68Ga-THP-PSMA: A PET Imaging Agent for Prostate Cancer Offering Rapid, Room-Temperature, 1-Step Kit-Based Radiolabeling

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The clinical impact and accessibility of 68Ga tracers for the prostate-specific membrane antigen (PSMA) and other targets would be greatly enhanced by the availability of a simple, 1-step kit-based labeling process. Radiopharmacy staff are accustomed to such procedures in the daily preparation of 99mTc radiopharmaceuticals. Currently, chelating agents used in 68Ga radiopharmaceuticals do not meet this ideal. The aim of this study was to develop and evaluate preclinically a 68Ga radiotracer for imaging PSMA expression that could be radiolabeled simply by addition of 68Ga generator eluate to a cold kit. **Methods:** A conjugate of a tris(hydroxypyridinone) (THP) chelator with the established urea-based PSMA inhibitor was synthesized and radiolabeled with 68Ga by adding generator eluate directly to a vial containing the cold precursors THP-PSMA and sodium bicarbonate, with no further manipulation. It was analyzed after 5 min by instant thin-layer chromatography and high-performance liquid chromatography. The product was subjected to in vitro studies to determine PSMA affinity using PSMA-expressing DU145-PSMA cells, with their nonexpressing analog DU145 as a control. In vivo PET imaging and ex vivo biodistribution studies were performed in mice bearing xenografts of the same cell lines, comparing 68Ga-THP-PSMA with 68Ga-HBED-CC-PSMA. **Results:** Radiolabeling was complete ($>95\%$) within 5 min at room temperature, showing a single radioactive species by high-performance liquid chromatography that was stable in human serum for more than 6 h and showed specific binding to PSMA-expressing cells (concentration giving 50% inhibition of 361 ± 60 nM). In vivo PET imaging showed specific uptake in PSMA-expressing tumors, reaching 5.6 ± 1.2 percentage injected dose per cubic centimeter at 40–60 min and rapid clearance from blood to kidney and bladder. The tumor uptake, biodistribution, and pharmacokinetics were not significantly different from those of 68Ga-HBED-CC-PSMA except for reduced uptake in the spleen. **Conclusion:** 68Ga-THP-PSMA has equivalent imaging properties but greatly simplified radiolabeling compared with other 68Ga-PSMA conjugates. THP offers the prospect of rapid, simple, 1-step, room-temperature syringe-and-vial radiolabeling of 68Ga radiopharmaceuticals.

Infrastructure in radiopharmacies has been developed around 99mTc-labeling protocols, in which a generator is eluted multiple times per day to produce diverse tracers by reconstituting commercially available cold kits compatible with good manufacturing practice (1). This is typically a high-throughput environment where speed, simplicity, volume, and reproducibility of radiolabeling are paramount. The growth of 18F and 11C PET tracers, with which a kit model is not compatible because of the need for an on-site cyclotron and more complex synthetic chemistry, has spawned more diverse, complex, and costly infrastructure to support PET. Nevertheless, the generator and kit approach retains its appeal and in principle, 68Ga generators compatible with good manufacturing practice now available are amenable to kit production if a simple, mild chelation step can be achieved (2,3). This would make 68Ga tracers widely available without the complex and costly infrastructure associated with 18F and 11C tracer production.

This concept was suggested more than 2 decades ago (4), but despite several recent reports of kit-based 68Ga tracer production (5,6), an ideal 1-step procedure for 68Ga radiolabeling, matching the simplicity typical of long-established 99mTc-labeling procedures requiring only the addition of generator eluate to the kit vial, has not yet been achieved. Toward this end, the chelator must meet several criteria: its labeling should reach completion ($>95\%$) quickly (<5 min) at room temperature and be unaffected by common trace metals, without additional steps to concentrate, buffer, or purify. Its complex should resist in vivo transchelation (e.g., by transferrin), and conjugation and radiolabeling should not induce mixtures of diastereomers, enantiomers, or geometric isomers, nor adverse pharmacokinetics, for example, delayed clearance or nonspecific binding. The current generation of 68Ga chelators do not meet these criteria. For example, the widely adopted macrocycle DOTA (7), while complexing Ga^{3+} with extraordinarily high kinetic stability, has very slow complexing kinetics, necessitating heat (e.g., 90°C, followed by a suitable cooling period), a large amount of the biomolecule, and low pH. Low yields ($<95\%$) necessitate a purification step. These factors add process complexity, limit specific activity, and may damage the

biomolecule. Conversely, the fast chelation rate of HBED-CC makes radiolabeling of ^{68}Ga-HBED-CC-PSMA (PSMA is prostate-specific membrane antigen) possible at room temperature, but produces an undesirable mixture of cis/trans geometric isomers distinguishable by high-performance liquid chromatography (HPLC) (5,8,9). Thus, a heating step is still required, to reduce the number of isomers and increase the yield of 1 to approximately 90% (9). Clinical radiosynthesis of tracers based on these chelators is currently performed on cartridge-based synthesis modules, taking 35 min and typically affording 80% ± 5% decay-corrected radiochemical yield (9).

Recently, several groups have introduced new [68]Ga³⁺ chelators that address these issues but none eliminate all of them. NOTA, TRAP, and DEDPA are promising but, like DOTA, require acidic conditions and are vulnerable to competition from contaminating trace metals. The DATA series of chelators show rapid, room-temperature labeling at pH 5; the DATA^{PPh} variant can be labeled in 15 min at pH 7 but requires preprocessed eluate (10). A class of chelator that promises to meet the requirements for kit-based labeling is the tris(hydroxypyridinone) (THP) system; it can complex ^{68}Ga rapidly at room temperature and neutral pH, with high yield and purity. Its performance has previously been evaluated against a range of common chelators (11), including HBED, demonstrating superior radiolabeling properties under milder conditions. THP has also been functionalized for conjugation to peptides and proteins while retaining the required mild radiolabeling and in vivo targeting properties (11–15).

Here we evaluate a THP bioconjugate targeting PSMA (overexpressed in prostate cancer), incorporating a small urea-linked dipeptide pharmacophore (Fig. 1) (7,8,16). A ^{68}Ga-labeled conjugate of this targeting moiety with HBED-CC has shown outstanding clinical promise in several trials in patients with prostate cancer (17,18) but is subject to the production difficulties outlined above. The aims of this work were to determine the potential of ^{68}Ga-THP-PSMA to achieve 1-step kit-based labeling of a radiopharmaceutical intended for PSMA imaging and to evaluate preclinically the resulting tracer.

