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A Multivalent Approach of Imaging Probe Design to Overcome an Endogenous Anion Binding Competition for Noninvasive Assessment of Prostate Specific Membrane Antigen

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Abstract

2[(3-amino-3-carboxypropyl)(hydroxy)(phosphinyl)-methyl]pentane-1,5-dioic acid) (GPI) is a highly potent inhibitor of prostate specific membrane antigen (PSMA) with a rapid *in vivo* clearance profile from non-target organs including kidneys, but its use for imaging of PSMA is blocked by an endogenous anion (serum phosphate) competition, which compromises its specific binding to the antigen. Multi-presentation of a targeting molecule on a single entity has been recognized as a practical way for imaging sensitivity enhancement. Herein, we demonstrate a multivalent approach based on a ⁶⁴Cu-specific bifunctional chelator scaffold to overcome the endogenous phosphate competition thus enabling the utility of GPI conjugates for *in vivo* detection of PSMA and imaging quantification. Both monomeric (H₂CBT1G) and dimeric (H₂CBT2G) conjugates were synthesized and labeled with ⁶⁴Cu for *in vitro* and *in vivo* evaluations. A 4-fold enhancement of PSMA binding affinity was observed for H₂CBT2G as compared to H₂CBT1G from the PSMA competitive binding assays performed on LNCaP cells. *In vivo* PET imaging studies were conducted on mouse xenograft models established with a PSMA⁺ cell line, LNCaP, and PSMA⁻ PC3 and H2009 cell lines. ⁶⁴Cu-CBT2G showed significantly higher LNCaP tumor uptake than ⁶⁴Cu-CBT1G at 1, 4, and 24 h post-injection (p.i.) ($p < 0.05$). In addition, tumor uptake of ⁶⁴Cu-CBT2G remained steady out to 24 h p.i. (1.46 ± 0.54 , 1.12 ± 0.56 , and 1.00 ± 0.50 %ID/g at 1, 4 and 24 h p.i., respectively), while ⁶⁴Cu-CBT1G showed a great decrease from 1 h to 4 h p.i. The PSMA imaging specificity of both H₂CBT1G and H₂CBT2G was demonstrated by their low uptake in PSMA⁻ tumors (PC3 and H2009) and further confirmed by a significant signal reduction in PSMA⁺ LNCaP tumors in the blockade study. In addition, the LNCaP tumor uptake (%ID/g) of ⁶⁴Cu-CBT2G was found to be in a positive linear correlation with the tumor size ($R^2 = 0.92$, 0.94 , and 0.93 for 1 h, 4 h, and 24 h p.i.). This may render the probe with potential application in the management of patients with prostate cancer.

Keywords

PSMA; prostate cancer; PET; ⁶⁴Cu; multivalency

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INTRODUCTION

Prostate cancer is the second leading cause of cancer death behind only lung cancer in American men. Noninvasive imaging techniques, such as ultrasound, x-ray, computed tomography (CT), magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT), and positron emission tomography (PET), have been used for the diagnosis of prostate cancer along with conventional methods. Despite the great success of FDG-PET scan (^{18}F -FDG: 2-deoxy-2-(^{18}F)fluoro-d-glucose) in other cancer types, its role in prostate cancer is limited by the fact that prostate cancer at early stages is not substantially up-regulating glucose metabolism and the prostate glands are in close proximity of the bladder, from which most of FDG activity is cleared.¹ To date, various PET radiotracers have been introduced for prostate cancer imaging.¹ For example, ^{11}C or ^{18}F -labeled choline showed promising results for detecting primary and metastatic prostate cancer but was inconsistent in finding densely sclerotic bone lesions;^{2, 3} ^{11}C -labeled acetate was reported with potential to detect local recurrences and regional lymph node metastases but still remains investigative;^{4, 5} radiolabeled peptides have been exploited for prostate cancer imaging by specifically targeting prostate cancer cell surface biomarkers or receptors.^{6, 7} However, noninvasive PET assessment of prostate cancer, local disease or distal metastasis, still remains a challenge.^{1, 8}

Prostate specific membrane antigen (PSMA) is a type II transmembrane glycoprotein that is over-expressed in prostate cancer but not in the vasculature of normal tissues.⁹ Moreover, up-regulation of PSMA expression is seen after androgen treatment and found in a positive correlation with lymph node metastases and prostate cancer grade.^{10, 11} Therefore, PSMA has been utilized as an effective target to develop imaging agents or therapeutics for prostate cancer. Indeed, the only U.S. Food and Drug Administration (US FDA) approved prostate cancer imaging agent is an ^{111}In -labeled anti-PSMA monoclonal antibody (^{111}In -capromab pendetide). However, the application of ^{111}In -capromab pendetide is limited because it only reacts with the intracellular epitope of PSMA. Other anti-PSMA antibodies (e.g. J591) that recognize the extracellular PSMA domain have become available, but antibody-mediated imaging suffers from the inherently slow pharmacokinetics and retarded washout rates of antibodies.^{12, 13} Therefore, while monoclonal antibodies such as J591 are more intended for PSMA-based radioimmunotherapy,¹⁴ small organic molecules have been developed for PSMA-targeted imaging and encouraging results have been seen with glutamate-urea-based and phosphoramidate-based PSMA-targeting ligands.^{15–19} A few years ago, 2[(3-amino-3-carboxypropyl)(hydroxy)(phosphinyl)-methyl]pentane-1,5-dioic acid (GPI) was reported as a potent inhibitor of PSMA with a high binding affinity to prostate cancer cells.²⁰ Impressively, GPI was found with a rapid *in vivo* clearance profile from non-target organs including kidneys, which can be regarded as a rather favorable property as compared to the glutamate-urea-based ligands.²⁰ However, it was also found that the PSMA binding of GPI conjugates was compromised by the serum phosphate anions. To overcome this endogenous competition,²¹ a multivalency approach was reported by presenting three copies of GPI on an adamantane scaffold. The adamantane-trimerized GPI conjugate was labeled with $^{99\text{m}}\text{Tc}$, which showed significantly enhanced *in vitro* binding affinity to PSMA but lacking a follow-up *in vivo* evaluation.^{22, 23}

