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⁶⁸Ga-THP-PSMA: a PET imaging agent for prostate cancer offering rapid, room temperature, one-step kit-based radiolabeling

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Running title: ^{68}Ga -THP-PSMA: Prostate cancer imaging

ABSTRACT

The clinical impact and accessibility of ^{68}Ga tracers for the prostate-specific membrane antigen (PSMA) and other targets would be greatly enhanced by the availability of a simple, one-step kit-based labeling process. Radiopharmacy staff are accustomed to such procedures in the daily preparation of $^{99\text{m}}\text{Tc}$ radiopharmaceuticals. Currently, chelating agents used in ^{68}Ga radiopharmaceuticals do not meet this ideal. **Aim:** To develop and evaluate preclinically a ^{68}Ga radiotracer for imaging PSMA expression that could be radiolabeled simply by addition of ^{68}Ga 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 ^{68}Ga 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 (iTLC) and high performance liquid chromatography (HPLC). The product was subjected to *in vitro* studies to determine PSMA affinity using PSMA-expressing DU145-PSMA cells, with their non-expressing analog DU145 as a control. *In vivo* positron emission tomography (PET) imaging and *ex vivo* biodistribution studies were carried out in mice bearing xenografts of the same cell lines, comparing with ^{68}Ga -HBED-CC-PSMA. **Results:** Radiolabeling was complete (>95%) within 5 min at room temperature, showing a single radioactive species by HPLC that was stable in human serum for >6 hours and showed specific binding to PSMA-expressing cells (IC_{50} of 361 ± 60 nM). *In vivo* PET imaging showed specific uptake in PSMA-expressing tumors, reaching 5.6 ± 1.2 % ID/ cm^3 at 40-60 min

and rapid clearance from blood to kidney and bladder. The tumor uptake, biodistribution and pharmacokinetics were not significantly different to those of ^{68}Ga -HBED-CC-PSMA except for reduced uptake in the spleen. **Conclusion:** ^{68}Ga -THP-PSMA has equivalent imaging properties but greatly simplified radiolabeling compared to other ^{68}Ga -PSMA conjugates. THP offers the prospect of rapid, simple, one-step, room temperature “syringe-and-vial” radiolabeling of ^{68}Ga radiopharmaceuticals.

KEY WORDS: PSMA receptor, PET imaging, gallium chelators, prostate cancer, radiopharmaceutical kit.

INTRODUCTION

Infrastructure in radiopharmacies has been developed around ^{99m}Tc labeling protocols, where 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 ^{18}F and ^{11}C PET tracers, with which a kit model is not compatible due to 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, ^{68}Ga 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 ^{68}Ga tracers widely available without the complex and costly infrastructure associated with ^{18}F and ^{11}C tracer production.

This concept was suggested more than two decades ago (4), but despite several recent reports of kit-based ^{68}Ga tracer production (5,6), an ideal one-step procedure for ^{68}Ga radiolabeling, matching the simplicity typical of long-established ^{99m}Tc -labeling procedures requiring only the addition of generator eluate to the kit vial, has not yet been achieved. Towards 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 e.g. delayed clearance or non-specific binding. The current generation of ^{68}Ga 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 kinetics of HBED-CC makes radiolabeling of ^{68}Ga -HBED-CC-PSMA possible at room temperature, but produces an undesirable mixture of cis/trans geometric isomers distinguishable by HPLC (5,8,9). Thus, a heating step is still required, to reduce the number of isomers and increase the yield of one to ~ 90 % (9). Clinical radiosynthesis of tracers based on these chelators is currently performed on cartridge-based synthesis modules, taking 35 min and typically affording $80\% \pm 5\%$ decay corrected radiochemical yield (9).

Recently, several groups have introduced new $^{68}\text{Ga}^{3+}$ 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 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 (over-expressed in prostate cancer), incorporating a small urea-linked dipeptide pharmacophore (7,8,16) (Fig.1). 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 one-step kit-based labeling of a radiopharmaceutical intended for PSMA imaging, and to evaluate preclinically the resulting tracer.

MATERIALS AND METHODS

Synthesis of THP-PSMA

THP-PSMA was synthesized as described in the Supplemental data.

Preparation of Lyophilized Kits

Kits for one-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.

⁶⁸Ga-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 are described in Supplemental data.

Initial ⁶⁸Ga radiolabeling optimization was performed with an Eckert and Ziegler (E&Z Radiopharma GmbH) ⁶⁸Ge/⁶⁸Ga generator producing 120 – 400 MBq ⁶⁸Ga. Eluates (0.1 M HCl, 5 mL, Sigma Aldrich HPCE grade) were fractionated (10 × 0.5 mL) but not preconditioned to concentrate ⁶⁸Ga or remove trace metal contaminants. Typically, ⁶⁸Ga³⁺ (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: ⁶⁸Ga-THP-PSMA R_t = 10.9 min; unbound ⁶⁸Ga R_t = 2.32 min. iTLC: ⁶⁸Ga-THP-PSMA R_f = 0.8-1; unbound ⁶⁸Ga R_f = 0. For *in vivo* studies THP-PSMA (2 µg) was labeled with ⁶⁸Ga as described above; >95% radiochemical purity was consistently achieved.

To evaluate labeling using the entire eluate without fractionation, E&Z ⁶⁸Ge/⁶⁸Ga generator eluate (5 mL 0.1M HCl, 122-202 MBq) or Galli E₀ (IRE ELiT)

$^{68}\text{Ge}/^{68}\text{Ga}$ generator eluate (1.1 ml eluate diluted to 5 mL with 0.1 M HCl, 600-660 MBq) as added directly into a vented freeze-dried kit vial. After mixing, 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 >95% (n = 3 per generator).

Log P_{OCT/PBS}

^{67}Ga labeled radiotracer (50 μL , 10 μM , specific activity 1.5 MBq/nmol) was added to a pre-equilibrated mixture of 500 μL octanol and 450 μL phosphate-buffered saline. The mixture was mixed for 30 min, and the phases separated by centrifugation (10,000 rpm, 10 min). Aliquots from each phase were gamma-counted.

Serum Stability

^{68}Ga -THP-PSMA was labeled at 5 MBq/nmol as described above. 20 μL was added to 180 μL 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 HLPC (details in Supplemental data). ^{68}Ga -THP-PSMA without serum and unchelated $^{68}\text{Ga}^{3+}$ 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), one day before the assay. Two minutes before incubation the medium was replaced with 245 μ L fresh medium at 37°C or 4°C, then 5 μ L of one of the ^{67}Ga PSMA tracers (specific activity 0.75-2.2 MBq/nmol) was 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 phosphate-buffered saline 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 gamma 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 $^{\text{nat}}\text{Ga}$ -THP-PSMA or $^{\text{nat}}\text{Ga}$ -HBED-CC-PSMA over a range of concentrations. Full details are described in Supplemental data.

Due to poor solubility of $^{\text{nat}}\text{Ga}$ -THP-PSMA above 0.25 mM, an alternative measure of affinity was developed, allowing the *relative* affinity of two gallium PSMA tracers to be determined simultaneously without the $^{\text{nat}}\text{Ga}$ 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 (non-internalizing conditions selected to best represent the equilibrium state from the cell uptake studies). The tracers compared were: $^{67/68}\text{Ga}$ -DOTA-PSMA, $^{67/68}\text{Ga}$ -HBED-CC-PSMA, and $^{67/68}\text{Ga}$ -THP-PSMA. Each ^{67}Ga tracer was compared to each ^{68}Ga tracer and affinity ratios were obtained by measuring total and non-specific binding for each tracer (gamma counting) and calculating the ratio of their specific binding. Full details are provided in the Supplemental data.

Mouse Model of Prostate Cancer

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

PET Scanning

PET imaging was performed on four groups of mice (n = 3 each) under isoflurane anesthesia with a BioScan nanoPET-CT PLUS (Mediso, Hungary). Mice were CT imaged before radiotracer administration and dynamic PET data were collected for the first hour post-injection. Mice were then euthanized and organs harvested, weighed and gamma-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

⁶⁸Ga-HBED-CC-PSMA (5-15 MBq, 50-140 μ L, 0.6-1.3 μ g, group 2) by tail vein injection. A third group (group 3) were co-administered ⁶⁸Ga-THP-PSMA and the PSMA-inhibitor 2-(phosphonomethyl)pentane-1,5-dioic acid (PMPA) (50 μ g, Enzo Life Sciences) (20,21). Mice bearing DU145 tumors were administered ⁶⁸Ga-THP-PSMA (group 4). Imaging protocol and image analysis methods are described in the Supplemental data.

Statistical Analysis

Data were analyzed in GraphPad Prism 5 (version 5.04) and expressed as mean \pm SD. Student t-tests were used to determine statistical significance with $P < 0.05$ considered significant.

