ppm), CDCl_3 ($\delta=7.26$ ppm), or D_2O ($\delta=4.87$ ppm). ^{13}C NMR chemical shifts are relative to CDCl_3 ($\delta=77.23$ ppm). ^{31}P NMR chemical shifts in CDCl_3 or D_2O were externally referenced to 85% H_3PO_4 ($\delta=0.00$ ppm) in CDCl_3 and D_2O . Aqueous buffered solutions for *in vitro* experiments and HPLC chromatography were prepared with deionized distilled water (Milli-Q water system, Millipore). Analysis and purification of $^{99\text{m}}\text{Tc}$ complex of compounds were conducted on a Hitachi D700 High Pressure Liquid Chromatograph (HPLC) equipped with a UV/Vis Series 200 detector, a Radiomatic 610TR detector, and a Varian Pursuit XRS 5 C18 250mm $\times$ 4.6mm column. Radioactivity from cell studies were counted on ORTEC manufactured NaI detector supplied with MAESTRO software for Windows: Model A65-B32, UMCBI Kernel Version 6.04 and connections-32 version 6.04. $^{99\text{m}}\text{TcO}_4$ saline solution was obtained from Cardinal Health IsoLinkTM vials were received as a gift from Covidien.

Chemical Syntheses

L2-([2-(4-Amino-4-carboxy-butyrylamino)-2-carboxyethoxy]-hydroxy-phosphorylamino)-pentanedioic acid pentapotassium salt (CTT-54, [7]) and 2-([2-(4-Amino-4-carboxy-butyrylamino)-2-carboxyethoxy]-hydroxy-phosphoryloxy) pentanedioic acid pentapotassium salt (CTT-54.2, [8]). Were synthesized as previously reported (19,28).

DBCO-PEG4-CTT-54 [5]—Compound **7** (16.7mg, 0.0264 mmol) was dissolved in 0.60 ml of 100mM K_2CO_3 buffer. DBCO-PEG₄-NHS (20 mg, 0.041 mmol) dissolved in 0.50 ml of DMSO was added to **7** and stirred 3 h. The percent yield was 98% as determined by HPLC and the crude material was used without further purification for the subsequent radiolabeling step. MALDI high-resolution mass spectrometry ($\text{M}+\text{H}$): calculated 1022.3647, found 1022.4085 for $\text{C}_{45}\text{H}_{58}\text{N}_5\text{O}_{20}\text{P}^+$.

DBCO-PEG4-CTT-54.2 [6]—Compound **8** (31.6 mg, 0.0229 mmol) was dissolved in 0.60 ml of 100 mM K_2CO_3 buffer. DBCO-PEG₄-NHS (25 mg, 0.036 mmol) dissolved in 0.40 ml of DMSO was added to **8** and stirred 3 h. The percent yield was 90% as determined by HPLC and the crude material used without further purification for the subsequent radiolabeling step. MALDI high-resolution mass spectrometry ($\text{M}-\text{H}+2\text{Na}$): calculated 1067.3121, found 1067.4208 for $\text{C}_{45}\text{H}_{58}\text{N}_4\text{Na}_2\text{O}_{21}\text{P}^+$.

DPA-azide [4]—1-propylazidoamine (105 mg, 1.05 mmol) was dissolved in 15 mL of dry, degassed tetrahydrofuran, and cooled to 0 °C. Following the slow addition of sodium triacetoxyborohydride (556 mg, 2.62 mmol), the mixture stirred 30 min. 2-pyridinecarboxaldehyde (247 mg, 2.30 mmol) was then added to the reaction mixture, which

was warmed to room temperature and stirred overnight. The solvent was removed *in vacuo* and the reaction was quenched by the addition of water (5 mL). The mixture was injected onto a preparative HPLC column, Varian pursuit XRs C18, and was eluted with a H₂O/CH₃OH gradient (R_t = 24.3 min). Beginning with H₂O gradually shifting to methanol: 0–3.0 min (100% H₂O, 0% MeOH), 3.1–14.0 min (85% H₂O, 15% MeOH, linear gradient), 14.1–25.0 min (66% H₂O, 34% MeOH, linear gradient), 25.1–35.0 min (0% H₂O, 100% MeOH, isocratic), at a flow rate of 15.0 mL/min. The collected fractions were basified and extracted with dichloromethane (3x). The organic layers were combined and dried over MgSO₄. The solvent was removed *in vacuo* to give a yellow oil. Yield: 72 mg, 24%. ¹H (300 MHz, CD₃OD) 8.45 (2 H, d), 7.81 (2 H, t), 7.62 (2 H, m), 7.28 (2 H, m), 3.78 (4H, s), 3.31 (2 H, p), 2.60 (2 H, t), 1.75 (2 H, p); ¹³C (75.5 MHz, CD₃OD) 159.3, 148.2, 137.5, 123.8, 122.7, 60.0, 51.3, 49.1, 26.2. λ_{max} (CH₃OH)/nm: 260 ($\Sigma/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ 8000); ν_{max} /cm⁻¹: 2342 (N=N=N). MALDI high-resolution mass spectrometry (M+H): calculated 283.167, found 283.1725 for C₁₅H₁₉N₆⁺.