The role of multivalency has also been recognized in the design of molecular imaging probes for detection sensitivity enhancement. A multivalent imaging probe can be constructed by either attaching a multimeric targeting vector to an imaging platform²⁴ or imparting the desired multivalency through multi-presentation of a target vector on the platform.^{25, 26} Indeed, the first multimeric PSMA-targeted imaging agent was synthesized by the former approach through coupling a PSMA binding glutamate-urea motif with a bifunctional chelator (DOTA: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) in

order to enhance the specific binding affinity.²⁷ However, the complicated synthetic method involved use of solid phase and click chemistry procedures. In the past decade, ⁶⁴Cu has garnered its popularity in both PET imaging and radiotherapy mainly due to its decay characteristics ($t_{1/2} = 12.7$ h, 17.4% β^+ , $E_{\beta^+max} = 0.656$ MeV) and its availability in large-scale quantities with high specific activity.²⁸ In this work, we used the latter approach to construct a dimeric PET imaging probe for PSMA detection by presenting two copies of GPI on a bifunctional chelator scaffold²⁵ derived from CB-TE2A (2,2'-(1,4,8,11-tetraazabicyclo[6.6.2]-hexadecane-4,11-diyl)diacetic acid), an ideal Cu(II) chelator for PET imaging. *In vivo* evaluations of the resulted ⁶⁴Cu radiotracers were performed in mice bearing LNCaP (PSMA⁺) and other PSMA⁻ tumor xenografts.

EXPERIMENTAL SECTION

General Methods and Materials

All chemicals, solvents, and reagents were purchased from Sigma-Aldrich and Fisher Chemical unless otherwise noted. All aqueous solutions were prepared in Milli-Q water. Mass spectrum characterization was performed by Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) using Voyager-DETM PRO Biospectrometry Workstation from Applied Biosystems. The ⁶⁴CuCl₂ solution was purchased from University of Wisconsin. The Na¹²⁵I solution was purchased from Perkin-Elmer. High performance liquid chromatography (HPLC) was performed on a Waters Xterra Shield RP18 Prep column (250×10 mm, 10 μ m) and read by a Waters 2996 Photodiode Array detector and an in-line Shell Jr. 2000 radio-detector. The mobile phase was H₂O with 0.1% TFA (solvent A) and acetonitrile with 0.1% TFA (solvent B). The gradient consisted of 0% B to 80 % B in 0 – 40 min at 4.0 mL/min flow rate. Radio-TLC analysis was performed on a Rita Star Radioisotope TLC Analyzer (Straubenhardt, Germany) to monitor the radiolabeling reaction using C18 reverse phase silica gel plate and 10%NH₄OAc:CH₃OH = 1:1 (v/v) as the mobile phase. GPI was synthesized by following the published procedures with minor modifications, and it remained as a racemic mixture upon the chiral center at the alpha position of the glutamic acid moiety.^{29–32} Both ^tBu-protected CB-TE2A-1DA (2-(11-(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecan-4-yl)pentanedioic acid) and CB-TE2A-2DA (2,2'-(1,4,8,11-tetraazabicyclo[6.6.2]hexadecane-4,11-diyl)dipentanedioic acid) were prepared as previously reported.²⁵

Cell Culture and Animal Model

LNCaP, PC3, and H2009 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The PC3 cell line was cultured in T-media (Invitrogen Corporation, CA) supplemented with 5% fetal bovine serum (FBS) and 1×Penicillin/Streptomycin (PS). LNCaP cells were cultured in RPMI 1640 media (HyClone, Thermo Scientific, IL), with 2.05 mM L-Glutamine supplemented with 10% FBS. H2009 cell line was cultured in DMEM media (Invitrogen Corporation, CA) supplemented with 5% FBS. All cells were cultured at 37 °C in an atmosphere of 5% CO₂ and passaged at 75 – 90 % confluency. Male SCID mice (6 – 8 weeks of age) were purchased from the Wakeland Colony at UT Southwestern. All animal studies were approved by UT Southwestern IACUC. For LNCaP subcutaneous tumor model, LNCaP cell suspension was injected subcutaneously (2.5×10^6 cells per injection with 75% BD Matrigel, injection volume 100 μ L) into the right and left shoulders of mice. For the PC3 and H2009 dual-tumor model, PC3 cell suspension was injected subcutaneously (2.0×10^6 cells per injection with 50% BD Matrigel, injection volume 100 μ L) into the right shoulder and H2009 cell suspension was injected subcutaneously (1.0×10^6 cells per injection, injection volume 100 μ L) into the left shoulder on the same mouse. After injection, animals were monitored twice a week by

general observations. Tumor volume (mm^3) was calculated using the ellipsoid formula ($\pi/6 \times \text{length} \times \text{width} \times \text{depth}$).

Preparation of H₂CBT1G

To a solution of ⁴Bu₂-CB-TE2A-1DA (0.025 g, 47.5 μmol) in anhydrous acetonitrile (0.7 mL) was added N-hydroxysuccinimide (NHS, 0.010 g, 82.8 μmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 0.016 g, 82.8 μmol). The resulting solution was stirred overnight at room temperature (r.t.). The solvent was then removed under vacuum to afford a crude product, which was purified by HPLC (Elution time: 26.7 min) and the resulting fractions lyophilized to give ⁴Bu₂-CB-TE2A-1NHS (0.013 g, 21.4 μmol , 45%) as a white solid. MALDI-TOF/MS m/z calculated for C₃₁H₅₃N₅O₈: 623.4; found: 624.6 [M + H]⁺.