RESULTS

Synthesis of THP-PSMA

Supplementary 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 semi-preparative HPLC afforded THP-PSMA as

a trifluoro acetic acid salt, yield 4 mg, 2.6 μmol , 5.2% yield from resin loading; $(\text{C}_{54}\text{H}_{77}\text{N}_{11}\text{O}_{19}(\text{CF}_3\text{COO})_3)$ MW 1526.33, HPLC: $R_t = 13.4$ min, >98% purity. LC-ESI-MS, ^1H 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. $^{68}\text{Ga}^{3+}$ (5-75 MBq) in aqueous HCl (0.1 M, 250 μL) was added to pre-prepared THP-PSMA (5-0.01 μg in 3 μL H_2O) in sodium bicarbonate solution (1 M, 27 μL). After five minutes 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 ^{68}Ga eluate, >95% radiochemical purity).

One-step Kit for Radiolabeling

Using kit vials containing lyophilized THP-PSMA (40 μg , 26 nmol), sodium bicarbonate (44 mg) and sodium phosphate buffer (17.5 mg), radiosynthesis of ^{68}Ga -THP-PSMA was achieved in one 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 five minutes, 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 post-reconstitution showed no instability or autoradiolysis (Supplemental Fig. 2).

Lipophilicity and Serum Stability

The log $P_{OCT/PBS}$ values of the three ^{68}Ga -PSMA complexes at pH 7.4 were very 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 three tracers showed time-dependent accumulation in PSMA-expressing cells, but very low uptake in non-PSMA-expressing cells, confirming uptake is PSMA-mediated. At 37°C uptake continued to increase with time, preventing measurement of equilibrium binding parameters such as 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 the K_d of $^{67/68}\text{Ga}$ -THP-PSMA was 10.4 ± 3.4 times higher than that of $^{67/68}\text{Ga}$ -HBED-CC-PSMA and 8.6 ± 3.4 times that of $^{67/68}\text{Ga}$ -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 and Supplemental Fig. 5).

PET Imaging and Biodistribution

PET/CT scanning of ^{68}Ga -THP-PSMA in SCID/beige mice bearing PSMA-positive xenografts (DU145-PSMA, group 1) showed that excretion of ^{68}Ga -THP-PSMA was rapid and exclusively renal, with the majority of activity associated with bladder and kidneys by 60 min (Fig. 4 and Supplemental Fig. 6). Blood clearance, represented by blood pool in the left ventricle, was rapid, decreasing to 1% ID/cm³ within 30 min (Fig. 5B). With images scaled between 0 and 25% ID/cm³, tumors were clearly delineated (Fig. 4). Analysis of the 40-60 min post-injection (PI) images gave a tumor uptake value of 5.6 ± 1.2 % ID/cm³ (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/cm³ ($p < 0.05$ compared to group 1), and by blocking experiments in mice bearing a DU145-PSMA tumor where PMPA was co-administered with ^{68}Ga -THP-PSMA (group 3) giving a tumor uptake of 1.0 ± 0.4 % ID/cm³ ($p < 0.05$ compared to 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 very similar blood clearance, tumor uptake and renal excretion (Fig. 5). Image analysis of 40-60 min PET images revealed that tumor uptake of ^{68}Ga -THP-PSMA (5.3 ± 0.1 %

ID/cm³) was not significantly different to that of ⁶⁸Ga-HBED-CC-PSMA (5.6 ± 1.2 % ID/cm³).

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 ⁶⁸Ga-THP-PSMA for PSMA expressing tumors. ⁶⁸Ga-THP-PSMA showed very similar *ex vivo* biodistribution to ⁶⁸Ga-HBED-CC-PSMA, apart from markedly lower spleen uptake (3.7 ± 1.3 and 17.6 ± 6.1 % ID/g 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 ⁶⁸Ga 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 ⁶⁸Ga (and ⁶⁷Ga). Radiolabeling yields of >95% and specific activity 15-45 MBq/nmol with unprocessed generator-produced ⁶⁸Ga were achieved in one step at pH 7 and ambient temperature within 5 min without further purification. Based on this we developed a lyophilized kit, which can be labeled/reconstituted simply by adding 5 mL of raw

generator eluate. One-step kits can only be used with generators with $<0.001\%$ ^{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-man studies. These radiolabeling properties demonstrate that kit-based radiolabeling of ^{68}Ga tracers, analogous to the simple manipulations to which radiopharmacy staff producing $^{99\text{m}}\text{Tc}$ 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 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 upon Ga^{3+} coordination: upon reverse-phase HPLC, ^{68}Ga -THP-PSMA elutes as a single radioactive species (Fig. 2B) shown by LCMS 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 to the timescale of HPLC and *in vivo* processes, and hence biologically irrelevant. The lipophilicity is low and comparable to that of established ^{68}Ga -PSMA ligands.

Although THP-PSMA was rationally designed based on 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 ^{68}Ga -THP-PSMA binds specifically to PSMA, although with weaker affinity than ^{68}Ga -HBED-CC-PSMA and ^{68}Ga -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 non-specific uptake of ^{68}Ga -THP-PSMA in non-target tissues and high, specific radioactivity concentration in PSMA-expressing tumors are clinically desirable features. In the pre-clinical model used here, tumor uptake and pharmacokinetic properties of ^{68}Ga -THP-PSMA and ^{68}Ga -HBED-CC-PSMA are indistinguishable except that spleen uptake of ^{68}Ga -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 spleen, and that ^{68}Ga -THP-PSMA has enhanced ability to distinguish PSMA from this alternative target.

CONCLUSION

Use of THP as the $^{68}\text{Ga}^{3+}$ chelator facilitates rapid chelation under mild conditions and produces a PSMA-targeted bioconjugate that can be labeled in one 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 post-synthesis purification, achieving >95 % radiochemical purity. *In vivo*, ^{68}Ga THP-PSMA accumulates in PSMA-expressing tumors, with good tumor to background ratios delineation of PSMA-positive tumor lesions similar to ^{68}Ga -HBED-CC-PSMA. Kit-based radiolabeling of ^{68}Ga radiopharmaceuticals is feasible with THP and would facilitate wider and more economical use of ^{68}Ga in hospitals, hence benefitting more patients.

DISCLOSURE:

PJB, RCH and GEM are named inventors on related patents. GEM and LKM are current employees of Theragnostics Ltd.

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REFERENCES

1. Zolle I, ed. *Technetium-99m pharmaceuticals*: Berlin, Germany: Springer; 2007:95-98.
2. Banerjee SR, Pomper MG. Clinical applications of gallium-68. *Appl Radiat Isot.* 2013;76:2-13.
3. Smith DL, Breeman WA, Sims-Mourtada J. The untapped potential of gallium 68-PET: the next wave of 68 Ga-agents. *Appl Radiat Isot.* 2013;76:14-23.
4. Deutsch E, Clinical PET: its time has come? *J Nucl Med.* 1993;34:1132–1133.
5. Ebenhan T, Vorster M, Marjanovic-Painter B, et al. Development of a single vial kit solution for radiolabeling of 68Ga-DKFZ-PSMA-11 and its performance in prostate cancer patients. *Molecules.* 2015;20:14860-14878.
6. Satpati D, Shinto A, Kamaleshwaran K, Sane S, Banerjee S. Convenient preparation of [68Ga] DKFZ-PSMA-11 using a robust single-vial kit and demonstration of its clinical efficacy. *Mol Imaging Biol.* 2016;18:420-427.
7. Benešová M, Schäfer M, Bauder-Wüst U, et al. Preclinical evaluation of a tailor-made DOTA-conjugated PSMA inhibitor with optimized linker moiety for imaging and endoradiotherapy of prostate cancer. *J Nucl Med.* 2015;56:914-920.

8. Eder M, Schäfer M, Bauder-Wüst U, et al. ^{68}Ga -complex lipophilicity and the targeting property of a urea-based PSMA inhibitor for PET imaging. *Bioconjug Chem.* 2012;23:688-697.
9. Eder M, Neels O, Müller M, et al. Novel preclinical and radiopharmaceutical aspects of [^{68}Ga] Ga-PSMA-HBED-CC: a new PET tracer for imaging of prostate cancer. *Pharmaceuticals.* 2014;7:779-796.
10. Seemann J, Waldron BP, Roesch F, Parker D. Approaching 'kit-type' labelling with ^{68}Ga : the DATA chelators. *ChemMedChem.* 2015;10:1019-1026.
11. Berry DJ, Ma Y, Ballinger JR, et al. Efficient bifunctional gallium-68 chelators for positron emission tomography: tris (hydroxypyridinone) ligands. *Chem Commun.* 2011;47:7068-7070.
12. Ma MT, Cullinane C, Imberti C, et al. New tris (hydroxypyridinone) bifunctional chelators containing isothiocyanate groups provide a versatile platform for rapid one-step labeling and PET imaging with $^{68}\text{Ga}^{3+}$. *Bioconjugate Chem.* 2015;27:309-318.
13. Ma MT, Cullinane C, Waldeck K, Roselt P, Hicks RJ, Blower PJ. Rapid kit-based ^{68}Ga -labelling and PET imaging with THP-Tyr 3-octreotate: a preliminary comparison with DOTA-Tyr 3-octreotate. *EJNMMI Res.* 2015;5:52.