^{99m}Tc radiolabeling

^{99m}Tc(CO)₃-DPA-DBCO-PEG₄-CTT-54 [1]. A 1 mL saline solution of Na^{99m}TcO₄ (20 mCi) was added to an IsoLink™ kit and heated for 30 min at 95°C. The solution was cooled then acidified with 0.20 mL of 1 M HCl after five minutes and neutralized with 0.25 mL of 1.0 M NaOH. The resulting *fac*-[^{99m}Tc(CO)₃(H₂O)₃]⁺ (0.2 mL) was added to a vial containing 0.1 mL of **4** (0.48 M) and 0.3 mL of phosphate buffer (0.01 M, pH 7). The mixture was heated at 95 °C for 30 min, cooled in an ice bath and the reaction progress was analyzed by radio-HPLC (R_t = 26.1 min) to yield **3** with greater than 99% radiochemical yield. Compound **5** (0.2 mL) was added to **3** and reacted for 30 min at 45 °C with vigorous stirring. Compound **1** was purified and isolated by HPLC (R_t = 13.2 min) using a reverse phase gradient system beginning with K₂CO₃ buffer (0.01 M, pH 8.3) gradually shifting to methanol: 0–1.0 min (75% K₂CO₃, 25% MeOH), 1.0–2.0 min (60% K₂CO₃, 40% MeOH, linear gradient), 2.0–8.0 min (60% K₂CO₃, 40% MeOH), 8.0–9.0 min (40% K₂CO₃, 60% MeOH, linear gradient), 9.0–16.0 min (40% K₂CO₃, 60% MeOH), 16.0–18.0 min (20% K₂CO₃, 80% MeOH, linear gradient), 18.0–22.0 min (20% K₂CO₃, 80% MeOH), 22.0–25.0 min (3% K₂CO₃, 97% MeOH, linear gradient), 25.0–30.0 min (3% K₂CO₃, 97% MeOH), 30.0–35.0 min (75% K₂CO₃, 25% MeOH), at a flow rate of 1.0 mL/min. The radiochemical purity of **1** was greater than 99%. ^{99m}Tc(CO)₃-DPA-DBCO-PEG₄-CTT-54.2 [2]. The procedure for the preparation of compound **2** was the same as that described above for **1**. Briefly, compound **3** was prepared as described in the ^{99m}Tc radiolabeling section above. Compound **6** (0.2 mL) was added to **3** and reacted for 30 min at 45 °C with vigorous stirring. Compound **2** was purified as described for **1** with the same retention time (R_t = 13.2 min). The radiochemical purity of **2** was greater than 99% as determined by HPLC.

IC₅₀ Determination

Inhibition studies were performed as described previously (29,30). Working solutions of the substrate (N-[4-(phenylazo)-benzoyl]-glutamyl-g-glutamic acid, PABGgG) and inhibitors were made in TRIS buffer (50 mM, pH 7.4 containing 1% Triton X-100). Working solutions (50 µg/mL) of purified PSMA (31) were diluted in TRIS buffer (50 mM, pH 7.4 containing

1% Triton X-100) to provide from 15% to 20% conversion of substrate to product in the absence of inhibitor. A typical incubation mixture (final volume 250 μ L) was prepared by the addition of either 25 μ L of an inhibitor solution or 25 μ L TRIS buffer (50 mM, pH 7.4 containing 1% Triton X-100) to 175 μ L TRIS buffer (50 mM, pH 7.4 containing 1% Triton X-100) in a test tube. PABGgG (25 μ L, 10 μ M) was added to the above solution. The enzymatic reaction was initiated by the addition of 25 μ L of the PSMA working solution. In all cases, the final concentration of PABGgG was 1 μ M while the enzyme was incubated with five serially diluted inhibitor concentrations providing a range of inhibition from 10% to 90%. The reaction was allowed to proceed for 15 min with constant shaking at 37 °C and was terminated by the addition of 25 μ L methanolic TFA (2% trifluoroacetic acid by volume in methanol) followed by vortexing. The quenched incubation mixture was quickly buffered by the addition of 25 μ L K_2HPO_4 (0.1 M), vortexed, and centrifuged (10 min at 7,000g). An 85 μ L aliquot of the resulting supernatant was subsequently quantified by HPLC as previously described (29,32). IC_{50} values were calculated using KaleidaGraph 3.6 (Synergy Software).