To a solution of the activated acid ⁴Bu₂-CB-TE2A-1NHS (0.005 g, 8.03 μmol) in anhydrous acetonitrile (0.5 mL) was added the solution of GPI (0.003 g, 9.64 μmol) in anhydrous acetonitrile (0.2 mL). To the resulting mixture was added a solution of triethylamine (0.001 g, 10.0 μmol) dissolved in anhydrous acetonitrile (0.2 mL). The resulting mixture was stirred overnight at r.t. The solvent was subsequently removed under vacuum to afford a crude product, which was purified by HPLC (Elution time: 16.1 min) and the resulting fractions lyophilized to give ⁴Bu₂-CB-TE2A-GPI₁ (0.004 g, 5.21 μmol , 65%) as a white solid. MALDI-TOF/MS m/z calculated for C₃₇H₆₆N₅O₁₃P: 819.4; found: 820.8 [M + H]⁺. To a solution of ⁴Bu₂-CB-TE2A-GPI₁ (0.004 g, 5.21 μmol) was added trifluoroacetic acid (0.4 mL). The resulting solution was stirred overnight at r.t. The solvent was then removed under vacuum to afford a crude product, which was purified by HPLC (Elution time: 9.1 min). The resulting fractions were lyophilized to give the free acid CB-TE2A-GPI₁ (H₂CBT1G) (0.003 mg, 1.22 μmol , 97%) as a white solid. MALDI-TOF/MS m/z calculated for C₂₉H₅₀N₅O₁₃P: 707.3; found: 708.8 [M + H]⁺.

Preparation of H₂CBT2G

To a solution of the acid ⁴Bu-CB-TE2A-2DA (0.035 g, 58.5 μmol) in anhydrous acetonitrile (1.0 mL) was added NHS (0.020 g, 165 μmol) and EDC (0.032 g, 165 μmol). The resulting solution was stirred overnight at r.t. The solvent was then removed under vacuum to afford a crude product, which was purified by HPLC (Elution time: 28.4 min) and the resulting fractions lyophilized to give ⁴Bu-CB-TE2A-2NHS (0.012 g, 14.6 μmol , 25%) as a white solid. MALDI-TOF/MS m/z calculated for C₃₈H₆₀N₆O₁₂: 792.4; found: 793.6 [M + H]⁺.

To a solution of the activated acid ⁴Bu₂-CB-TE2A-2NHS (0.002 g, 2.43 μmol) in anhydrous acetonitrile (0.2 mL) was added the solution of GPI (0.003 g, 9.64 μmol) dissolved in anhydrous acetonitrile (0.2 mL). To the resulting mixture was added a solution of triethylamine (0.001 g, 10.0 μmol) dissolved in anhydrous acetonitrile (0.1 mL). The resulting solution was stirred overnight at r.t. The solvent was subsequently removed under vacuum to afford a crude product, which was purified by HPLC (Elution time: 16.8 min) and the resulting fraction lyophilized to give ⁴Bu₂-CB-TE2A-GPI₂ (0.002 g, 1.09 μmol , 45%) as a white solid. MALDI-TOF/MS m/z calculated for C₅₀H₈₆N₆O₂₂P₂: 1184.5; found: 1186.0 ([M + H]⁺). To a solution of protected acid ⁴Bu₂-CB-TE2A-GPI₂ (0.002 g, 1.09 μmol) was added trifluoroacetic acid (0.4 mL). The resulting solution was stirred overnight at r.t. The solvent was then removed under vacuum to afford a crude product, which was purified by HPLC (Elution time: 10.4 min). The resulting fractions were lyophilized to give the free acid CB-TE2A-GPI₂ (H₂CBT2G) (0.001 mg, 0.76 μmol , 97%) as a white solid. MALDI-TOF/MS m/z calculated for C₄₂H₇₀N₆O₂₂P₂: 1072.4; found: 1074.0 [M + H]⁺.

Preparation of ^{64}Cu -CBT1G and ^{64}Cu -CBT2G

To a 1.5 mL vial containing 0.5 – 1 μg of $\text{H}_2\text{CBT1G}$ or $\text{H}_2\text{CBT2G}$ in 50 μL of 0.4 M NH_4OAc (pH = 6.5) solution, 37 – 74 MBq of $^{64}\text{Cu}^{2+}$ in 0.1 M HCl was added. The reaction mixture was incubated at 75 °C for 30 min. The radiolabeling yields were determined by radio-TLC. The unreacted $^{64}\text{Cu}^{2+}$ stayed at the bottom of TLC plate, while ^{64}Cu -CBT1G or ^{64}Cu -CBT2G moved to the solvent front.

In Vitro and *In Vivo* Stability

The *in vitro* stability test was performed in rat serum. Briefly, ^{64}Cu -CBT1G or ^{64}Cu -CBT2G (0.74 MBq, 5 μL) was added into 100 μL of rat serum (n=3). A 50 μL of sample was taken out and mixed with 200 μL of ethanol, after 4 h and 24 h incubation at 37 °C, respectively. The solution was vortexed and centrifuged for 5 min at 21,000 *g*. The supernatant was then analyzed by radio-TLC. For *in vivo* stability evaluation, male SCID mice were injected with 3.7 MBq of ^{64}Cu -CBT1G or ^{64}Cu -CBT2G in 100 μL of saline via the tail vein. Urine samples were collected within 1 h and 24 h p.i., and then analyzed by radio-TLC.