14. Imberti C, Terry SY, Cullinane C, et al. Enhancing PET signal at target tissue in vivo: dendritic and multimeric tris (hydroxypyridinone) conjugates for molecular imaging of $\alpha v\beta 3$ integrin expression with gallium-68. *Bioconjugate Chem.* 2017;28:481.
15. Dubey RD, Klippstein R, Wang JT-W, et al. Novel hyaluronic acid conjugates for dual nuclear imaging and therapy in CD44-expressing tumors in mice in vivo. *Nanotheranostics.* 2017;1:59-79.
16. Kozikowski AP, Nan F, Conti P, et al. Design of remarkably simple, yet potent urea-based inhibitors of glutamate carboxypeptidase II (NAALADase). *J Med Chem.* 2001;44:298-301.
17. Eiber M, Maurer T, Souvatzoglou M, et al. Evaluation of hybrid ^{68}Ga -PSMA ligand PET/CT in 248 patients with biochemical recurrence after radical prostatectomy. *J Nucl Med.* 2015;56:668-674.
18. Afshar-Oromieh A, Zechmann CM, Malcher A, et al. Comparison of PET imaging with a ^{68}Ga -labelled PSMA ligand and ^{18}F -choline-based PET/CT for the diagnosis of recurrent prostate cancer. *Eur J Nucl Med Mol Imaging.* 2014;41:11-20.
19. Kampmeier F, Williams JD, Maher J, Mullen GE, Blower PJ. Design and preclinical evaluation of a $^{99\text{m}}\text{Tc}$ -labelled diabody of mAb J591 for SPECT imaging of prostate-specific membrane antigen (PSMA). *EJNMMI Res.* 2014;4:13.

20. Jackson PF, Tays KL, Maclin KM, et al. Design and pharmacological activity of phosphinic acid based NAALADase inhibitors. *J Med Chem.* 2001;44:4170-4175.
21. Chen Y, Foss CA, Byun Y, et al. Radiohalogenated prostate-specific membrane antigen (PSMA)-based ureas as imaging agents for prostate cancer. *J Med Chem.* 2008;51:7933-7943.
22. Zhou T, Neubert H, Liu DY, et al. Iron binding dendrimers: a novel approach for the treatment of haemochromatosis. *J Med Chem.* 2006;49:4171-4182.
23. Cusnir R, Imberti C, Hider RC, Blower PJ, Ma MT. Hydroxypyridinone chelators: from iron scavenging to radiopharmaceuticals for PET Imaging with gallium-68. *Int J Mol Sci.* 2017;18:116.
24. Hillier SM, Maresca KP, Lu G, et al. ^{99m}Tc-labeled small-molecule inhibitors of prostate-specific membrane antigen for molecular imaging of prostate cancer. *J Nucl Med.* 2013;54:1369-1376.
25. Chen Y, Pullambhatla M, Banerjee SR, et al. Synthesis and biological evaluation of low molecular weight fluorescent imaging agents for the prostate-specific membrane antigen. *Bioconjug Chem.* 2012;23:2377-2385.
26. Bacich DJ, Pinto JT, Tong WP, Heston WDW. Cloning, expression, genomic localization, and enzymatic activities of the mouse homolog of prostate-specific membrane antigen/ NAALADase/folate hydrolase. *Mamm Genome.* 2001;12:117-123.

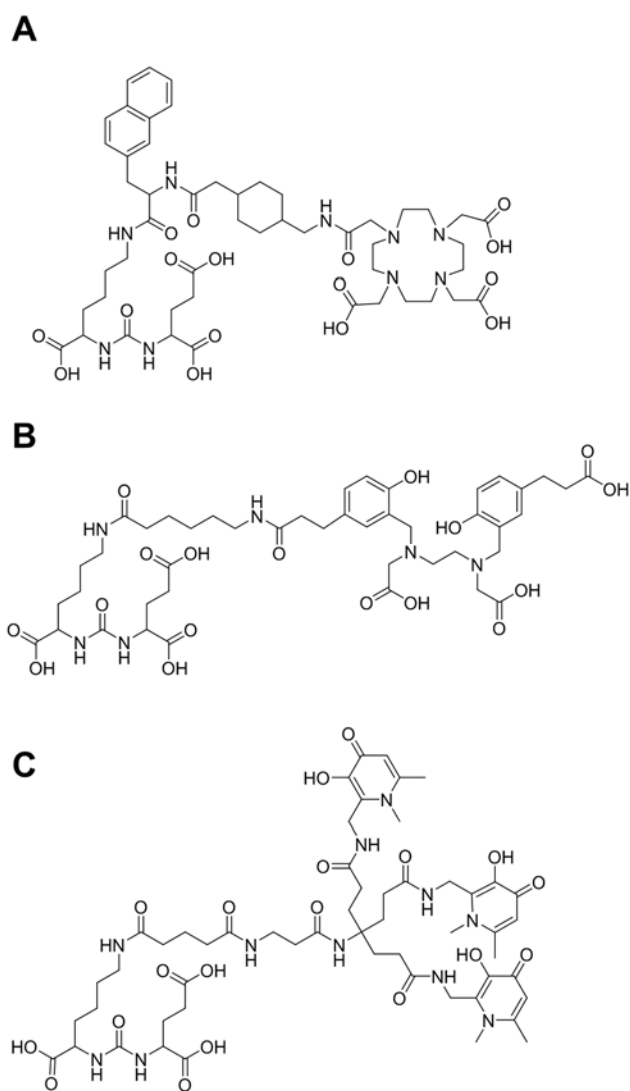


FIGURE 1. Structure of **(A)** DOTA-PSMA (PSMA-617) (7); **(B)** HBED-CC-PSMA (DKFZ-PSMA-11) (8,11); **(C)** THP-PSMA

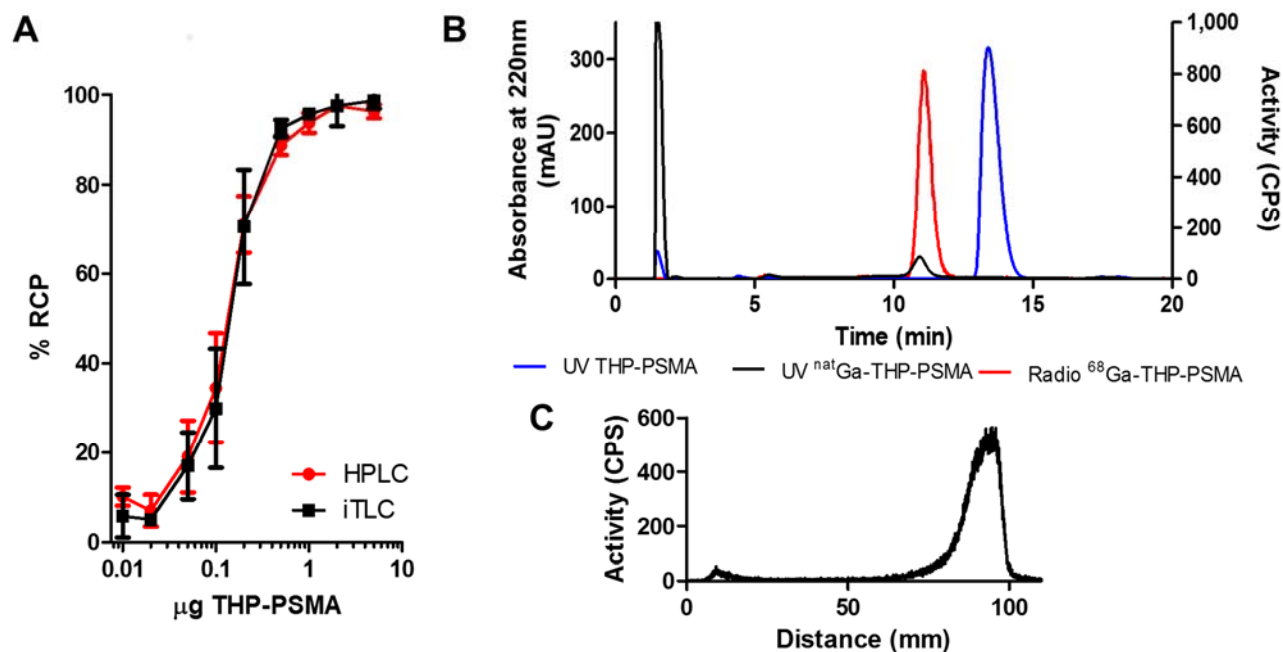


FIGURE 2 (A) Dependence of radiochemical purity of ^{68}Ga -THP-PSMA on mass of THP-PSMA in 280 μL , as measured by HPLC (red) and iTLC (black) after 5 min; $n = 3$, mean \pm SD. (B) HPLC ($\lambda = 220$ nm) of THP-PSMA (blue, $R_T = 13.38$ min) and $^{\text{nat}}\text{Ga}$ -THP-PSMA (black, $R_T = 10.92$ min, excess $\text{Ga}(\text{NO}_3)_3$ is present with $R_T = 1.47$ min), and radio-HPLC of ^{68}Ga -THP-PSMA (red, $R_T = 11.05$ min) labeled using the one-step kit and analyzed 10 min post-reconstitution. (C) iTLC of ^{68}Ga -THP-PSMA labeled using one-step kit, analyzed 5 min post-reconstitution (R_f unchelated $^{68}\text{Ga} = 0$, R_f ^{68}Ga -THP-PSMA = 0.8-1).