MATERIALS AND METHODS

All reagents and consumables were purchased from Sigma Aldrich or Fischer Scientific, with the exception of Fmoc-Lys-Dde-COOH (Bachem AG), PMPA (Enzo Life Sciences), DOTA-PSMA (PSMA-617), and HBED-CC-PSMA DKFZ-11 (ABX). Severe combined immunodeficient (SCID)/beige mice were purchased from Charles River. These cells were cultured in RMPI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin/streptomycin. To prepare for experiments, cells were grown at 37°C in an incubator with humidified air equilibrated with 5% CO₂.

^{1}H- and ^{13}C-NMR spectra were acquired on a Bruker Avance III 700 spectrometer operating at 700 MHz (^{1}H frequency), equipped with a quadruple-resonance QCI cryoprobe. High-resolution liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) was performed in positive-ion mode on an Agilent 6520 Accurate-Mass Q-TOF LC/MS connected to an Agilent 1200 HPLC. All HPLC utilized an Agilent 1200 LC with in-line radio (sodium iodide γ-detector) and ultraviolet detection (220 nm analytical, 280 nm preparative). Data were analyzed using Laura software (version 4.0.2.75; LabLogic Systems Ltd.).

HPLC Methods

The chemical purity of THP-PSMA and radiolabeling of [68/67]Ga-THP-PSMA were assessed using an Agilent Eclipse XDB C₁₈ 5-μm 4.6 × 150 mm column with an isocratic mobile phase (87.5% H₂O, 12.5% acetonitrile [ACN], 0.075% trifluoroacetic acid [TFA], 0.05% triethylamine), with a flow rate of 1 mL/min. [68/67]Ga radiolabeling of HBED-CC-PSMA and DOTA-PSMA was assessed with the same HPLC equipment but an alternative mobile phase: A = H₂O with 0.05% TFA, B = ACN with 0.05% TFA, gradient: 0–5 min = 90% A, 5–15 minutes = ramp to 50% B (4%/min), 15–20 min = 90% A, with a flow rate of 1 mL/min. Serum stability was assessed using size-exclusion chromatography with a Phenomenex BioSep 5-μm size-exclusion chromatography ₛ2000 column (300 × 7.8 mm) with phosphate-buffered saline (PBS) as the mobile phase and a flow rate of 1 mL/min.

Instant Thin-Layer Chromatography (iTLC) Methods

Radiolabeling for all [67/68]Ga-PSMA radiotracers was assessed by iTLC utilizing Varian ITLC SGI0001 strips (10-cm length) with a mobile phase of 1 M ammonium acetate in water/methanol (1:1). iTLC plates were scanned with a Lab-Logic mini-Scan TLC reader and analyzed with Laura software. Radioactivity counting was performed with a γ-counter (LKB Wallac; PerkinElmer) for cell studies and in vivo biodistribution.

Conversion of ^{67}Ga-Citrate Solution to ^{67}Ga-Chloride in 0.1 M HCl

Conversion of ^{67}Ga-citrate to ^{67}Ga-chloride is required because the presence of citrate reduces the radiolabeling efficiency of ^{67}Ga with a number of chelators. ^{67}Ga-citrate (6.49 mM citrate; Mallinckrodt) was passed over a Silica Light Sep-Pak cartridge (120-mg sorbent, 55- to 105-μm particle size) at 1 mL/min to capture the radiometal on the cartridge. This process was repeated 3 times whereupon approximately 90% of the activity remained on the cartridge. After being washed with 5 mL of H₂O, the ^{67}Ga was eluted with 400 μL of 0.1 M HCl and collected in 50-μL fractions. Fractions with the highest activity concentration (75 MBq/50 μL) were used for labeling (activity concentration, 1.5 GBq/mL).

Synthesis of THP-PSMA

Synthesis of H-Lys-(Dde)-2CT-Resin (Compound 3). 2-chlorotrityl resin (350-mg equivalent to a loading of 0.35 mmol) was inserted in a
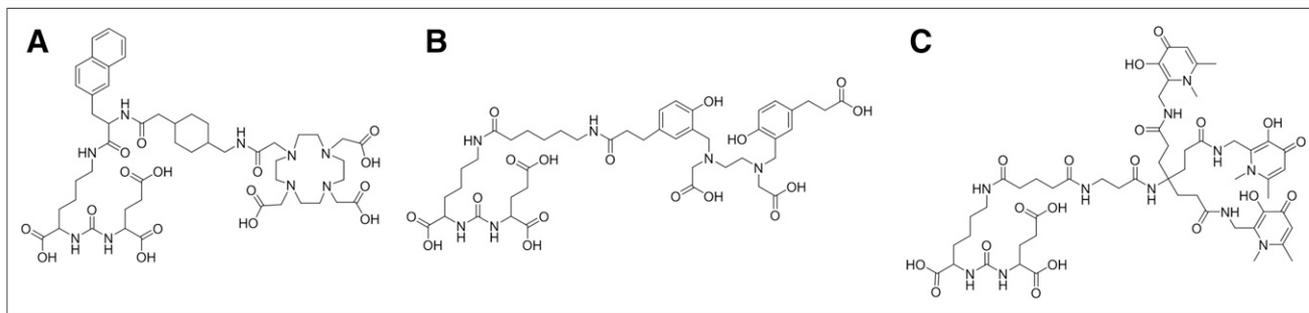

FIGURE 1. Structure of DOTA-PSMA (PSMA-617) (7) (A), HBED-CC-PSMA (DKFZ-PSMA-11) (8,11) (B), and THP-PSMA (C).

fritted polypropylene syringe, and a solution of Fmoc-Lys-Dde-COOH (600 mg, 1.1 mmol) (**1**, Supplemental Fig. 1 [supplemental materials are available at http://jnm.snmjournals.org]) and excess *N,N*-diisopropylethylamine (DIPEA) in dichloromethane (DCM) was added. After being stirred overnight to form compound **2**, the resin was washed with DCM and subsequently treated with methanol for 10 min. Thereafter, the resin was washed with dimethylformamide (DMF) and DCM and finally dried under vacuum. Resuspension of the beads in a solution of piperidine/DMF (20%:80%) for 20 min followed by separation of the resin from the supernatant by suction filtration and washing with DMF and DCM produced resin-bound compound **3**.