1-Octanol/Water Distribution Coefficient

PBS (phosphate buffered saline, 1 $\times$, pH 7.4) and 1-octanol were pre-mixed a day before the experiment. Approximately 0.111 MBq (10 μL) of ^{64}Cu -CBT1G or ^{64}Cu -CBT2G was added to the pre-mixed PBS (490 μL) and 1-octanol (500 μL). The mixture was vigorously vortexed for 1 min at r.t. After centrifugation at 21,000 *g* for 5 min, 100 μL aliquots of both layers were measured using a γ -counter. Then $\log D_{\text{oct/water}}$ values were calculated. The experiment was performed in quintuplicates.

Preparation of ^{125}I -GPI

To a solution of N-Sulfosuccinimidyl-3-(4-hydroxyphenyl)propionate (Sulfo-SHPP, water soluble Bonton-Hunter reagent: 7 mg, 19.3 μmol) in 0.2 mL phosphate buffer (0.1 M, pH 8.0) was added the solution of GPI (3 mg, 9.64 μmol , 0.01 mL of H_2O). The resulting solution was stirred overnight at r.t. The product was purified by HPLC and the collected fractions lyophilized to give GPI modified with a Bolton-Hunter moiety (BH-GPI: 1.8 mg, 3.86 μmol , 40%) as a white solid. MALDI-TOF/MS *m/z* calculated for $\text{C}_{19}\text{H}_{26}\text{NO}_{10}\text{P}$: 459.4; found: 460.2 $[\text{M} + \text{H}]^+$. A Pierce pre-coated iodination tube was wetted with 1 mL of Tris buffer (pH 7.5). To the pre-wetted tube was added 100 μL of Tris buffer, followed by 5 μL (37 MBq) of Na^{125}I (Perkin-Elmer). The iodide was activated for 6 min at r.t. and then added to the BH-GPI solution (0.5 μg in 0.025 mL H_2O). After 9 min at r.t., the mixture was directly applied to semi-preparative HPLC. HPLC fractions of ^{125}I -GPI were collected between 20 and 21 min and then concentrated by a Sep-Pak® Light C18 cartridge.

In Vitro Cell Binding Assay

The PSMA binding affinities of $\text{H}_2\text{CBT1G}$, and $\text{H}_2\text{CBT2G}$ were determined by a competitive cell-binding assay using ^{125}I -GPI as the radioligand. Suspended LNCaP cells in Tris-buffered saline (TBS) were seeded on multi-well DV plates (Millipore) with 5×10^4 cells per well, and then incubated with ^{125}I -GPI (33,000 cpm/well) in the presence of increasing concentrations (0 – 10,000 nM) of GPI, $\text{H}_2\text{CBT1G}$, and $\text{H}_2\text{CBT2G}$ at r.t. for 2 h (n=4). The final volume in each well was maintained at 200 μL . At the end of incubation, unbound ^{125}I -GPI was removed by filtration followed by five-time rinses with cold TBS buffer. The filters were collected and their radioactivity was measured. The best-fit IC_{50} values (inhibitory concentration where 50% of the ^{125}I -GPI bound on LNCaP cells were displaced) of GPI, $\text{H}_2\text{CBT1G}$, and $\text{H}_2\text{CBT2G}$ were calculated by fitting the data with nonlinear regression using GraphPad Prism 5.0. The same experiments were performed in triplicates.

Cell Uptake and Internalization

Cell uptake and internalization of ^{64}Cu -CBT1G and ^{64}Cu -CBT2G was measured according to the reference.³³ Briefly, LNCaP cells were seeded into 48-well plates (BD BioCoat™, Poly-D-Lysine) at a density of 2.5×10^5 cells/well and incubated at 37°C, 5% CO₂ overnight. After washing the cells once with 0.5 mL of TBS buffer at 37°C, approximately 150,000 cpm of ^{64}Cu -CBT1G or ^{64}Cu -CBT2G was added to cells in 0.2 mL of TBS buffer and incubated at 37°C, 5% CO₂ for 1, 10, 30, 60, and 120 min (n = 6). There were three additional wells co-incubating ^{64}Cu -CBT1G or ^{64}Cu -CBT2G with 2 µg of GPI for nonspecific binding assay. The unbound radioligands were removed by rinsing with 0.5 mL of ice-cold TBS buffer per well. The surface bound radioligand were removed by incubating with 0.5 mL of ice-cold low-pH stripping buffer (50 mM glycine and 0.15 M NaCl, pH 3.0) for 10 min at 4°C. Then the stripping buffer media were collected and counted with a γ-counter to determine the surface bound radioligand level. At last, 0.5 mL of 1 M NaOH was added to each well to solubilize cells. The activity was counted to quantify the internalized radioligand.

Anion Competition Assay in Different Solutions

LNCaP cells were seeded into 48-well plates (BD BioCoat™, Poly-D-Lysine) at a density of 2.5×10^5 cells/well and incubated at 37°C, 5% CO₂ for overnight. After washing the cells with 0.5 mL of TBS buffer, PBS buffer, or LNCaP cell culture medium, approximately 150,000 cpm of ^{64}Cu -CBT1G or ^{64}Cu -CBT2G was added to the cells in different buffers or media and incubated at 37°C, 5% CO₂ for 2 h (n = 3). The unbound radioligand was removed from cells by washing with 0.5 mL of ice-cold corresponding buffer per well. The cells were then solubilized in 1 M NaOH for radioactivity measurement by γ-counter. To measure the non-specific binding, 2 µg of GPI was co-loaded with ^{64}Cu -CBT1G or ^{64}Cu -CBT2G in the TBS buffer condition (n = 3).