Mode of inhibition Study

Mode of inhibition study followed the procedure describe in our previous work (27). Briefly the concentration of PSMA (2.5 μ g/mL) was 100-fold greater than used in the typical ezyme activity assays. The enzyme was pre-incubated for 10 minutes with 0.1 μ M of inhibitor (40 μ L), at approximately 10-fold greater than the IC_{50} value. The solution was diluted with 1 mM of substrate in 50 mM tris + 1% triton buffer (100-fold, total volume 3960 μ L). The formation of product was monitored every 5 minutes for 1 hour. A control sample was defined as incubation described here without inhibitor. Progress curves of product formation were generated to monitor the recovery of enzymatic activity for inhibited PSMA and compared to a control sample in which no inhibitor was added (Figure 1).

In Vitro Studies

Cell Lines and Cell culture—LNCaP and PC-3 cells were grown in T-75 flasks with complete growth medium [RPMI 1640 containing 10% heat-inactivated fetal calf serum (FBS), 100 units of penicillin and 100 μ g/mL streptomycin] in a humidified incubator at 37 °C and 5% CO_2 .

In Vitro Cell Uptake Studies—Confluent LNCaP and PC-3 cells were detached with 0.25% trypsin-0.53 mM EDTA solution. The cells were washed three times with 1% FBS 1X-RPMI 1640 phosphate-free medium. The cells were subdivided into microcentrifuge tubes to contain approximately 2 million cells each in 1 ml of 10% FBS 1X-RPMI 1640 phosphate-free medium and 25 μ L ethanol to reduce aggregation of **1** and **2**. Separate solutions of either **1** or **2** (0.11 μ Ci, approximately 0.1 mL) was added to microcentrifuge tubes containing one of the following: LNCaP cells (n=5) in 1% FBS 1X-RPMI 1640 phosphate-free medium, PC-3 cells (n=5) in 1% FBS 1X-RPMI 1640 phosphate-free medium, 1 mL of 1% FBS 1X-RPMI 1640 phosphate-free medium (n=3), and 1 ml PBS (n=3); the latter two solutions served as references for the total activity incubated with the cells. Two additional sets of microcentrifuge tubes were also prepared which contained the following: 1 ml 1% FBS 1X-RPMI 1640 phosphate-free medium (n=3) and 1 ml PBS buffer

(n=3); as no activity was added to these tubes, they served as references for background correction of activity. All samples above were incubated at 37 °C for 30 min, 2 hours, and 4 hours. At each time point the samples were centrifuged (4 min at 4000g) and media was removed. The cell pellets were washed with PBS buffer (1 mL), centrifuged (4 min at 4000g), and the buffer removed; this was repeated twice. The radioactivity of the cell pellets were counted, and compared to the total activity references. All samples and references were adjusted for background and decay. Percent uptake is equal to radiotracer bound to cell divided by unbound and bound radiotracer and represents the fraction of the radiotracer (**1** or **2**) associated with the cells compared to the total amount of the radiotracer incubated with the cells.

Cell Viability—An aliquot (10µl) of LNCaP cells incubated with either **1** or **2** at 37 °C was removed at 30 minutes, 2 hours and 4 hours. Each aliquot was mixed with 10µl of trypan blue then 10µl of the mixture was placed on a hemocytometer. Both live and dead cells were counted and recorded separately.

Internalization Studies—Internalization studies followed the method describe for the *in vitro cell uptake studies* with a minor modification. Following the incubation periods (30 min, 2 hr, 4 hr) the cells were washed as described in the uptake studies with PBS buffer cooled to 4°C. The cells were then treated with a 50 mM glycine, 100 mM NaCl, pH = 3 solution for 2 minutes at 37 °C. The cells were then centrifuged (4 min at 4000g) and the supernatants were collected. This treatment was repeated twice more and the combined supernatants were counted for external binding while the cell pellet was counted for internalization (19,33)

RESULTS AND DISCUSSION

Preparation of the PSMA-targeted SPECT radiotracers

The PSMA inhibitors **7** and **8** were conjugated through their *N*-terminal amine to the clickable moiety, DBCO using the amine-reactive DBCO-PEG₄-NHS reagent. Compound **4** was labeled rapidly and efficiently with *fac*-[^{99m}Tc(CO)₃(H₂O)₃]⁺ to yield **3** in greater than 99%. Without purification, compound **3** was subsequently clicked to **5** and **6**. The resulting radiotracers were isolated and purified by HPLC with high radiochemical yields.