Synthesis of Resin-Bound THP-PSMA (Compound 9). Bis(tert-butyl) ester L-glutamate hydrochloride (444 mg, 1.5 mmol) was dissolved in anhydrous DCM (100 mL) containing DIPEA (750 μL, 4.3 mmol). This solution was added dropwise to a 3-neck round bottom flask containing a solution of triphosgene (150 mg, 0.5 mmol) in anhydrous DCM (5 mL) at 0°C under a nitrogen atmosphere. The reaction solution was then allowed to warm to room temperature and stirred for 1 h. The previously prepared H-Lys-(Dde)-2CT-resin (**3**, Supplemental Fig. 1) (250 mg, equivalent to a loading of 0.25 mmol) was added and stirred overnight to form compound **6** (Supplemental Fig. 1). The resin was separated from the supernatant by suction filtration in a fritted polypropylene syringe, washed, and dried. The resin was suspended in a mixture of 2% (v/v) hydrazine hydrate in 2 mL of DMF and stirred for 15 min, and then washed with DMF. This step was repeated 4 times to produce compound **7** (Supplemental Fig. 1). Compound **7** (100-mg equivalent to a loading of 0.1 mmol) was added to a solution of glutaric anhydride (114 mg, 1 mmol) and DIPEA (358 μL, 2 mmol) dissolved in 2 mL of DMF, and the mixture was stirred for 3 h to produce compound **8** (Supplemental Fig. 1). Compound **8** (50 mg, equivalent to a loading of 0.05 mmol) was suspended in a solution of 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (20 mg, 0.05 mmol) and DIPEA (18 μL, 0.1 mmol) in anhydrous DMF (0.5 mL), and in situ activation of the carboxylate was allowed to proceed for 10 min. A solution of THP-NH$_2$ (70 mg, 0.09 mmol), synthesized by a previously published method (*12–14*), in DMF/DMSO (2.5 mL/2 mL) containing DIPEA (18 μL, 0.1 mmol) was then added to this suspension and stirred for 36 h, to furnish compound **9** (Supplemental Fig. 1).

Cleavage of THP-PSMA (Compound 10) from Resin. Cleavage and removal of the side-chain protecting groups were performed by treating the resin-bound compound **9** with TFA for 3 h at room temperature in the presence of phenol (5% w/v), water (5% v/v), and triisopropylsilane (2% v/v) as scavengers. The filtrate was collected and the resin washed with TFA and DCM. The solution was concentrated to less than 0.5 mL at 40°C under a gentle stream of nitrogen. Ice-cold diethyl ether was added to precipitate THP-PSMA (**10**, Supplemental Fig. 1). The suspension was centrifuged, supernatant decanted off, precipitate washed with diethyl ether, and finally dissolved in aqueous ACN and lyophilized. Crude THP-PSMA was purified by semipreparative reversed-phase HPLC to give a TFA salt ($C_{54}H_{77}N_{11}O_{19}$.(CF$_3$COO)$_3$) (molecular weight, 1,526.33). Semipreparative reversed-phase HPLC was conducted using an Agilent Eclipse XDB C$_{18}$ 5-μm 21.2 × 150 mm column with the concentration of mobile phase B increasing at a rate of 1%/min (A = H$_2$O with 0.2% TFA, B = ACN with 0.2% TFA, starting from 100% A at time 0; flow rate, 5 mL/min). The yield of THP-PSMA(CF$_3$COO)$_3$ was 4 mg, 2.6 μmol, 5.2% yield from resin loading. The analytical HPLC R_t was 13 m 23 s, and purity was greater than 98%. The "Results" section provides analytical data.

Preparation of Lyophilized Kits

Kits for 1-step radiolabeling were prepared by lyophilizing an aqueous solution (5.25 mL) containing sodium bicarbonate (44 mg), sodium phosphate monobasic (8.6 mg), sodium phosphate dibasic

heptahydrate (8.9 mg), and THP-PSMA (40 μg, 26 nmol) in a plastic vial affording a white powder.

^{68}Ga-THP-PSMA Radiolabeling

Radiolabeling and radioanalysis (HPLC and iTLC) of $^{67/68}$Ga-DOTA-PSMA (PSMA-617) (*8*), $^{67/68/nat}$Ga-HBED-CC-PSMA (DKFZ-PSMA-11) (*9,10*), and $^{67/nat}$Ga-THP-PSMA were performed as follows.

Initial ^{68}Ga radiolabeling optimization was performed with an Eckert and Ziegler (E&Z Radiopharma GmbH) ^{68}Ge/^{68}Ga generator, producing 120–400 MBq of ^{68}Ga. Eluates (0.1 M HCl, 5 mL [Sigma Aldrich], HPCE grade) were fractionated (10 × 0.5 mL) but not preconditioned to concentrate ^{68}Ga or remove trace metal contaminants. Typically, ^{68}Ga^{3+} (5–75 MBq, 250 μL of the hottest fraction) was added to a mixture containing THP-PSMA in a range of concentrations (5–0.01 μg in 3 μL) in sodium bicarbonate solution (1 M, 26 μL), producing a solution with a pH of 6.5–7.5. Radiochemical yield was evaluated after 5 min at room temperature using HPLC and iTLC (HPLC: ^{68}Ga-THP-PSMA R_t = 10.9 min, unbound ^{68}Ga R_t = 2.32 min; iTLC: ^{68}Ga-THP-PSMA R_f = 0.8–1, unbound ^{68}Ga R_f = 0). For in vivo studies, THP-PSMA (2 μg) was labeled with ^{68}Ga as described above; a greater than 95% radiochemical purity was consistently achieved.

To evaluate labeling using the entire eluate without fractionation, E&Z ^{68}Ge/^{68}Ga generator eluate (5 mL 0.1 M HCl, 122–202 MBq) or Galli E$_O$ (IRE ELiT) ^{68}Ge/^{68}Ga generator eluate (1.1 mL eluate diluted to 5 mL with 0.1 M HCl, 600–660 MBq) was added directly into a vented freeze-dried kit vial. After being mixed, carbon dioxide evolution visibly ceased after 15 s, providing a transparent, colorless solution at pH 6–7 with a final concentration of 5.25 μM THP-PSMA. iTLC was performed 5 min and HPLC 10 min after reconstitution. Decay-corrected radiochemical yield determined by each method was greater than 95% (n = 3 per generator).