Small Animal PET/CT Imaging

Small animal PET/CT imaging was performed with a Siemens Inveon PET/CT Multimodality System in tumor-bearing SCID mice intravenously received *ca.* 3.7 MBq of ^{64}Cu -CBT1G or ^{64}Cu -CBT2G in 100 µL via the tail vein. Prior to imaging, the mouse was sedated on the imaging bed under 2% isoflurane anesthesia for the duration of imaging. Immediately after the CT data acquisition that was performed at 80 kV and 500 µA with a focal spot of 58 µm, static PET scans were conducted at the given time points post-injection (p.i.) (1 h, 4 h, and 24 h) for 15 min. For the blockade study, the radiotracer was co-administrated with 2-(phosphonomethyl)pentanedioic acid (2-PMPA) at the dose of 30 mg/kg of mouse body weight. Both CT and PET images were reconstructed with manufacturer's software. Reconstructed CT and PET images were fused for quantitative data analysis; regions of interest (ROIs) were drawn as guided by CT and quantitatively expressed as percent injected dose per gram of tissue (%ID/g).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism. A *p* value less than 0.05 (unpaired *t* test) was considered statistically significant. All results are presented as mean ± standard deviation.

RESULTS

Preparation of H₂CBT1G and H₂CBT2G

CB-TE2A-1DA and CB-TE2A-2DA were prepared according to our previously reported procedures²⁵ (Figure 1). The inner and outer carboxylate groups of CB-TE2A-1DA and CB-

TE2A-2DA were orthogonally protected. In this work, their outer carboxylate groups were deprotected for the conjugation with GPI while the inner carboxylate groups remained protected with t -Bu group. The GPI conjugation was carried out by a typical NHS/EDC procedure. The NHS esters, t Bu₂-CB-TE2A-1NHS and t Bu₂-CB-TE2A-2NHS, were synthesized and subsequently treated with amine containing GPI under basic conditions. The synthesis of these NHS-activated esters was reported in our previous work, in which the ester purification was performed by extraction.²⁵ However, the use of extracted t Bu₂-CB-TE2A-1NHS and t Bu₂-CB-TE2A-2NHS led to poor yields and difficult purification in this present work. The problem was especially evident when using t Bu₂-CB-TE2A-2NHS. To address this unexpected issue, we performed the ester purification via HPLC so that the impurities and side products could be efficiently removed. The relevant fraction from HPLC were collected and immediately lyophilized. Each time only freshly prepared α -NHS ester intermediates were used for GPI conjugation. The conjugation of t Bu₂-CB-TE2A-1NHS and t Bu₂-CB-TE2A-2NHS with 1 and 4 equivalents of GPI in the presence of triethylamine provided t Bu-protected conjugates in 45 – 65% yields. Finally, the α -carboxylate group was deprotected using 95% TFA to provide H₂CBT1G and H₂CBT2G, each containing two internal α -carboxylic acids for ⁶⁴Cu chelating.

Radiochemistry

Both H₂CBT1G and H₂CBT2G were efficiently labeled by ⁶⁴Cu at 75 °C in 0.4 M NH₄OAc buffer within 30 min as monitored by radio-TLC. For *in vitro* and *in vivo* evaluations, the radiochemical purities of ⁶⁴Cu-CBT1G and ⁶⁴Cu-CBT2G, were maintained at over 99% as determined by radio-HPLC. Their specific activities were in the range of 50 – 80 GBq/ μ mol. Both ⁶⁴Cu-CBT1G and ⁶⁴Cu-CBT2G are highly hydrophilic, as indicated by their low logD values (⁶⁴Cu-CBT1G: -3.25 ± 0.15 ; ⁶⁴Cu-CBT2G: -2.70 ± 0.03). The serum stability test and the urine sample analysis showed that both ⁶⁴Cu-CBT1G and ⁶⁴Cu-CBT2G remained intact within 24 h without detectable demetallization, which is a great advantage of using our CB-TE2A derived chelator scaffold for ⁶⁴Cu-labeling.

In Vitro Cell Study

The high PSMA binding affinity ($K_i = 9$ nM) of GPI reported in the literature was measured by using ^{99m}Tc-labeled GPI as the PSMA specific radioligand.^{20, 22} In this work, we designed and synthesized an ¹²⁵I-labeled GPI analog (Figure 2) as the radioligand for the competitive cell binding assay of GPI, H₂CBT1G, and H₂CBT2G. Because GPI has no available functional group for direct radioiodination, a Bolton-Hunter moiety was introduced. To obtain the highest achievable specific activity of ¹²⁵I-GPI, an HPLC purification method was applied. The *in vitro* PSMA binding affinities of GPI, H₂CBT1G, and H₂CBT2G were measured by the required concentration to displace 50% of LNCaP cell bound ¹²⁵I-GPI ($n = 3$), where GPI served as the positive control. All three compounds inhibited the binding of ¹²⁵I-GPI to LNCaP cells in a dose-dependent manner (Figure 3A). The calculated IC₅₀ values were 460 ± 13 nM (GPI), 578 ± 81 nM (H₂CBT1G), and 129 ± 17 nM (H₂CBT2G). No significant difference was observed between GPI and H₂CBT1G, indicating the conjugation had negligible effect on the PSMA binding affinity of GPI. The significant increase of H₂CBT2G's PSMA binding affinity reflects the anticipated multivalent effect.

The PSMA-mediated uptake and internalization of ⁶⁴Cu-CBT1G and ⁶⁴Cu-CBT2G was evaluated using LNCaP cells with and without presence of excess GPI in TBS buffer. Both ⁶⁴Cu-CBT1G and ⁶⁴Cu-CBT2G displayed a similar and appreciable level of internalization in a time-dependent manner in the absence of GPI (Figure 3B), while the cell uptake amount of ⁶⁴Cu-CBT2G was about two times higher than that of ⁶⁴Cu-CBT1G

starting from 10 min (Figure 3C). The presence of GPI at the saturating concentration nearly abolished cell uptake (down to < 0.5%), indicating that cell uptake was mediated by PSMA.