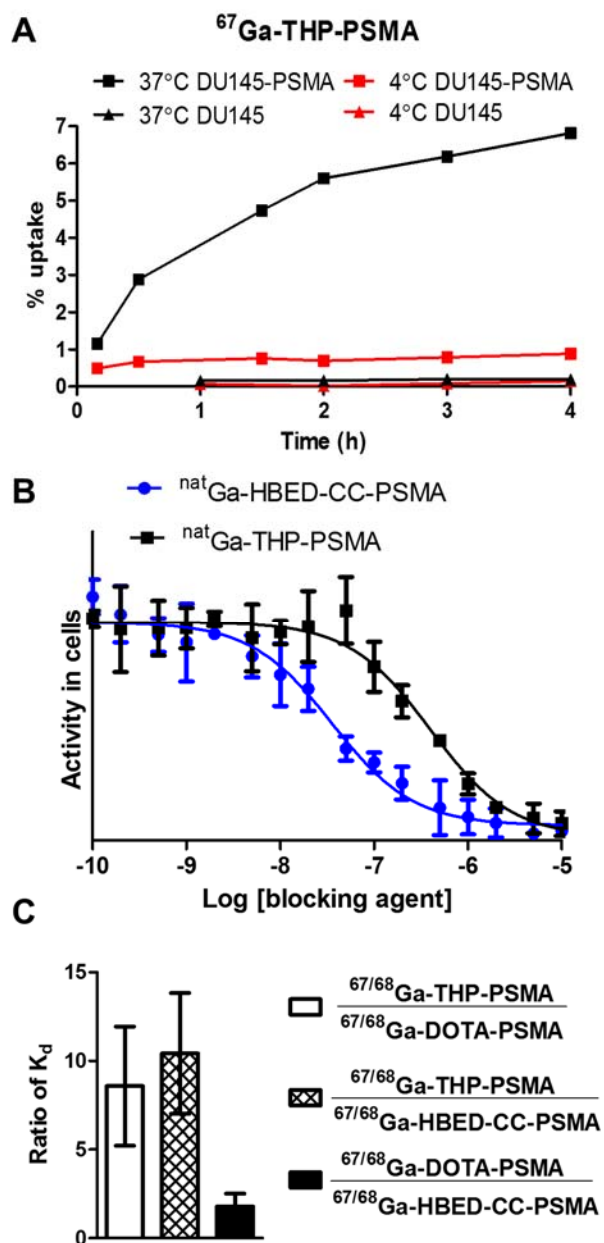


FIGURE 3: (A) ^{67}Ga -THP-PSMA uptake over time at 4°C and 37°C, 1×10^6 cells/mL with DU145-PSMA or DU145 cells. (B) Representative IC_{50} experiment for $^{\text{nat}}\text{Ga}$ -THP-PSMA (black) or $^{\text{nat}}\text{Ga}$ -HBED-CC-PSMA (blue) with 1 nM ^{68}Ga -DOTA-PSMA

as the probe (n = 3 for each concentration). IC₅₀ values in main text are the mean of at least 3 experiments (C) Ratio of K_d of two tracers incubated at 1 nM with DU145-PSMA cells at 4°C for 2 h. The ratios were calculated from the specific binding obtained by incubating the ⁶⁷Ga version of a tracer in the same well as the ⁶⁸Ga 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.

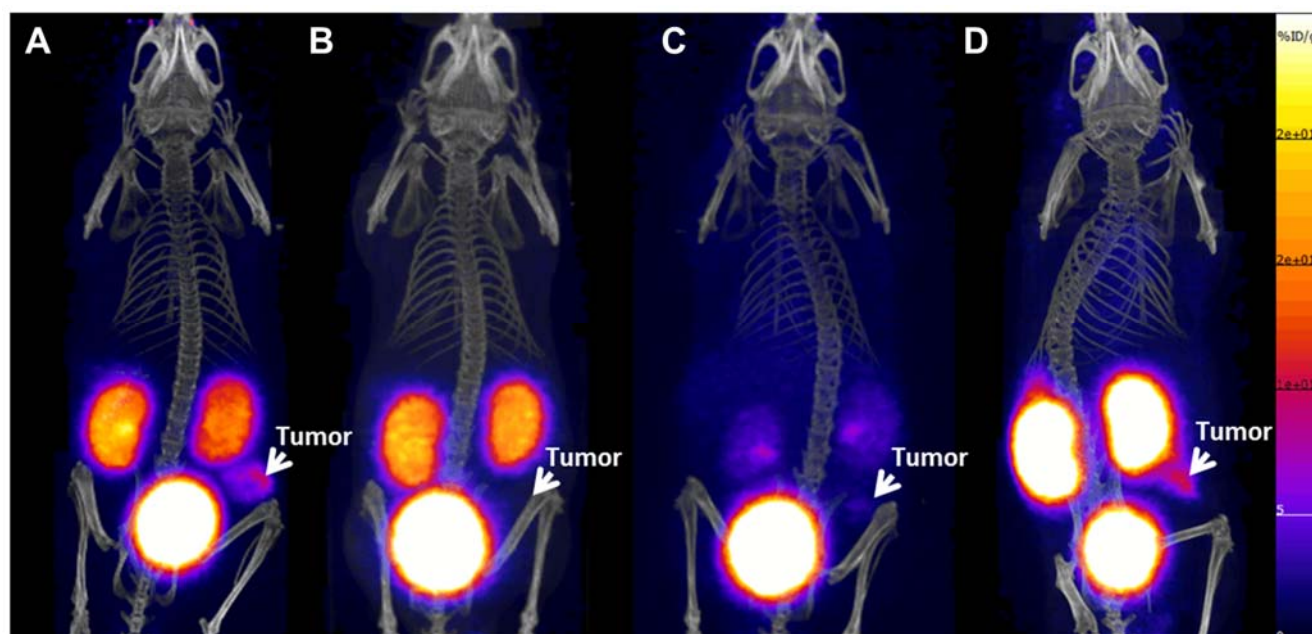


FIGURE 4. Representative PET/CT images of mice bearing xenografts at 40-60 min PI with PET images scaled from 0-25 % ID/cm³. **(A)** ⁶⁸Ga-THP-PSMA in DU145-PSMA tumor (group 1); **(B)** ⁶⁸Ga-THP-PSMA in DU145 tumor (group 4); **(C)** ⁶⁸Ga-THP-PSMA in DU145-PSMA tumor blocked with PMPA (group 3); **(D)** ⁶⁸Ga-HBED-CC-PSMA in DU145-PSMA tumor (group 2).

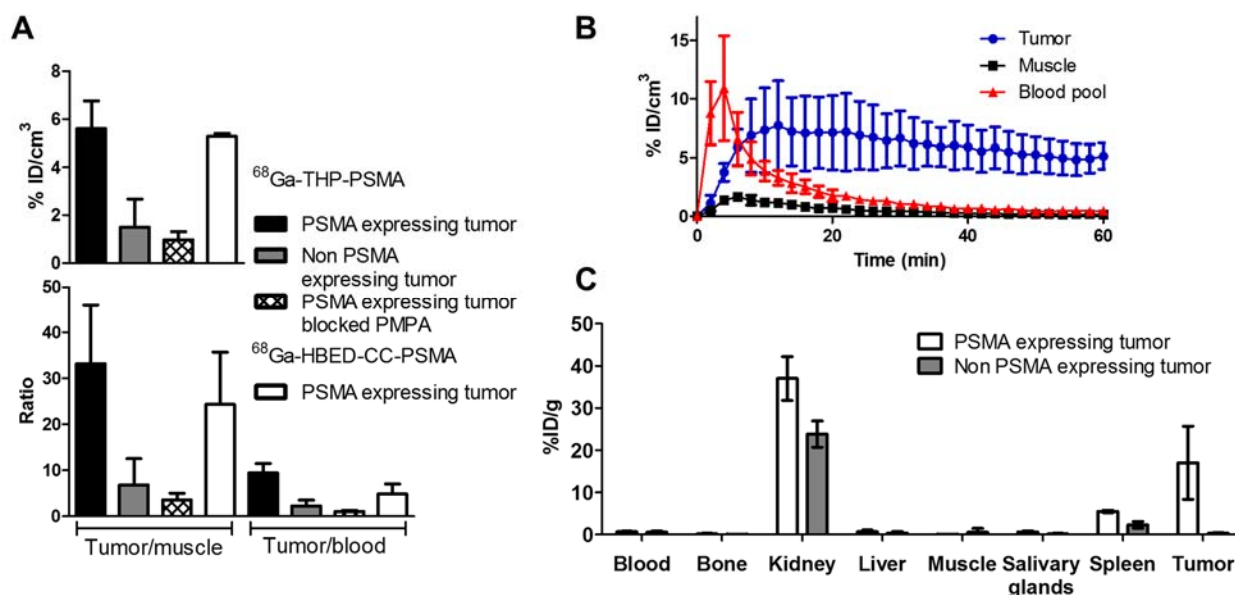


FIGURE 5. (A) PET/CT-derived % ID/cm³ in tumor and tumor/blood and tumor/muscle ratio 40-60 min PI. ⁶⁸Ga-THP-PSMA in DU145-PSMA tumor (black), ⁶⁸Ga-THP-PSMA in DU145-PSMA tumor blocked with PMPA (hatched), ⁶⁸Ga-THP-PSMA in DU145 tumor (gray) or ⁶⁸Ga-HBED-CC-PSMA in DU145-PSMA tumor (white); (n = 3, mean ± SD); (B) PET-derived time-activity curves of mice bearing DU145-PSMA tumors, imaged with ⁶⁸Ga-THP-PSMA for 1 h PI: 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 a DU145-PSMA (white) or a DU145 tumor (gray), culled 1 hour post injection (n = 3, mean ± SD). Unlike PET-imaged animals, these mice were not anesthetized between injection and euthanasia.