Preparation of ^{67}Ga and ^{68}Ga PSMA Tracers for Biological Evaluation. For ^{67}Ga-THP-PSMA for in vitro studies, 10 μL of a 2 mg/mL solution (20 μg, 13.1 nmol THP-PSMA [molecular weight, 1,526.33]) was added to 50 μL of ^{67}Ga in 0.1 M HCl (converted from ^{67}Ga citrate) and the pH adjusted to 6.5–7.5 with 4.5 μL of 1 M sodium bicarbonate. Labeling was assessed after 5 min at room temperature by iTLC and HPLC. Before being added to cells, the tracer was diluted to 50 nM in PBS.

For DOTA-PSMA for in vitro studies, 50 μL of ^{67}Ga or ^{68}Ga in 0.1 M HCl, 5.25 μL of 1 M HCl, and 20 μL of 2.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were combined, producing a solution at pH 3.5. For ^{67}Ga labeling, 20 μg (13.3 nmol) of DOTA-PSMA (ABX; molecular weight, 1,498.18) were added; for ^{68}Ga labeling, 2 μg (1.3 nmol) of DOTA PSMA were added. The solution was heated to 95°C for 30 min, and then labeling was assessed by iTLC and HPLC. Before being added to cells, the tracer was diluted to 50 nM in PBS.

For HBED-CC-PSMA for in vitro studies, 50 μL of ^{67}Ga or ^{68}Ga in 0.1 M HCl, 5 μL of 1 M HCl, and 50 μL of 2.1 M HEPES were combined, producing a solution at pH 4.5. For ^{67}Ga labeling, 29 μg (20.6 nmol) of HBED-CC-PSMA (ABX; molecular weight, 1,403.09) were added; for ^{68}Ga labeling, 2 μg (1.4 nmol) of HBED-CC-PSMA were added. The solution was heated to 95°C for 10 min, and then labeling was assessed by iTLC and HPLC. Before being added to cells, the tracer was diluted to 50 nM in PBS.

For HBED-CC-PSMA for in vivo studies, ^{68}Ga eluate (200 μL, 35–50 MBq) was added to a pre-prepared solution of HBED-CC-PSMA (15 μL, 105 μM, 2.2 μg of HBED-CC-PSMA) and sodium bicarbonate (19 μL, 1 M) and heated at 90°C for 10 min (pH 3–4). A radiochemical yield of greater than 95% was consistently achieved.

Preparation of ^{nat}Ga Chelated PSMA Tracers. For ^{nat}Ga-THP-PSMA, 124 μL of 0.93 mM THP-PSMA were added to 93 μL of 6.4 mg/mL ^{nat}Ga(NO$_3$)$_3$.X(H$_2$O) dissolved in 0.1 M HCl (a 20-fold excess of gallium

over THP-PSMA). The pH was adjusted to 6.5–7.5 with 14 μL of 1 M sodium bicarbonate, and 231 μL of PBS were then added. The solution was sonicated for 15 min, producing a final solution of 0.25 mM natGa-THP-PSMA. This solution was diluted in PBS to produce the range of concentrations required for the concentration giving 50% inhibition (IC_{50}). This complex was characterized with LC-ESI-MS: m/z $[C_{54}H_{74}N_{11}O_{19}Ga + H]^+$ observed monoisotopic peak = 1250.45092, calculated = 1250.44909. $[C_{54}H_{74}N_{11}O_{19}Ga + 2H]^{2+}$ observed monoisotopic peak = 625.72846, calculated = 625.72818.

For natGa-HBED-CC-PSMA, 124 μL of 0.93 mM HBED-CC-PSMA were added to 93 μL of 6.4 mg/mL natGa(NO₃)₃.X(H₂O) dissolved in 0.1 M HCl (a 20-fold excess of gallium over HBED-CC-PSMA). The pH was adjusted to 4 with 13 μL of 1 M sodium bicarbonate, and the solution was heated to 95°C for 10 min. The pH was adjusted to 6.5–7.5 with 1 M sodium bicarbonate followed by sonication for 15 min, producing a final solution of 0.5 mM natGa-HBED-CC-PSMA. This solution was diluted in PBS to produce the range of concentrations required for the IC_{50}.

Log P_OCT/PBS

[67]Ga-labeled radiotracer (50 μL, 10 μM; specific activity, 1.5 MBq/nmol) was added to a preequilibrated mixture of 500 μL of octanol (OCT) and 450 μL of PBS. The mixture was mixed for 30 min and the phases separated by centrifugation (10,000 rpm, 10 min). Aliquots from each phase were γ-counted.

Serum Stability

^{68}Ga-THP-PSMA was labeled at 5 MBq/nmol as described above. Twenty microliters were added to 180 μL of human serum, giving a final THP-PSMA concentration of 2.5 μM. Samples were incubated at 37°C and monitored over 6 h by size-exclusion HPLC. ^{68}Ga-THP-PSMA without serum and unchelated [68]Ga³⁺ incubated with serum were analyzed similarly.

Cell Uptake and Binding Affinity Assays

To determine the cellular uptake of each tracer at 37°C and 4°C over time, and whether a plateau (equilibrium) state is reached, PSMA-expressing cells DU145-PSMA and non–PSMA-expressing cells DU145 (19) were seeded in a 24-well plate (0.25×10^6 cells/well), 1 d before the assay. Two minutes before incubation, the medium was replaced with 245 μL of fresh medium at 37°C or 4°C, then 5 μL of one of the ^{67}Ga PSMA tracers (specific activity, 0.75–2.2 MBq/nmol) were added, giving a final concentration of 1 nM. Plates were incubated at 37°C or 4°C. At each time point, supernatant was removed and cells were washed with 3×0.25 mL of PBS to determine the unbound fraction, followed by an acid wash (0.5 M glycine, pH 2.5, at 4°C, 5 min) to determine cell surface–bound activity. Cells were lysed with 1 M NaOH to determine activity internalized by the cells. Fractions were γ-counted.

To determine the IC_{50}, competitive binding studies were performed with DU145-PSMA cells with 1 nM ^{68}Ga-DOTA-PSMA as the probe, blocking with natGa-THP-PSMA or natGa-HBED-CC-PSMA over a range of concentrations. Cells (0.25×10^6 per well) were seeded in a 24-well plate, 1 d before the assay. Increasing concentrations of natGa-THP-PSMA or natGa-HBED-CC-PSMA followed by 1 nM ^{68}Ga-DOTA-PSMA (specific activity, 5–7.5 MBq/nmol, diluted in PBS) were added to the cells (total volume, 250 μL). After 30-min incubation at 37°C, the cells were washed with PBS (3×0.25 mL), lysed with NaOH (1 M, 0.25 mL), and the wells washed with PBS (0.25 mL). The activity present in supernatant and lysate was measured by γ-counting. Data were analyzed using GraphPad Prism (GraphPad Software) and a 1-site–fit log IC_{50} algorithm.