The multimeric design of ^{64}Cu -CBT2G was intended to address the binding competition of GPI with the endogenous phosphate anions in serum.²² In order to evaluate the multivalent effect on this competition,²¹ both ^{64}Cu -CBT1G and ^{64}Cu -CBT2G were tested to bind with LNCaP cells in no phosphate-containing TBS buffer and phosphate-containing PBS buffer and LNCaP cell culture medium. Shown in Figure 3C, both phosphate-containing buffers, PBS and cell culture medium, significantly inhibited the PSMA binding of ^{64}Cu -CBT1G and ^{64}Cu -CBT2G as compared to TBS. However, while the monomeric ^{64}Cu -CBT1G's PSMA binding was reduced to the nonspecific binding level when exposed to phosphate anions, the dimeric ^{64}Cu -CBT2G showed a significant improvement of its PSMA binding in competing with phosphates ($p < 0.05$).

Small Animal PET/CT Imaging

Small animal PET/CT imaging studies were conducted in SCID mice bearing LNCaP (PSMA⁺) or PC3 and H2009 (PSMA⁻) tumors. To increase the sample size for statistics, LNCaP tumors were implanted on both sides of shoulder. Representative coronal PET/CT images are presented in Figure 4. LNCaP tumors were clearly visualized by ^{64}Cu -CBT2G at 1, 4, and 24 h p.i., and their intensity maintained steady throughout the 24 h-period (Figure 4A). LNCaP tumors were also visible by ^{64}Cu -CBT1G but with much lower contrast than ^{64}Cu -CBT2G at all time points. Both ^{64}Cu -CBT1G and ^{64}Cu -CBT2G exhibited a rapid clearance profile through the urinary route, which is desirable for good tumor to background contrast. Besides PSMA⁺ tumors, kidneys and bladder (urine) were other major organs showing significant activity accumulation at 1 h and 4 h p.i. Once urine was out of the body, the bladder was nearly invisible especially at 24 h p.i.

The quantitative imaging analysis is shown in Figure 5. The LNCaP tumor accumulation of ^{64}Cu -CBT2G remained rather stable within 24 h (1.46 ± 0.54 , 1.12 ± 0.56 , and 1.00 ± 0.50 %ID/g at 1, 4 and 24 h p.i., respectively). In contrast, ^{64}Cu -CBT1G showed much lower LNCaP tumor uptake (0.81 ± 0.12 , 0.46 ± 0.10 , and 0.35 ± 0.11 %ID/g at 1, 4 and 24 h p.i., respectively) than ^{64}Cu -CBT2G (one tailed $p < 0.05$ at each time point) and a significant tumor uptake drop from 1 h to 4 h p.i. (43%, one tailed $p < 0.05$). The significantly enhanced tumor uptake and imaging signal retention of the dimeric ^{64}Cu -CBT2G can be attributed to the multivalent effect. Due to their negatively charged and highly hydrophilic nature, both ^{64}Cu -CBT1G and ^{64}Cu -CBT2G showed low liver uptake (< 1.0 %ID/g). However, relatively high kidney uptake was observed for both GPI conjugates. This is consistent with the previous report of PSMA expression in proximal renal tubules^{34, 35} (Figure 5B). Of note, ^{64}Cu -CBT1G and ^{64}Cu -CBT2G displayed a similar uptake difference in PSMA⁺ tumors and kidneys, indicative of their imaging specificity of PSMA that was verified by a blockade study using a known PSMA inhibitor, 2-PMPA. In the blockade study, LNCaP tumor uptake of both ^{64}Cu -CBT1G and ^{64}Cu -CBT2G at 24 h p.i. was reduced by approximately five times (Figure 5A). A similar magnitude of uptake decrease was also observed in kidneys at 24 h p.i. The PSMA imaging specificity was further confirmed by using two PSMA⁻ tumor models, PC3 and H2009. At the time point as early as 1 h p.i., PC3 and H2009 tumors were barely visible by either ^{64}Cu -CBT1G or ^{64}Cu -CBT2G.

DISCUSSION

The cell membrane bound PSMA is a valuable biomarker that has drawn considerable interest in the development of diagnostics and therapeutics of prostate and other cancer types, given the well recognized fact that its up-regulation in primary prostate cancer and

metastases and its expression level has positive correlations with prostatic cancer stages, especially in castration resistance prostate cells.^{10, 11, 34, 36} In addition to antibody-based imaging agents (i.e., US FDA approved capromab pendetide), a different class of urea based small organic compounds, initially developed for the inhibition of PSMA activities, has been extensively utilized for PET and SPECT imaging probe development for non-invasive assessment of PSMA expression in prostate cancer. For instance, ¹²³I-MIP-1072 ((S)-2-(3-((S)-1-carboxy-5-((4-¹²³I-iodobenzyl)amino)pentyl)ureido)pentanedioic acid) has been in clinical trials.³⁷ Reported with a high binding affinity (9 nM) to PSMA, GPI chemically resembles the glutamate-urea compounds and the phosphoramidate ligands as well, due to the similar binding pentanedioic acid moiety. A major obstacle that impedes the use of GPI for *in vivo* PSMA imaging is the endogenous binding competition caused by phosphate anions in serum. Based on the assumption that multivalency can enhance the desired specific binding affinity and decrease the off-rate of the specific ligand, indeed a multivalency strategy was reported to overcome the hurdle by presenting three copies of GPI on an adamantane. Although the *in vitro* data demonstrated a great success as indicated by the significantly enhanced PSMA binding affinity and tolerance to the endogenous binding competition,²² the *in vivo* performance of such multivalent GPI radiolabeled agents has not been reported.