Supplemental Data

⁶⁸Ga-THP-PSMA: a PET imaging agent for prostate cancer offering rapid, room temperature, one-step kit-based radiolabeling

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SUPPLEMENTAL METHODS

All reagents and consumables were purchased from Sigma Aldrich (Dorset, UK) or Fischer Scientific (Loughborough, UK), with the exception of Fmoc-Lys-Dde-COOH (Bachem AG, Bubendorf Switzerland), PMPA (Enzo Life Sciences, Exeter, UK), DOTA-PSMA (PSMA-617) and HBED-CC-PSMA DKFZ-11, (ABX, Radeberg, Germany). SCID/Beige mice were purchased from Charles River, Margate, UK. DU145 and DU145-PSMA cells were provided by Dr Florian Kampier (1). These cells

were cultured in RMPI 1640 medium supplemented with 10 % FBS, 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.

Instrumentation

¹H and ¹³C NMR spectra were acquired on a Bruker Avance III 700 spectrometer operating at 700 MHz (¹H frequency), equipped with a quadruple-resonance QCI cryoprobe.

High resolution 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 gamma detector) and UV detection (220 nm analytical, 280 nm preparative). Data were analyzed using Laura software (V.4.0.2.75 LabLogic Systems Ltd.).

HPLC methods: Chemical purity of THP-PSMA and radiolabeling of ^{68/67}Ga-THP-PSMA were assessed using an Agilent Eclipse XDB C₁₈ 5 micron 4.6 x 150 mm column with an isocratic mobile phase (87.5 % H₂O, 12.5 % ACN, 0.075 %TFA, 0.05% TEA), 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 minutes = 90 % A, 5-15 minutes = ramp to 50 % B (4 % / min), 15-20 minutes = 90 % A, with a flow rate of 1 mL/min.

Serum stability was assessed using size exclusion chromatography using a Phenomenex BioSep 5 μ m SEC-_s2000 column (300 \times 7.8 mm) with PBS as the mobile phase and a flow rate of 1 mL/min.

iTLC plates were scanned with a Lab-Logic mini-Scan TLC reader and analyzed with Laura software. Radioactivity counting was performed with a gamma counter (LKB Wallac, PerkinElmer, UK) for cell studies and in vivo biodistribution.

iTLC methods: Radiolabeling for all $^{67/68}\text{Ga}$ -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).

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 fritted polypropylene syringe and a solution of Fmoc-Lys-Dde-COOH (600 mg 1.1 mmol) (**1**, Supplemental Fig. 1) and excess DIPEA in DCM was added. After stirring 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 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**.

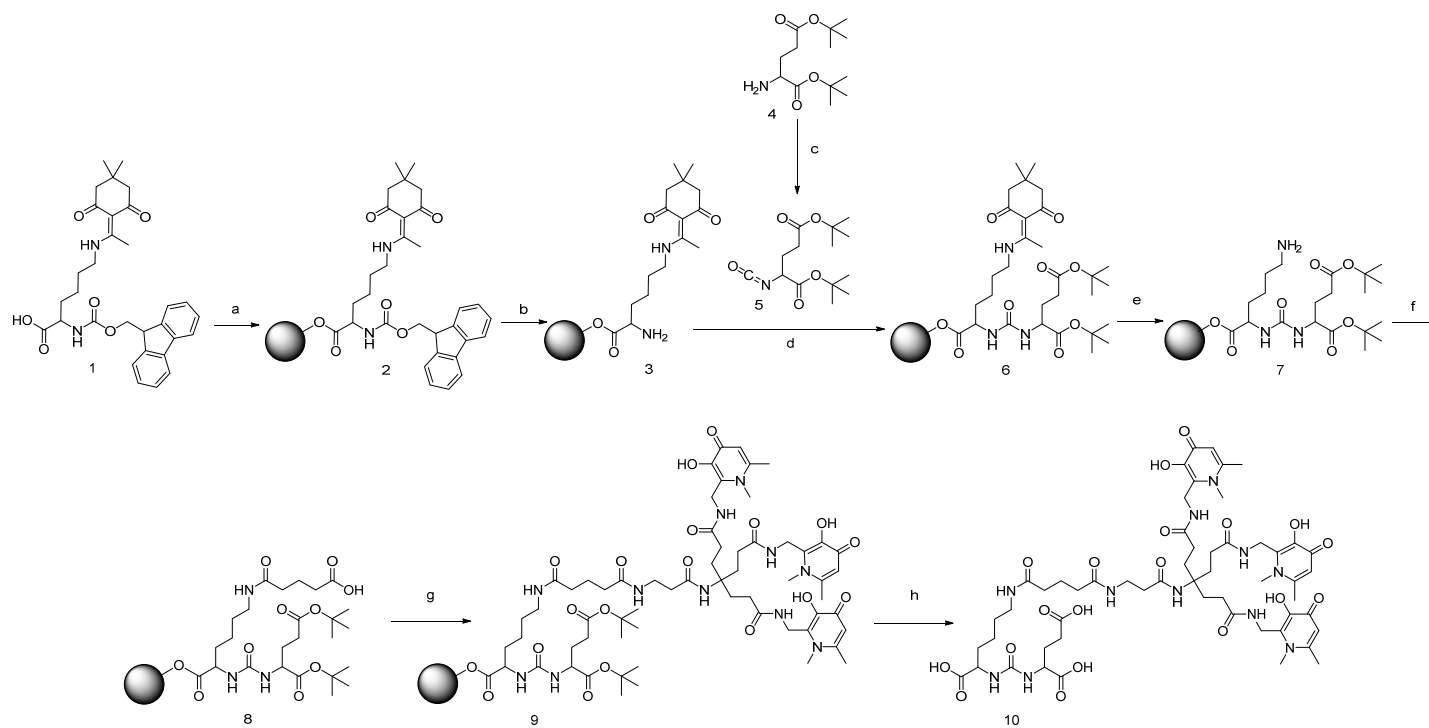
Synthesis of resin-bound THP-PSMA (compound 9). Bis(tert-butyl) ester L-glutamate hydrochloride (444 mg, 1.5 mmol) was dissolved in anhydrous DCM (100 mL) containing DIPEA (750 μ L, 4.3 mmol). This solution was added dropwise to a 3-neck round bottom flask containing a solution of triphosgene (150 mg, 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 hour. 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 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 μ L, 2 mmol) dissolved in 2 mL DMF, and the mixture stirred for 3 hours 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 HATU (20 mg, 0.05 mmol) and DIPEA (18 μ L, 0.1 mmol) in anhydrous DMF (0.5 mL) and in situ activation of the carboxylate was allowed to proceed for 10 minutes. A solution of THP-NH₂ (70 mg, 0.09 mmol), synthesized by a previously published method (2), in DMF/DMSO (2.5 mL/2 mL)

containing DIPEA (18 μ L, 0.1 mmol) was then added to this suspension, and stirred for 36 hours, to furnish compound **9** (Supplemental Fig. 1).

Cleavage of THP-PSMA (compound 10) from the resin. Cleavage and removal of the side-chain protecting groups were performed by treating the resin-bound compound **9** with TFA for 3 hours at RT in the presence of phenol (5 % w/v), water (5 % v/v) and TIPS (2 % v/v) as scavengers. The filtrate was collected and the resin washed with TFA and DCM. The solution was concentrated to < 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, the supernatant decanted off, the precipitate washed with diethyl ether, and finally dissolved in aqueous acetonitrile and lyophilized. Crude THP-PSMA was purified by semi-preparative RP-HPLC to give a TFA salt ($C_{54}H_{77}N_{11}O_{19} \cdot (CF_3COO)_3$) MW = 1526.33. Semi-preparative reverse phase HPLC was conducted using an Agilent Eclipse XDB C_{18} 5 micron 21.2 x 150 mm column with the concentration of mobile phase B increasing at a rate of 1%/min (A = H₂O with 0.2% TFA, B = acetonitrile (ACN) with 0.2% TFA, starting from 100% A at time 0, flow rate 5 mL/min).

Yield of THP-PSMA(CF_3COO)₃: 4 mg, 2.6 μ mol, 5.2% yield from resin loading.

Analytical HPLC R_t = 13m 23s, > 98% purity. For analytical data see results section below.