Because of the poor solubility of natGa-THP-PSMA above 0.25 mM, an alternative measure of affinity was developed, allowing the relative affinity of 2 gallium PSMA tracers to be determined simultaneously without the natGa complex, mitigating solubility difficulties and minimizing variance across samples arising from different cell numbers or radiotracer batches. Two different PSMA tracers, one labeled with ^{67}Ga and the other with ^{68}Ga, were simultaneously incubated with DU145-PSMA cells in a single well at 4°C for 2 h (noninternalizing conditions selected to best represent the equilibrium state from the cell uptake studies). The tracers compared were [67/68]Ga-DOTA-PSMA, [67/68]Ga-HBED-CC-PSMA, and [67/68]Ga-THP-PSMA. Each ^{67}Ga tracer was compared with each ^{68}Ga tracer, and affinity ratios were obtained by measuring total and nonspecific binding for each tracer (γ-counting) and calculating the ratio of their specific binding.

Mouse Model of Prostate Cancer

Animal studies complied with the guideline on responsibility in the use of animals in bioscience research of the U.K. Research Councils' and Medical Research Charities', under U.K. Home Office project and personal licenses. Subcutaneous prostate cancer xenografts were produced in SCID/beige mice (male, 5–12 wk) by injecting 4×10^6 DU145-PSMA or DU145 cells in the right flank. Imaging was performed once the tumor had reached 5–10 mm in diameter (1–4 wk after inoculation).

PET Scanning

PET imaging was performed on 4 groups of mice ($n = 3$ each) under isoflurane anesthesia with a BioScan nanoPET-CT PLUS (Mediso). Mice were imaged with CT before radiotracer administration, and dynamic PET data were collected for the first hour after injection. Mice were then euthanized and organs harvested, weighed, and γ-counted. Mice bearing DU145-PSMA tumors were administered either ^{68}Ga-THP-PSMA (5–15 MBq, 50–140 μL, 0.4–0.9 μg, group 1) or ^{68}Ga-HBED-CC-PSMA (5–15 MBq, 50–140 μL, 0.6–1.3 μg, group 2) by tail vein injection. A third group (group 3) was coadministered ^{68}Ga-THP-PSMA and the PSMA-inhibitor 2-(phosphonomethyl)pentane-1,5-dioic acid (PMPA) (50 μg, Enzo Life Sciences) (20,21). Mice bearing DU145 tumors were administered ^{68}Ga-THP-PSMA (group 4).

Imaging Protocol

PET/CT Acquisition. The PET/CT scans were obtained on a BioScan nanoPET-CT PLUS (Mediso) scanner using their proprietary acquisition software (Nucline, version 2.00). CT was performed with an x-ray tube voltage of 45 kVp, 600 ms of exposure time, and 360° projections. This scan took 10 min to obtain. Dynamic PET scans were acquired within a 94.7-mm field of view from 0 to 60 min after tail vein injection of the ^{68}Ga-PSMA tracer. Acquisition took place in 1–5 coincidence mode with a coincidence window of 5 ns and a 400- to 600-keV energy window. The dynamic PET data were reconstructed using Nucline software (version 2.00). Data were allocated to both 20- and 2-min bins using 0.4 mm³ voxels for PET and 0.21 mm³ for CT. Image processing and analysis were performed using Vivoquant software (version 1.23). Before analysis, both CT and PET images were realigned and processed to a voxel size of 0.21 mm³ and the PET output calibrated to display MBq per voxel. Regions of interest (ROIs) for each data file were produced using 3 different techniques: fixed-volume ROIs, Otsu thresholding, and freehand segmentation from the CT image. Fixed-volume ROIs were used for muscle (17.2 mm³ sphere within the thigh muscle) and blood pool (2.2 mm³ sphere in left ventricle). These ROIs were positioned manually and drawn in triplicate within each organ. Otsu thresholding was used to determine the total activity deriving from the kidneys or the bladder. This method was not suitable for tumor uptake because of the close proximity of kidney signal. Total tumor volume was drawn manually from the CT image, defined 3 times per animal, and the average value from these 3 ROIs determined. Percentage injected dose (%ID) per cubic centimeter (%ID/cm³) values for each ROI were calculated using the activity and volume of the ROI and the ID as the total activity within the image, excluding activity within the tail. Time–activity

curves were produced from the 2-min bins, and total uptake was determined from the 20-min binned data at the 40- to 60-min time point. Static images were produced from dynamic data between 40 and 60 min and scaled between 0 and 25 %ID/cm^3.

Biodistribution Studies

Additional ^{68}Ga (THP-PSMA) biodistribution and blocking studies were performed in mice bearing DU145 or DU145-PSMA tumors without imaging or continuous anesthesia. ^{68}Ga (THP-PSMA) (3.5–12.7 MBq, 0.7–0.9 μg THP-PSMA) was administered via tail vein injection under isoflurane anesthesia. After injection anesthesia ceased and 1 h after injection the animals were euthanized by cervical dislocation and organs harvested, weighed, and counted ($n = 3$ for each group).

Statistical Analysis

Data were analyzed in GraphPad Prism 5 (version 5.04) and expressed as mean ± SD. Student t tests were used to determine statistical significance, with a P value of less than 0.05 considered significant.

RESULTS

Synthesis of THP-PSMA

Supplemental Figure 1 shows the route used to synthesize THP-PSMA. The resin-bound PSMA inhibitor (compound **7**) was prepared by in situ formation of a bis(tert-butyl) glutamate isocyanate (compound **5**) followed by coupling with resin-bound protected lysine (compound **3**). After deprotection and coupling with glutaric anhydride, the intermediate bearing a pendant carboxylate (compound **8**) was activated and formed an amide bond with the free amine of a THP derivative (22). Cleavage from the solid support and glutamate deprotection using trifluoro acetic acid formed THP-PSMA (Fig. 1). Purification by semipreparative HPLC afforded THP-PSMA as a trifluoro acetic acid salt, yield 4 mg, 2.6 μmol, 5.2% yield from resin loading; ($C_{54}H_{77}N_{11}O_{19}.(CF_3COO)_3$) molecular weight, 1526.33, HPLC: $R_t = 13.4$ min, greater than 98% purity. LC-ESI-MS, ^{1}H, and ^{13}C NMR data are shown in Supplemental Table 1.