In vitro performance of the PSMA-targeted SPECT tracers. *IC₅₀ Determination.* The IC₅₀ values of **5** and **6** were expected to be similar based on our previous work with phosphoramidate and phosphate based inhibitors of PSMA (27,28). The IC₅₀ values for **5** and **6** were 1.0 nM and 6.6 nM, respectively. The similarity of the IC₅₀ values suggested that any observed differences in uptake would likely be due to the mode of inhibition and not exclusively inhibitory potency.

Mode of inhibition—Based on our previous studies with phosphoramidate and phosphate based inhibitors of PSMA, it was expected that the mode of binding for **5** would exhibit irreversible inhibition of PSMA while **6** would exhibit slowly-reversible inhibition (19,27,28). To determine the reversibility of PSMA inhibition for these compounds, we monitored the recovery of enzyme activity following rapid dilution of the enzyme inhibitor-

complex as described previously (34). If the inhibitor is rapidly reversible, the progress curve should be linear with a slope nearly equal the slope of an uninhibited control sample. If the inhibitor is irreversible, then only <9% residual activity can be measured after dilution. If the inhibitor is moderately reversible on the time scale of the activity assay, the progress curves will be curvilinear and increase with time. As expected, the results from the inhibition reversibility experiments for **5** confirmed that it was an irreversible inhibitor of PSMA and **6** was a slowly reversible inhibitor of PSMA [Figure 1]. These findings were consistent with our earlier observations for analogous phosphoramidate-based inhibitors (19,27,28). Based on our previous studies, PSMA inhibitors that exhibited an irreversible mode of inhibition were also shown to induce internalization of the PSMA enzyme-inhibitor complex into the PSMA(+) LNCaP cells to a greater extent than slowly reversible or reversible PSMA inhibitors (27).

Specificity for PSMA-Positive cells—The radiotracers **1** and **2** were incubated separately with both the PSMA(+) LNCaP and PSMA negative PC-3 cell lines. The percent uptake was determined at 30 min, 2 h and 4 h [Table 1]. Specifically, LNCaP and PC-3 cells, media, and PBS buffer were incubated at 37 °C with approximately 0.1 μ Ci of the radiotracers for each time point; media and PBS served as reference samples. In addition, media and PBS buffer were incubated at 37 °C at each time point in the absence of the radiotracers to serve as controls for the correction of background activity. The radioactivity of each cell incubation sample was counted and the percent uptake was determined relative to the total activity from the reference samples.

Both radiotracers exhibited increasing uptake in LNCaP cells over 4 h. In contrast, the PC-3 cells exhibited negligible uptake as expected due to the absence of PSMA expression in this cell line. The percent uptake of **1** in LNCaP cells approximately doubled from 0.5 to 2 h with an additional 23% increase in uptake from 2 to 4 h. This logarithmic uptake was consistent with what we observed for the previous reported radiotracer $^{99m}\text{Tc}(\text{CO})_3\text{-DTPA-CTT-54}$, which utilized the same irreversible phosphoramidate targeting moiety (19). It is interesting to note that radiotracer **1** displayed higher uptake than $^{99m}\text{Tc}(\text{CO})_3\text{-DTPA-CTT-54}$. However, with the exception of the PSMA targeting core, radiotracer **1** is too great a departure from the $^{99m}\text{Tc}(\text{CO})_3\text{-DTPA-CTT-54}$ in terms of structural modifications (e.g., PEG linker, chelate structure, DBCO group) to develop a reasonable SAR to explain the differences in *in vitro* performance. The rate of uptake of **2** in LNCaP cells was considerably less with linear uptake out to 4 h [Figure 2]. With similar IC_{50} values for both **1** and **2** the difference in the extent and nature of the uptake is likely due to differences in the mode of binding to PSMA.