SUPPLEMENTAL FIGURE 1: Reaction scheme for synthesis of THP-PSMA. a. 2-chlorotriyl polymer bound resin beads, DIPEA, DCM; b. Piperidine, DMF; c. Triphosgene, DIPEA, DCM, 0 °C; d. Compound **3**, Compound **5**, DCM; e. Hydrazine hydrate, DMF; f. Glutaric anhydride, DIPEA, DMF; g. THP-NH₂, HATU, DIPEA, DMF, DMSO; h. TFA, phenol, TIPS, H₂O

Radiolabeling

$^{68/67}\text{Ga}$ radiolabeling of HBED-CC-PSMA and DOTA-PSMA was assessed with the same HPLC equipment but an alternative mobile phase: A = H_2O with 0.05 % TFA, B = ACN with 0.05 % TFA, gradient: 0-5 minutes = 90 % A, 5-15 minutes = ramp to 50 % B (4 % / min), 15-20 minutes = 90 % A, with a flow rate of 1 mL/min.

iTLC methods: Radiolabeling for all $^{67/68}\text{Ga}$ -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).

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 as the presence of citrate reduces the radiolabeling efficiency of ^{67}Ga with a number of chelators (3,4). ^{67}Ga -citrate (6.49 mM citrate Mallinckrodt, Switzerland) was passed over a Silica Light Sep-Pak cartridge (120 mg sorbent, 55-105 μm particle size) at 1 mL/min to capture the radio-metal on the cartridge. This process was repeated 3 times whereupon ~ 90% of the activity remained on the cartridge. After washing with 5 mL dH_2O the ^{67}Ga was eluted with 400 μL 0.1M 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).

Preparation of ^{67}Ga and ^{68}Ga PSMA tracers:

Before addition to cells the tracer was diluted to 50 nM in PBS

^{67}Ga -THP-PSMA for in vitro studies: 10 μL 2 mg/mL solution (20 μg , 13.1 nmol THP-PSMA (MW 1526.33)) was added to 50 μL ^{67}Ga in 0.1M HCl (converted from ^{67}Ga citrate), and the pH adjusted to 6.5 - 7.5 with 4.5 μL 1M sodium bicarbonate. Labeling was assessed after 5 min at room temperature by iTLC and HPLC. Before addition to cells the tracer was diluted to 50nM in PBS.

DOTA-PSMA for in vitro studies: 50 μL ^{67}Ga or ^{68}Ga in 0.1M HCl, 5.25 μL 1M HCl and 20 μL 2.1M HEPES were combined, producing a solution at pH 3.5. For ^{67}Ga labeling 20 μg (13.3 nmol) DOTA-PSMA (ABX, MW 1498.18) was added; for ^{68}Ga labeling 2 μg (1.3 nmol) DOTA PSMA was added. The solution was heated to 95°C for 30 minutes and then labeling was assessed by iTLC and HPLC. Before addition to cells the tracer was diluted to 50nM in PBS.

HBED-CC-PSMA for in vitro studies: 50 μL ^{67}Ga or ^{68}Ga in 0.1M HCl, 5 μL 1M HCl and 50 μL 2.1M HEPES were combined, producing a solution at pH 4.5. For ^{67}Ga labeling 29 μg (20.6 nmol) HBED-CC-PSMA (ABX, MW 1403.09) was added; for ^{68}Ga labeling 2 μg (1.4 nmol) HBED-CC-PSMA was added. The solution was heated to 95°C for 10 minutes and then labeling was assessed by iTLC and HPLC. Before addition to cells the tracer was diluted to 50nM in PBS.

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 HBED-CC-PSMA) and sodium bicarbonate (19 μL , 1 M), and heated at 90 $^{\circ}\text{C}$ for 10 min (pH 3 – 4). A radiochemical yield of > 95% was consistently achieved.

Preparation of ^{nat}Ga chelated PSMA tracers:

^{nat}Ga -THP-PSMA: 124 μL of 0.93 mM THP-PSMA was added to 93 μL 6.4 mg/mL $^{nat}\text{Ga}(\text{NO}_3)_3 \cdot \text{X}(\text{H}_2\text{O})$ dissolved in 0.1M HCl (a 20-fold excess of gallium over THP-PSMA). The pH was adjusted to 6.5-7.5 with 14 μL 1M sodium bicarbonate, and 231 μL PBS was then added. The solution was sonicated for 15 minutes. This produced a final solution of 0.25 mM ^{nat}Ga -THP-PSMA. This solution was diluted in PBS to produce the range of concentrations required for the IC_{50} . This complex was characterized with LC-ESI-MS: m/z $[\text{C}_{54}\text{H}_{74}\text{N}_{11}\text{O}_{19}\text{Ga} + \text{H}]^+$ observed monoisotopic peak = 1250.45092 calculated = 1250.44909. $[\text{C}_{54}\text{H}_{74}\text{N}_{11}\text{O}_{19}\text{Ga} + 2\text{H}]^{2+}$ observed monoisotopic peak = 625.72846, calculated = 625.72818.

^{nat}Ga -HBED-CC-PSMA: 124 μL of 0.93 mM HBED-CC-PSMA was added to 93 μL of 6.4 mg/mL $^{nat}\text{Ga}(\text{NO}_3)_3 \cdot \text{X}(\text{H}_2\text{O})$ dissolved in 0.1M HCl (a 20-fold excess of gallium over HBED-CC-PSMA). The pH was adjusted to 4 with 13 μL 1M sodium bicarbonate and the solution was heated to 95 $^{\circ}\text{C}$ for 10 min. The pH was adjusted to 6.5-7.5 with 1M sodium bicarbonate followed by sonication for 15 minutes. This produced a final solution of 0.5 mM ^{nat}Ga -HBED-CC-PSMA. This solution was diluted in PBS to produce the range of concentrations required for the IC_{50} .

IC₅₀ measurement protocol

0.25 x 10⁶ cells/well were seeded in a 24-well plate, one day before the assay.

Increasing concentrations of ^{nat}Ga-THP-PSMA or ^{nat}Ga-HBED-CC-PSMA followed by 1nM ⁶⁸Ga-DOTA-PSMA (specific activity 5 - 7.5MBq/nmole, diluted in PBS) was added to the cells (total volume 250 μ L). After 30 minutes incubation at 37 °C, the cells were washed with PBS (3 \times 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 were measured by gamma counting. Data was analyzed using Graph Pad Prism and a one site – fit log IC₅₀ algorithm.

Relative affinity measurements

DU145-PSMA cells were seeded with 1.5 x 10⁶ cells/well in a 6 well plate, one day before the assay. Two minutes prior to incubation with tracer, the cell medium was replaced by 1410 μ L fresh medium at 4°C. 30 μ L of ⁶⁷Ga-PSMA tracer (50 nM, specific activity 0.75 - 2.2 MBq/nmol), and 30 μ L ⁶⁸Ga-PSMA tracer (50 nM, specific activity 4 - 26 MBq/nmol, diluted in PBS) and 30 μ L PBS were added, giving a total volume of 1.5 mL and final concentrations of 1 nM ⁶⁷Ga PSMA tracer and 1nM ⁶⁸Ga PSMA tracer (total labeled and unlabeled PSMA conjugate concentration 2 nM).

Additionally non-specific uptake was determined by using non-PSMA expressing cells (DU145) or by blocking PSMA expressing cells (DU145-PSMA) with 750 μ M PMPA. After 2 hours incubation at 4°C, the cells were washed with 3 \times 0.25 mL 4°C PBS to determine the unbound fraction, then incubated with 0.5 M glycine pH 2.5 at 4°C for 5

minutes to determine the cell surface bound fraction, and then lysed with 1 M NaOH and the well washed with PBS to determine the fraction internalized by the cells. The activity from the ^{67}Ga -PSMA tracer and the ^{68}Ga -PSMA tracer present in each fraction was measured by gamma counting (^{68}Ga values were corrected for ^{67}Ga component and ^{67}Ga was measured once ^{68}Ga had decayed > 10 half-lives). Specific binding was calculated by subtracting the non-specific binding (binding of the tracer to DU145 cells or to DU145-PSMA cells in the presence of PMPA) from the total binding percentage of activity associated with the DU145-PSMA cells when no blocking agent is present. Ratios of specific binding were calculated pair-wise for tracers incubated in the same well.

Imaging protocol:

PET-CT acquisition: The PET-CT scans were performed on a BioScan nanoPET-CT PLUS (Mediso, Hungary) scanner using their proprietary acquisition software (Nucline v 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 minutes to perform. Dynamic PET scans were performed within a 94.7 mm field of view from 0 min to 60 min post 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 400–600 keV energy window.

Image analysis:

The dynamic PET data were reconstructed using Nucline software (v 2.00). Data were allocated to both 20 min and 2 min bins using 0.4 mm^3 voxels for PET and 0.21 mm^3 for CT. Image processing and analysis were performed using Vivoquant software (v 1.23). Before analysis both CT and PET images were realigned, processed to a voxel size of 0.21 mm^3 and the PET output calibrated to display MBq per voxel. Regions of interest (ROIs) for each data file were produced using three different techniques: Fixed volume ROIs, Otsu thresholding and freehand segmentation from the CT image. Fixed volume ROIs were used for muscle (17.2 mm^3 sphere within the thigh muscle), and blood pool (2.2 mm^3 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 due to the close proximity of kidney signal. Total tumor volume was drawn manually from the CT image, defined three times per animal and the average value from these three ROIs determined. $\%ID/\text{cm}^3$ 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 - 60 min time point. Static images were produced from dynamic data between 40 - 60 min and scaled between 0 and 25 $\% \text{ ID} / \text{cm}^3$.