Radiolabeling

The THP chelator enabled radiolabeling with unmodified generator eluate, in a single step. ^{68}Ga^{3+} (5–75 MBq) in aqueous HCl (0.1 M, 250 μL) was added to preprepared THP-PSMA (5–0.01 μg in 3 μL of H$_2$O) in sodium bicarbonate solution (1 M, 27 μL). After 5 min, pH was 6.5–7.5. Radiochemical purity, determined by HPLC and iTLC as a function of THP-PSMA concentration is shown in Figure 2. Specific activities between 15 and 45 MBq/nmol were consistently achieved with the labeling conditions used for in vivo work (2 μg, 1.3 nmol THP-PSMA, 250 μL 20–60 MBq of ^{68}Ga eluate, >95% radiochemical purity).

1-Step Kit for Radiolabeling

When kit vials containing lyophilized THP-PSMA (40 μg, 26 nmol), sodium bicarbonate (44 mg), and sodium phosphate buffer (17.5 mg) were used, radiosynthesis of ^{68}Ga-THP-PSMA was achieved in 1 step by direct addition of 5 mL of generator eluate (0.1 M HCl, 122–202 MBq from the E&Z generator or 600–660 MBq from the IRE generator). After 5 min, the pH was 6–7 and iTLC confirmed radiochemical purity above 95% with specific activities of up to 22 MBq/nmol using the IRE generator (Fig. 2; Supplemental Fig. 2). iTLC analysis up to 3 h after reconstitution showed no instability or autoradiolysis (Supplemental Fig. 2).

Lipophilicity and Serum Stability

The log $P_{OCT/PBS}$ values of the 3 ^{68}Ga-PSMA complexes at pH 7.4 were similar: -5.35 ± 0.1 for ^{67}Ga-THP-PSMA ($n = 6$), -5.40 ± 0.2 for ^{67}Ga-HBED-CC-PSMA ($n = 6$), and -5.40 ± 0.1 for ^{67}Ga-DOTA-PSMA ($n = 5$), indicating that all tracers are hydrophilic and lipophilicity is unlikely to underlie differences in in vivo performance. In serum, ^{68}Ga-THP-PSMA (Supplemental Fig. 3) showed minimal transchelation (<2%) to proteins after 6-h incubation.

In Vitro Cell Uptake and Binding Affinity Assays

Uptake of ^{67}Ga-DOTA-PSMA, ^{67}Ga-HBED-CC-PSMA, and ^{67}Ga-THP-PSMA in DU145-PSMA and DU145 cells at 37°C and 4°C is shown in Figures 3A and Supplemental Figure 4. All 3 tracers showed time-dependent accumulation in PSMA-expressing cells, but low uptake in non–PSMA-expressing cells, confirming that uptake is PSMA-mediated. At 37°C, uptake continued to increase with time, preventing measurement of equilibrium binding parameters such as the dissociation constant (K_d), but at 4°C, a plateau was reached for all tracers after approximately 2 h. Relative affinity was therefore measured after 2 h at 4°C. Results showed that the K_d of $^{67/68}$Ga-THP-PSMA was 10.4 ± 3.4 times higher than that of $^{67/68}$Ga-HBED-CC-PSMA and 8.6 ± 3.4 times that of $^{67/68}$Ga-DOTA-PSMA, in agreement with directly measured IC$_{50}$ values of Ga-THP-PSMA (361 ± 60 nM) and Ga-HBED-CC-PSMA (34.3 ± 4.1 nM) (Fig. 3B; Supplemental Fig. 5).

PET Imaging and Biodistribution

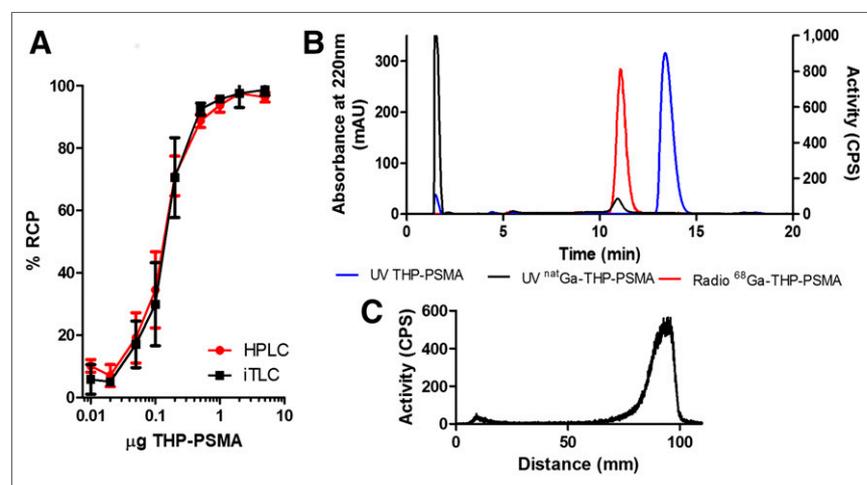PET/CT scanning of ^{68}Ga-THP-PSMA in SCID/beige mice bearing PSMA-positive