Cell Viability—To confirm that the cell uptake and internalization of the radiotracers **1** and **2** in LNCaP cells was not due to the cytotoxic loss of cell membrane integrity, cell viability studies using trypan blue were conducted on LNCaP cells for each incubation time point at 37 °C. For **1** the cell viability of LNCaP was 96% (0.5h), 91% (2h) and 89% (4h). For **2** the cell viability of LNCaP was 98% (0.5h), 98% (2h) and 93% (4h). The cell viability study is supportive of the conclusion that the cellular accumulation of the probe was due to interactions with PSMA and not through passive diffusion into permeabilized cells.

Internalization into PSMA-positive cells—The uptake of the radiotracers in LNCaP cells were further evaluated for the extent internalization. Following the incubation periods (30 min, 2 and 4 h) the cells were washed and stripped of cell-surface bound radiotracers by treatment with a solution of 50 mM glycine buffer, 100mM NaCl, pH=3 as described previously. Both the wash solutions and the cell pellets were counted for radioactivity to determine the percent internalization of the probe.

While the percent internalization of both radiotracers were similar at 30 min (Table 1), it tended to decrease for **2** over 4 h. This was in contrast to **1** for which the internalization increased. The decreased internalization observed for the radiotracer **2**, a slowly reversible phosphate-based PSMA inhibitor core, compared to that of **1**, an irreversible phosphoramidate-based PSMA inhibitor core, is consistent with our earlier findings correlating mode of binding and internalization of PSMA (27).

CONCLUSION

We have demonstrated that a PSMA-targeted SPECT agent can be assembled efficiently using copper-free click chemistry. This was accomplished by outfitting a small-molecule PSMA inhibitor with the strained alkyne group DBCO and a radiolabeled azide-bearing chelate structure (DPA). In addition, it was revealed that mode of binding has an effect on internalization and percent uptake of PSMA-targeted SPECT agents; with the irreversible targeting agent demonstrating superior uptake and internalization in PSMA+ cells. Finally, in comparison to our first generation PSMA-targeted SPECT agent $^{99m}\text{Tc}(\text{CO})_3\text{-DTPA-CTT-54}$, the uptake data for **1** suggests that assembling the labeled agent through click chemistry does not diminish the *in vitro* performance. The DBCO scaffold described in this work now supports a modular approach for the assembly of PSMA-targeted click-ready imaging and therapeutic agent, such as MRI contrast, fluorescence dyes, near IR dyes, chemotherapeutics, quantum dots, and alternative radionuclides (35–37).

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ABBREVIATIONS

DBCO	Dibenzylcyclooctyne
DPA	Di-(2-picolyl) amine
DTPA	Diethylenetriaminepentaacetic acid
PEG	Polyethylene glycol
PSMA	Prostate Specific Membrane Antigen
SPECT	Single-Photon Emission Computed Tomography

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Figure 1.
Mode of Inhibition: Enzymatic activity recovery profile for PSMA inhibition.

Figure 2. Percent Uptake of PSMA-targeted SPECT imaging agent in LNCaP cells. Standard deviations are listed in Table 1.

SCHEME 1.

a. DBCO-PEG₄-NHS, 0.1M K₂CO₃ b. *fac*-[^{99m}Tc(CO)₃(H₂O)₃]⁺, 0.01M Phosphate Buffer pH=7, 95C, 0.5 hr c. 0.01M Phosphate Buffer pH=7, RT, 1 hr

Table I

Uptake and Internalization

^{99m} Tc(CO) ₃ -DPA-DBCO-PEG ₄ -CTT-54			
Cell Line	Time (h)	% Uptake *	% Internalization *
LNCaP	0.5	7.95 (±0.35)	42 (±1.7)
LNCaP	2.0	19.06 (±0.65)	51 (±5.4)
LNCaP	4.0	24.85 (±0.41)	52 (±3.0)
PC-3	0.5	0.33 (±0.07)	N/A
PC-3	2.0	0.89 (±0.28)	N/A
PC-3	4.0	1.28 (±0.22)	N/A
^{99m} Tc(CO) ₃ -DPA-DBCO-PEG ₄ -CTT-54.2			
Cell Line	Time (h)	% Uptake *	% Internalization *
LNCaP	0.5	1.46 (±0.66)	40 (±3.9)
LNCaP	2.0	4.79 (±0.19)	30 (±3.1)
LNCaP	4.0	9.16 (±0.26)	27 (±4.2)
PC-3	0.5	0.35 (±0.20)	N/A
PC-3	2.0	0.21 (±0.09)	N/A
PC-3	4.0	0.13 (±0.04)	N/A

*
standard deviation in parentheses