Among nonstandard PET nuclides, ⁶⁴Cu is of great clinical potential owing to its low positron range, commercial availability, and reasonably long decay half-life.²⁸ Recently we have reported a bifunctional chelator scaffold, CB-TE2A-2DA, for multivalent PET probe design aimed for the use of ⁶⁴Cu.²⁵ As compared to the commonly used chelator as DOTA, this chelator scaffold features an intact CB-TE2A core to form a stable and neutral complex with ⁶⁴Cu and two peripheral functional groups for anchoring of targeting molecules.²⁵ In this work, we presented two copies of GPI on CB-TE2A-2DA for ⁶⁴Cu PET probe design with the goal of developing a practical approach for noninvasive imaging of PSMA.

Measured by an *in vitro* competitive cell binding assay, the dimeric GPI conjugate, H₂CBT2G, indeed showed the anticipated enhancement of PSMA binding affinity. The multivalent enhancement ratios calculated by dividing IC₅₀ values were 3.6 (GPI/H₂CBT2G) and 4.5 (H₂CBT1G/H₂CBT2G), consistent with the multivalent effect. As mentioned above, increased PSMA binding affinities can be attributed to the multivalent effect, which might be explained by the structure of PSMA. It was reported that PSMA exists in both noncovalently dimeric and monomeric forms; and the homodimerization occurs in the extracellular domain of PSMA.³⁸ Interestingly, it is the PSMA dimer that maintains the native conformation and possesses a higher level enzymatic activity than monomeric PSMA.³⁸ These findings provide further rationales in support of multivalent imaging probe design for PSMA detection.

In the absence of antibody binding, PSMA is constitutively internalized at a fairly consistent rate of ~60%.³⁹ Therefore, it is critically important for GPI-based imaging probes to reach the target for imaging signal build-up. In other words, they must be able to out-compete endogenous phosphate anions for PSMA binding. Not surprisingly, phosphate anions showed a strong effect on PSMA binding of both monomeric ⁶⁴Cu-CBT1G and dimeric ⁶⁴Cu-CBT2G (Figure 3C), whereas the dimeric one displayed a significant improvement of PSMA binding when in competition with phosphate anions. This improvement might be attributed to the decreased off-rate of ⁶⁴Cu-CBT2G from PSMA targets.

Given the structural similarities between GPI and other PSMA inhibitors of phosphoramidate peptidomimetics, ⁶⁴Cu-CBT2G observed similar internalization profiles as reported,⁴⁰ which can be characterized by two features: time dependence before reaching the

steady retention state (Figure 3B) and no obvious effect on PSMA internalization.⁴¹ In contrast, anti-PSMA antibodies, such as J591, are capable of accelerating PSMA internalization up to 3 folds in a dose dependent manner.³⁹ Upon the mode of PSMA inhibition ⁶⁴Cu-CBT2G likely exhibited rapidly reversible profiles as 2-PMPA due to the lack of a phosphoramidate P-N linkage in GPI.⁴² This reversible profiles have been suggested less favorable for *in vivo* PSMA targeting,⁴³ which might be another reason of relatively low PSMA⁺ tumor uptake for ⁶⁴Cu-CBT2G.

The imaging signal amplification resulting from the dimeric presentation in ⁶⁴Cu-CBT2G was evaluated in a PSMA⁺ mouse xenograft model established by LNCaP cell line. As shown in Figure 4A, the visual contrast of LNCaP tumors was strikingly enhanced by ⁶⁴Cu-CBT2G as compared to ⁶⁴Cu-CBT1G during the period of study. The corresponding imaging quantification revealed the signal amplification was approximately two-fold at all three imaging time points (Figure 5A). The PSMA imaging specificity was verified by two different methods. One used the conventional blocking approach by co-administering the radiotracer with a blocking dose of a known PSMA inhibitor (Figure 4B); and the other employed two PSMA⁻ tumor xenografts, PC3 and H2009 (Figure 4C). The significant imaging signal reduction as shown in both methods clearly demonstrated the PSMA imaging specificity for both ⁶⁴Cu-CBT1G and ⁶⁴Cu-CBT2G. It is noteworthy that no bone uptake was observed for ⁶⁴Cu-CBT1G or ⁶⁴Cu-CBT2G, indicating that the GPI-based PSMA imaging probes may find potential application for detection of prostate cancer metastases, which are preferentially found in bone. In addition, it was found that the bladder radioactivity was voided at 24 h p.i., while the tumor imaging intensity was maintained with ⁶⁴Cu-CBT2G. This late time point imaging, which was made possible by the reasonably long decay half-life of ⁶⁴Cu, provides an opportunity of imaging localized prostate cancer with ⁶⁴Cu-CBT2G. Both ⁶⁴Cu-CBT1G and ⁶⁴Cu-CBT2G showed an appreciable level of renal uptake. This is not a surprise because the proximal renal tubules express PSMA.¹⁷ Of note, the renal uptake level of ⁶⁴Cu-CBT2G was much lower at all three time points compared to the reported Glu-urea based imaging agents^{15–18, 27} (Figure 5B). This is obvious a desirable feature for PSMA targeted agents.