FIGURE 2. (A) Dependence of radiochemical purity of ^{68}Ga-THP-PSMA on mass of THP-PSMA in 280 μL, as measured by HPLC (red) and iTLC (black) after 5 min; $n = 3$, mean ± SD. (B) HPLC (λ = 220 nm) of THP-PSMA (blue, R_T = 13.38 min) and ^{nat}Ga-THP-PSMA (black, R_T = 10.92 min, excess Ga(NO$_3$)$_3$ is present with R_T = 1.47 min), and radio-HPLC of ^{68}Ga-THP-PSMA (red, R_T = 11.05 min) labeled using 1-step kit and analyzed 10 min after reconstitution. (C) iTLC of ^{68}Ga-THP-PSMA labeled using 1-step kit, analyzed 5 min after reconstitution (Rf unchelated ^{68}Ga = 0, Rf ^{68}Ga-THP-PSMA = 0.8–1).
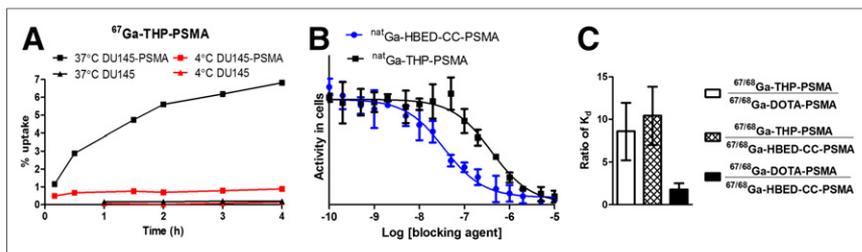
FIGURE 3. (A) ^{67}Ga-THP-PSMA uptake over time at 4°C and 37°C, 1×10^6 cells/mL with DU145-PSMA or DU145 cells. (B) Representative IC_{50} experiment for ^{nat}Ga-THP-PSMA (black) or ^{nat}Ga-HBED-CC-PSMA (blue) with 1 nM ^{68}Ga-DOTA-PSMA as probe ($n = 3$ for each concentration). IC_{50} values in main text are mean of at least 3 experiments. (C) Ratio of K_d of 2 tracers incubated at 1 nM with DU145-PSMA cells at 4°C for 2 h. Ratios were calculated from specific binding obtained by incubating ^{67}Ga version of tracer in same well as ^{68}Ga version of its comparator, and vice versa. Total wells ($n = 34$) for comparison of $^{67/68}$Ga-THP-PSMA with $^{67/68}$Ga-HBED-CC-PSMA or $^{67/68}$Ga-DOTA-PSMA and $n = 18$ for comparison of $^{67/68}$Ga-HBED-CC-PSMA with $^{67/68}$Ga-DOTA-PSMA; mean ± SD.

xenografts (DU145-PSMA, group 1) showed that excretion of ^{68}Ga-THP-PSMA was rapid and exclusively renal, with most activity associated with bladder and kidneys by 60 min (Fig. 4; Supplemental Fig. 6). Blood clearance, represented by blood pool in the left ventricle, was rapid, decreasing to 1 %ID/cm^3 within 30 min (Fig. 5B). With images scaled between 0 and 25 %ID/cm^3, tumors were clearly delineated (Fig. 4). Analysis of the 40- to 60-min postinjection images gave a tumor uptake value of 5.6 ± 1.2 %ID/cm^3 (Fig. 5B). Specificity was confirmed both by mice bearing a PSMA-negative but otherwise similar tumor (DU145, group 4) with uptake of 1.5 ± 1.2 %ID/cm^3 ($P < 0.05$ compared with group 1), and by blocking experiments in mice bearing a DU145-PSMA tumor where PMPA was coadministered with ^{68}Ga-THP-PSMA (group 3), giving a tumor uptake of 1.0 ± 0.4 %ID/cm^3 ($P < 0.05$ compared with group 1).

For comparison with an established PET tracer, mice bearing DU145-PSMA tumors were imaged with ^{68}Ga-HBED-CC-PSMA (group 2) (8,9,17). Time–activity curves for ^{68}Ga-HBED-CC-PSMA (group 2) and ^{68}Ga-THP-PSMA (group 1) showed similar blood clearance, tumor uptake, and renal excretion (Fig. 5). Image

analysis of 40- to 60-min PET images revealed that tumor uptake of ^{68}Ga-THP-PSMA (5.3 ± 0.1 %ID/cm^3) was not significantly different from that of ^{68}Ga-HBED-CC-PSMA (5.6 ± 1.2 %ID/cm^3).

Ex vivo biodistribution data, summarized in Figure 5C and Supplemental Table 2, were consistent with PET image analysis, confirming renal excretion and excellent specificity of ^{68}Ga-THP-PSMA for PSMA-expressing tumors. ^{68}Ga-THP-PSMA showed ex vivo biodistribution similar to ^{68}Ga-HBED-CC-PSMA, apart from markedly lower spleen uptake (3.7 ± 1.3 and 17.6 ± 6.1 %ID dose per gram, respectively). Importantly, anesthesia appeared to severely affect tracer uptake in the kidney and excretion to bladder, with large variation in kidney uptake across all groups, so comparison of kidney and bladder activity should be interpreted with caution.

DISCUSSION

The aim of this study was to develop a ^{68}Ga radiotracer that targets PSMA with simple and high-yielding radiolabeling procedures suitable for development into a single-step kit-formulated radiopharmaceutical compatible with good manufacturing practice, requiring only addition of unprocessed, unfractionated generator eluate to a single vial. The latter requirement was met by incorporating THP. A THP-PSMA conjugate has been synthesized and characterized, and could be readily radiolabeled with ^{68}Ga (and ^{67}Ga). Radiolabeling yields of greater than 95% and specific activity of 15–45 MBq/nmol with unprocessed generator-produced ^{68}Ga were achieved in 1 step at pH 7 and ambient temperature within 5 min without further purification. On the basis of this, we developed a lyophilized kit, which can be labeled/reconstituted simply by adding 5 mL of raw generator eluate. 1-step kits can be used only with generators with less than 0.001% ^{68}Ge breakthrough; of these, the E&Z has the largest elution volume (5 mL, 0.1 M HCl) and the kit was designed accordingly.

Within the range used, specific activity was limited only by the activity available from the generator; the highest observed was 22 MBq/nmol with an elution of 660 MBq in 5 mL. Higher specific activities have been obtained in a clinical setting and will be reported alongside first-in-human studies. These radiolabeling properties demonstrate that kit-based radiolabeling of ^{68}Ga tracers, analogous to the simple manipulations to which radiopharmacy staff producing ^{99m}Tc radiopharmaceuticals are accustomed, and requiring equipment no more complex than a shielded syringe and vial, is entirely feasible with an appropriate chelator. As well as rapid and simple radiolabeling, the coordination properties of THP endow its conjugates with other properties well suited to radiopharmaceutical application. It is highly selective for tripositive metal ions with ionic radius similar to that of Fe^{3+} and Ga^{3+} (23), potentially reducing the need
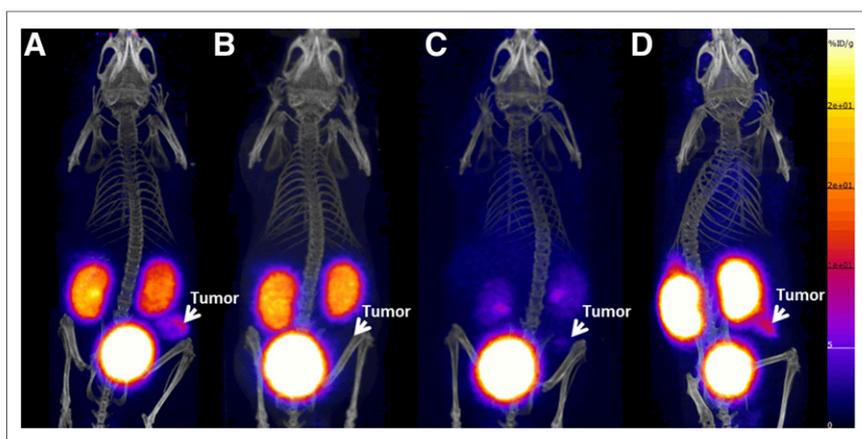

FIGURE 4. Representative PET/CT images of mice bearing xenografts at 40–60 min after injection with PET images scaled from 0%–25 %ID/cm^3. (A) ^{68}Ga-THP-PSMA in DU145-PSMA tumor (group 1). (B) ^{68}Ga-THP-PSMA in DU145 tumor (group 4). (C) ^{68}Ga-THP-PSMA in DU145-PSMA tumor blocked with PMPA (group 3). (D) ^{68}Ga-HBED-CC-PSMA in DU145-PSMA tumor (group 2).
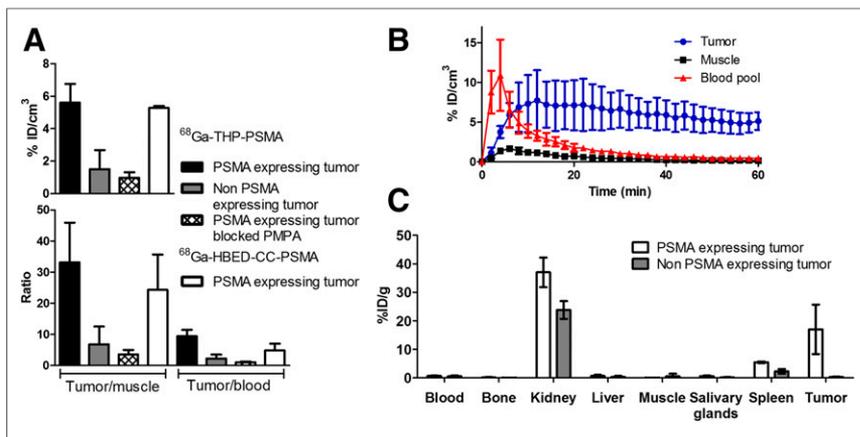
FIGURE 5. (A) PET/CT-derived %ID/cm³ in tumor and tumor-to-blood and tumor-to-muscle ratio 40–60 min after injection. ⁶⁸Ga-THP-PSMA in DU145-PSMA tumor (black), ⁶⁸Ga-THP-PSMA in DU145-PSMA tumor blocked with PMPA (hatched), ⁶⁸Ga-THP-PSMA in DU145 tumor (gray), or ⁶⁸Ga-HBED-CC-PSMA in DU145-PSMA tumor (white) (n = 3, mean ± SD). (B) PET-derived time–activity curves of mice bearing DU145-PSMA tumors, imaged with ⁶⁸Ga-THP-PSMA for 1 h after injection: blood pool (left ventricle) (red), tumor (blue), and leg muscle (black); (n = 3, mean ± SD). (C) Ex vivo biodistribution of ⁶⁸Ga-THP-PSMA in mice with DU145-PSMA (white) or DU145 tumor (gray), culled 1 h after injection (n = 3, mean ± SD). Unlike PET-imaged animals, these were not anesthetized between injection and euthanasia.

to remove contaminating metal ions in raw generator eluate. Unlike HBED-CC, the tripodal design of THP restricts the number of geometric isomers that form on Ga³⁺ coordination: on reversed-phase HPLC, ⁶⁸Ga-THP-PSMA elutes as a single radioactive species (Fig. 2B) shown by LC-MS to be a 1:1 complex with gallium (Supplemental Table 1). Any isomerism (such as the Δ/Λ isomerism possible with tripodal complexes) is subject to equilibration that is rapid compared with the timescale of HPLC and in vivo processes, and hence biologically irrelevant. The lipophilicity is low and comparable to that of established ⁶⁸Ga-PSMA ligands.

Although THP-PSMA was rationally designed on the basis of previous literature (8,21,24,25), we have not yet attempted to optimize the specific target affinity of THP-PSMA bioconjugates. In vitro experiments demonstrate that ⁶⁸Ga-THP-PSMA binds specifically to PSMA, although with weaker affinity than ⁶⁸Ga-HBED-CC-PSMA and ⁶⁸Ga-DOTA-PSMA. The simplicity of radiolabeling, excellent serum stability, and in vitro PSMA binding justified further evaluation of the new tracer in vivo, which showed that incorporation of THP into bioconjugates confers no THP-specific adverse pharmacokinetics. The rapid renal excretion, low nonspecific uptake of ⁶⁸Ga-THP-PSMA in nontarget tissues, and high, specific radioactivity concentration in PSMA-expressing tumors are clinically desirable features. In the preclinical model used here, tumor uptake and pharmacokinetic properties of ⁶⁸Ga-THP-PSMA and ⁶⁸Ga-HBED-CC-PSMA are indistinguishable except that spleen uptake of ⁶⁸Ga-THP-PSMA is lower by almost a factor of 5. Although PSMA-targeting radiopharmaceuticals generally appear to share high uptake in spleen in mice, murine spleen is not believed to express PSMA (26). It seems likely therefore that some other target capable of binding the PSMA tracers is present in the spleen and that ⁶⁸Ga-THP-PSMA has enhanced ability to distinguish PSMA from this alternative target.

CONCLUSION

Use of THP as the ⁶⁸Ga³⁺ chelator facilitates rapid chelation under mild conditions and produces a PSMA-targeted bioconjugate

that can be labeled in 1 step by reconstitution of a kit with unprocessed generator eluate. Labeling requires only a generator, a cold-kit vial, a syringe, quality control facilities, and shielding. Kit-based labeling can be performed in a few minutes, using the full volume of unprocessed generator eluate (5 mL), without postsynthesis purification, achieving a greater than 95% radiochemical purity. In vivo, ⁶⁸Ga THP-PSMA accumulates in PSMA-expressing tumors, with good tumor-to-background ratio delineation of PSMA-positive tumor lesions similar to ⁶⁸Ga-HBED-CC-PSMA. Kit-based radiolabeling of ⁶⁸Ga radiopharmaceuticals is feasible with THP and would facilitate wider and more economical use of ⁶⁸Ga in hospitals, hence benefitting more patients.

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