High sensitivity aside, PET is capable of imaging quantification. Therefore, an ideal PET imaging probe is expected to enable both detection and quantification of a target biomarker. This is of great clinical significance because it offers a noninvasive approach for molecular profiling of disease status, which can potentiate personalized treatment of disease by stratifying patients for tailored therapy. Indeed, endeavors to quantify the PSMA expression with noninvasive imaging approaches have been seen in the literature^{18, 44} as represented by ⁶⁴Cu-J591 enabled PET⁴⁴ and ¹²³I-MIP-1072 enabled SPECT.¹⁸ Given the PSMA detection capability proved for ⁶⁴Cu-CBT2G, we further evaluated its potential to quantitatively delineate the expression level of PSMA in tumors at various stages. In our evaluation, PSMA⁺ LNCaP tumors were allowed to grow to different sizes (weights). Both ⁶⁴Cu-CBT1G and ⁶⁴Cu-CBT2G were used to image tumors. As shown in Figure 6, ⁶⁴Cu-CBT2G displayed a much better linear correlation ($R^2 = 0.92$ at all three time points) between the tumor uptake (%ID/g) and the tumor size than ⁶⁴Cu-CBT1G ($R^2 = 0.01, 0.56, 0.54$ at 1 h, 4 h, and 24 h, respectively). Of note, the unit of %ID/g in Figure 6 is a standardized radiotracer uptake value obtained from the quantitative PET imaging analysis on the assumption that 1 cubic centimeter of tumor tissue equals 1 gram. Given that the target of GPI is PMSA, we believe that the radiotracer concentration (%ID/g) found in LNCaP tumors reflects the PMSA density expressed by the tumors during the course of tumor-growth. Of course, this must be further verified by immunohistochemistry. Interestingly, the slope of the linear correlation, which represents the detection sensitivity and accuracy of PSMA level change, was much greater for ⁶⁴Cu-CBT2G than for ⁶⁴Cu-

CBT1G. This observation clearly indicates the imaging quantification potential of PSMA in prostate cancer when ^{64}Cu -CBT2G is used.

CONCLUSION

A promising PSMA targeted imaging probe, ^{64}Cu -CBT2G, was successfully designed and synthesized by presenting two copies of GPI on a bifunctional chelator scaffold. Our results indicate that the multivalent approach of imaging probe design was able to minimize the endogenous serum phosphate competition that hampers the PSMA binding of GPI and thus afforded the desired imaging sensitivity for *in vivo* detection of PSMA. Given the positive linear relationship observed between the tumor uptake of ^{64}Cu -CBT2G and the tumor size, ^{64}Cu -CBT2G is expected to potentiate the quantitative assessment of PSMA expression in prostate cancer noninvasively.

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ABBREVIATIONS

PSMA	prostate specific membrane antigen
CT	computed tomography
MRI	magnetic resonance imaging
SPECT	single-photon emission computed tomography
PET	positron emission tomography
^{18}F-FDG	2-deoxy-2-(^{18}F)fluoro-d-glucose
FDA	food and drug administration
GPI	2[(3-amino-3-carboxypropyl)(hydroxy)(phosphinyl)-methyl]pentane-1,5-dioic acid)
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
CB-TE2A	2,2'-(1,4,8,11-tetraazabicyclo[6.6.2]-hexadecane-4,11-diyl)diacetic acid
%ID/g	percentage of the injected dose per gram of tissue
MALDI-TOF	Matrix-assisted laser desorption/ionization time of flight
HPLC	high performance liquid chromatography
TLC	thin layer chromatography
CB-TE2A-1DA	2-(11-(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecan-4-yl)pentanedioic acid
CB-TE2A-2DA	2,2'-(1,4,8,11-tetraazabicyclo[6.6.2]hexadecane-4,11-diyl)dipentanedioic acid
FBS	fetal bovine serum
IACUC	Institutional Animal Care and Use Committee

NHS	N-Hydroxysuccinimide
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
TBS	Tris-buffered saline
ROI	regions of interest
2-PMPA	2-(phosphonomethyl)pentanedioic acid)

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FIGURE 1.
Preparation of H₂CBT1G and H₂CBT2G conjugates

FIGURE 2.
Preparation of ^{125}I -GPI

FIGURE 3.

In vitro cell binding assay. (A) The PSMA binding affinities of GPI, H₂CBT1G, and H₂CBT2G were measured by a competitive cell-binding assay using LNCaP cells (PSMA positive) where ¹²⁵I-GPI was employed as the PSMA-specific radioligand. The IC₅₀ values of GPI, H₂CBT1G, and H₂CBT2G, were determined to be 460 ± 13; 578 ± 81; and 129 ± 17 nM, respectively (n = 3). (B) Cell internalization of ⁶⁴Cu-CBT1G and ⁶⁴Cu-CBT2G. (C) Cell uptake kinetics in LNCaP cells with ⁶⁴Cu-CBT1G and ⁶⁴Cu-CBT2G (n = 6) and their non-specific internalization levels by co-incubating with 2 μg of GPI per well (n = 3). (D) LNCaP cell binding assay in TBS buffer, PBS buffer, and cell culture medium. Non-specific binding assay was performed by co-incubating with 2 μg of GPI in TBS buffer. **p* > 0.05; §*p* < 0.05.

FIGURE 4.

Representative whole-body coronal PET/CT images of ^{64}Cu -CBT1G and ^{64}Cu -CBT2G in (A) LNCaP tumor bearing mice at 1, 4 and 24 h p.i.; (B) LNCaP tumor bearing mice at 24 h p.i. with blockade (coadministration with 30 mg/kg of 2-PMPA); and (C) PC3 and H2009 tumor bearing mice at 1 h p.i. All the images are presented at the same signal intensity scale (0 – 2.2 %ID/g).

FIGURE 5. Comparative uptake of ^{64}Cu -CBT1G and ^{64}Cu -CBT2G at 1 h, 4 h, 24 h, and 24 h blockade in LNCaP tumors (A) and kidneys (B) obtained from quantitative imaging analysis. Data are presented as %ID/g $\pm$ s.d. (n = 3).

FIGURE 6.

Correlations between LNCaP tumor uptake and tumor size (mm^3) at 1 h, 4 h, 24 h p.i. A – C: ^{64}Cu -CBT1G; D – F: ^{64}Cu -CBT2G.