Biodistribution studies

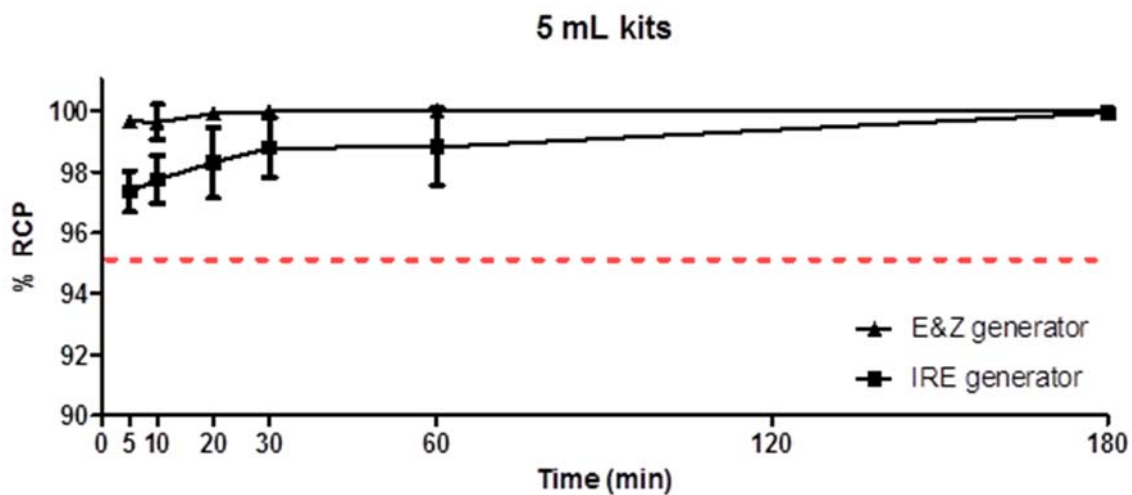
Additional ^{68}Ga (THP-PSMA) biodistribution and blocking studies were performed in mice bearing DU145 or DU145-PSMA tumors without imaging or continuous anesthesia. ^{68}Ga (THP-PSMA) (3.5 – 12.7 MBq, 0.7 -0.9 μg THP-PSMA) was administered via tail vein injection under isoflurane anesthesia. After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

SUPPLEMENTAL RESULTS

¹ H NMR: (D ₂ O, 700 MHz)	1.37 (m, 2H), 1.50 (m, 2H), 1.7 (m, 1H), 1.78 (m, 2H), 1.85 (m, 1H), 1.92 (t, <i>J</i> = 8.09, 6H), 1.96 (m, 1H), 2.14 (m, 1H), 2.18 (q, <i>J</i> = 6.59, 4H), 2.22 (t, <i>J</i> = 8.09, 6H), 2.35 (t, <i>J</i> = 6.64, 2H), 2.49 (t, <i>J</i> = 7.49, 2H), 2.57 (s, 9H), 3.13 (t, <i>J</i> = 6.98, 2H), 3.36 (t, <i>J</i> = 6.64, 2H), 3.91 (s, 9H), 4.15 (dd, <i>J</i> = 8.99, 5.59 1H), 4.23 (dd, <i>J</i> = 8.70, 4.97 1H), 4.67 (s, 9H), 7.00 (s, 3H)
¹³ C NMR: (D ₂ O, pH 3, 175 MHz)	20.5, 21.9, 22.4, 26.5, 27.8, 29.3, 29.4, 30.1, 30.7, 34.8, 35.5, 35.9, 36.1, 38.5, 39.0, 52.8, 113.6, 138.1, 143.2, 149.9, 159.4, 161.0, 173.2, 175.5, 175.7, 176.2, 176.6, 177.4
ESI-MS: m/z [C ₅₄ H ₇₇ N ₁₁ O ₁₉ + H] ⁺	observed monoisotopic peak = 1184.5494 calculated = 1184.5475
ESI-MS: m/z [C ₅₄ H ₇₇ N ₁₁ O ₁₉ + 2H] ⁺²	observed monoisotopic peak = 592.7768, calculated = 592.7788
ESI-MS: m/z [C ₅₄ H ₇₇ N ₁₁ O ₁₉ + 3H] ⁺³	observed monoisotopic peak = 395.5202, calculated = 395.5221
m/z [C ₅₄ H ₇₄ N ₁₁ O ₁₉ Ga + H] ⁺	observed monoisotopic peak = 1250.45092 calculated = 1250.449102
m/z [C ₅₄ H ₇₄ N ₁₁ O ₁₉ Ga + 2H] ⁺²	observed monoisotopic peak = 625.72846, calculated = 625.72819

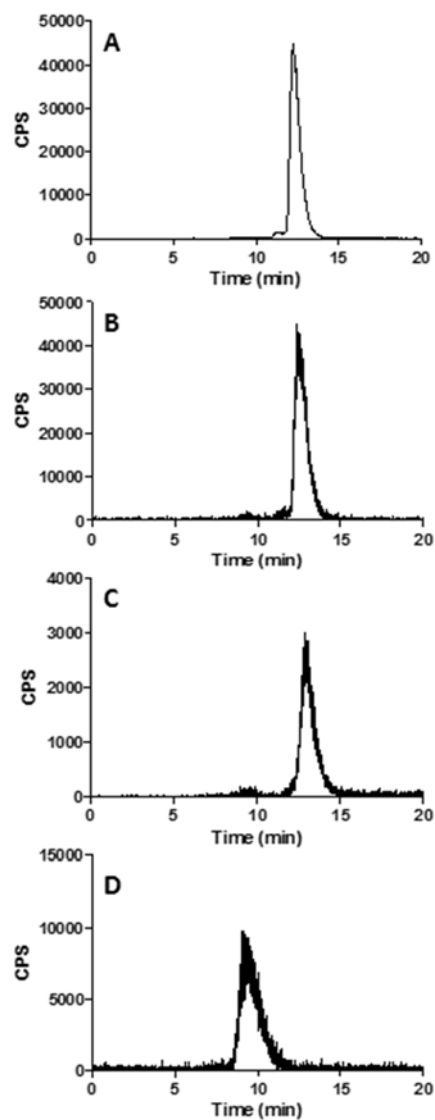
SUPPLEMENTAL TABLE 1: ¹H NMR, ¹³C NMR and ESI-MS data for THP-PSMA

confirming its identity.



SUPPLEMENTAL FIGURE 2: Radiochemical purity as measured by iTLC of ^{68}Ga -THP-PSMA labeled with 5mL one step kits as a function of incubation time at room temperature, with both an IRE generator and an E&Z generator. Broken red line represents quality control threshold of 95% (n=3, mean \pm SD)

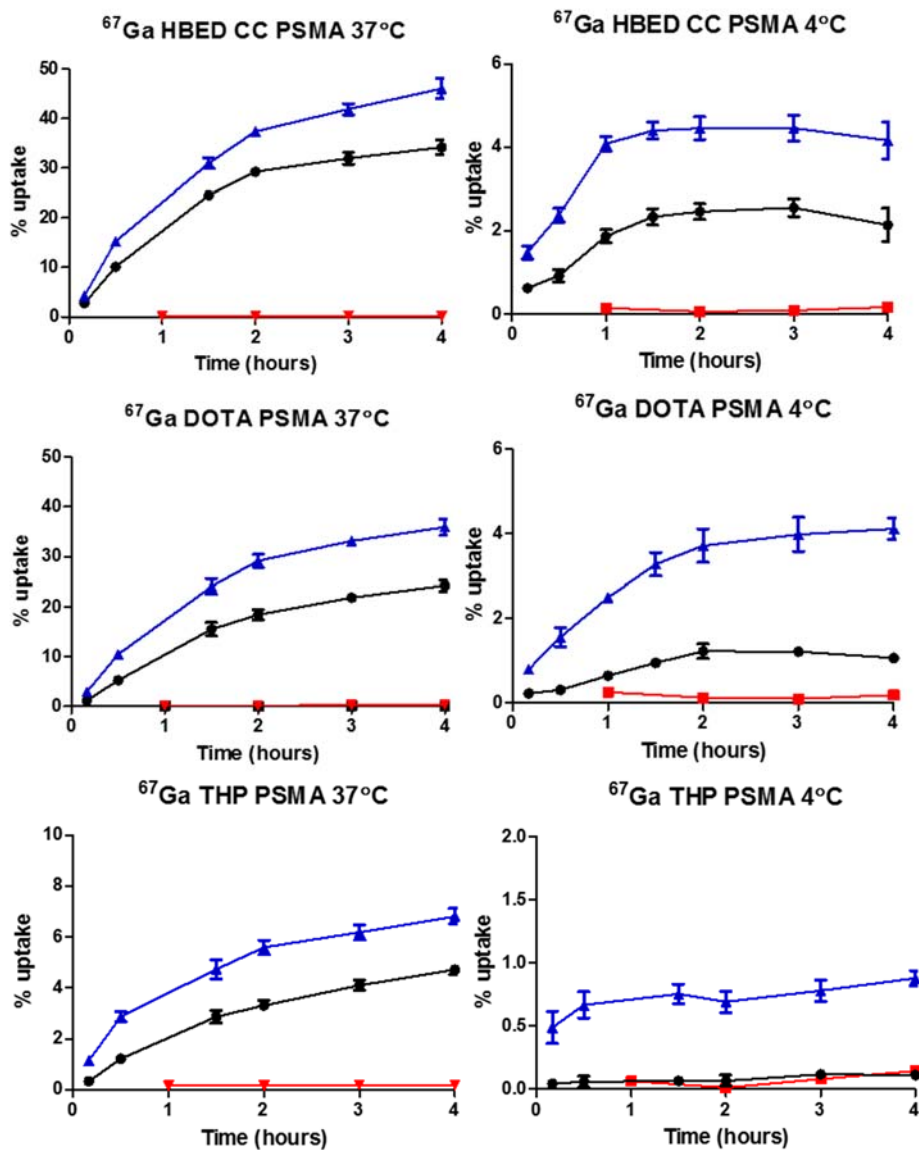
Serum Stability:



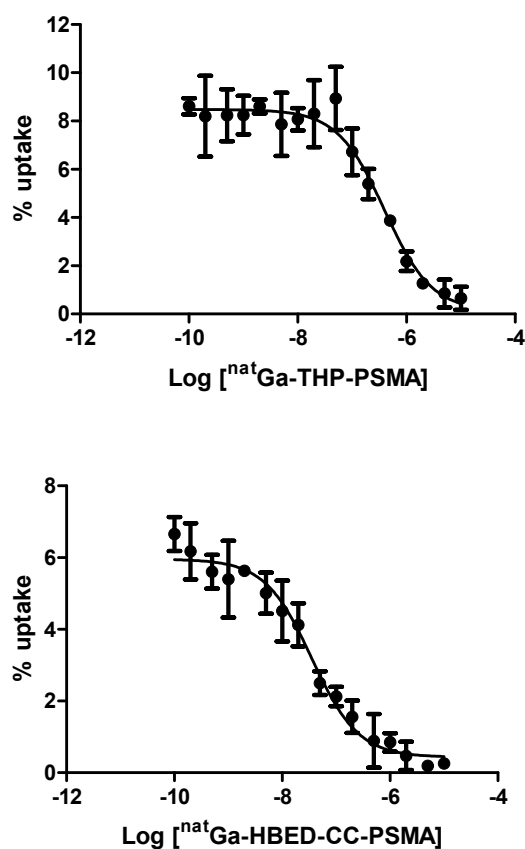
SUPPLEMENTAL FIGURE 3. Size exclusion HPLC chromatograms of (A) ^{68}Ga -THP-PSMA; (B) ^{68}Ga -THP-PSMA after 6 hours incubation in human serum at 37°C; <2% transchelation to serum (C) ^{67}Ga -THP-PSMA after 8 days incubation in human

serum at 37°C <5% transchelation to serum (D) $^{68}\text{Ga}^{3+}$ incubated in human serum for 6 hours. All chromatograms have been decay corrected.

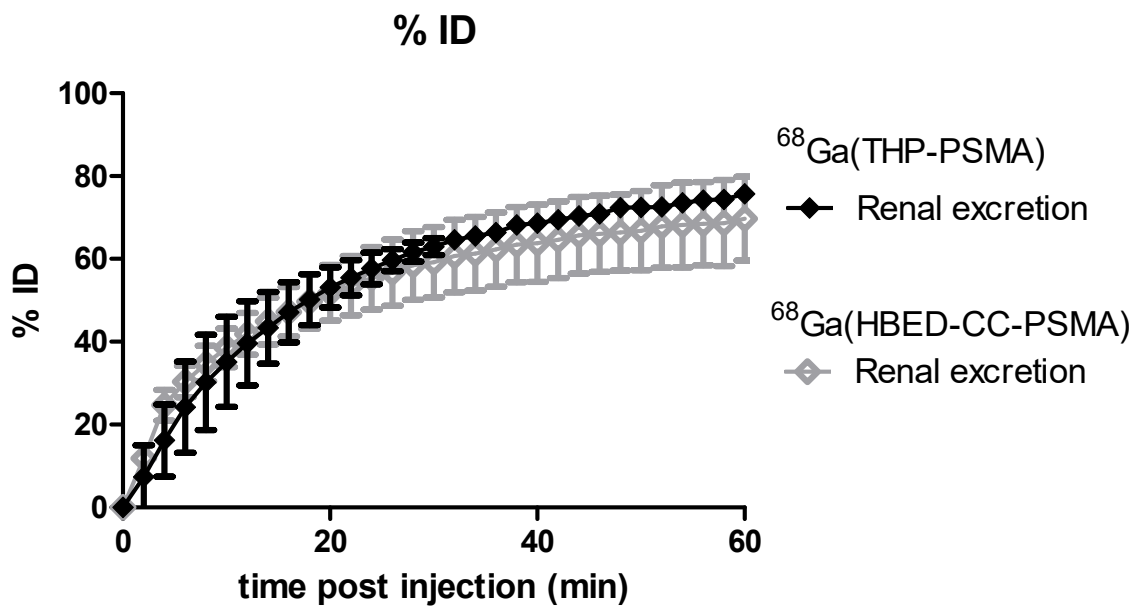
Cell Uptake and Binding Affinity Assays



SUPPLEMENTAL FIGURE 4: Uptake of 1nM ^{67}Ga -HBED-CC-PSMA ^{67}Ga -DOTA-PSMA and ^{67}Ga -THP-PSMA over time at 4°C and 37°C, 1×10^6 cells/mL. Combined internalized and cell surface bound activity DU145-PSMA cells (blue); internalized activity DU145-PSMA cells (black); internalized and cell surface bound activity DU145 cells (red). (n=4, mean \pm SD) per time point per tracer.



SUPPLEMENTAL FIGURE 5: Representative IC_{50} experiments for ^{nat}Ga -THP-PSMA and ^{nat}Ga -HBED-CC-PSMA with 1nM ^{68}Ga -DOTA-PSMA as the probe. (n=4) for each concentration. IC_{50} values in main text are the mean of at least 3 experiments.



SUPPLEMENTAL FIGURE 6. Time activity curves of SCID beige mice bearing DU145-PSMA tumors, derived from PET imaging data from mice imaged with ^{68}Ga -THP-PSMA or ^{68}Ga -HBED-CC-PSMA during one hour post injection. Plotted data is the mean and standard deviation of three mice calculated from the total % ID in the kidneys and bladder combined.

Tumor type	⁶⁸ Ga-THP-PSMA			⁶⁸ Ga-HBED-CC-PSMA
	Group 1	Group 4	Group 3	Group 2
	DU145-PSMA	DU145	DU145-PSMA Blocked PMPA	DU145-PSMA
	(n=3)	(n=3)	(n=3)	(n=3)
Tumor	16.4 ± 7.4	0.69 ± 0.7	1.40 ± 0.2	19.5 ± 7.6
Blood	0.73 ± 0.4	1.23 ± 1.7	1.11 ± 0.7	2.18 ± 1.2
Intestines	0.35 ± 0.1	0.64 ± 0.6	0.71 ± 0.3	1.20 ± 0.5
Kidney	40.4 ± 34	52.3 ± 77	5.20 ± 5.0	63.4 ± 23
Liver	0.45 ± 0.2	0.59 ± 0.7	0.60 ± 0.2	0.69 ± 0.1
Lungs	0.50 ± 0.2	0.94 ± 1.3	0.72 ± 0.3	1.29 ± 0.4
Muscle	0.51 ± 0.4	0.31 ± 0.4	0.21 ± 0.1	0.26 ± 0.1
Salivary glands	0.28 ± 0.1	0.48 ± 0.7	0.33 ± 0.2	0.76 ± 0.1
Spleen	3.70 ± 1.3	3.61 ± 3.1	0.75 ± 0.1	17.6 ± 6.1
Tumor to organ ratios				
Tumor to blood	30.8 ± 28	0.93 ± 0.4	1.64 ± 0.9	11.0 ± 6.0
Tumor to muscle	44.6 ± 28	3.16 ± 1.1	9.79 ± 6.8	77.0 ± 34
Tumor to spleen	4.38 ± 0.8	0.17 ± 0.1	1.87 ± 0.3	1.16 ± 0.42

SUPPLEMENTAL TABLE 2. Ex vivo biodistribution at 1 hour post injection for PET-imaged mice. Mice bearing DU145-PSMA tumors were injected with ⁶⁸Ga-THP-PSMA, ⁶⁸Ga-THP-PSMA plus blocking agent PMPA, or ⁶⁸Ga-HBED-CC-PSMA. Mice bearing control DU145 tumors were imaged with ⁶⁸Ga-THP-PSMA only.

ABBREVIATIONS

ACN - acetonitrile

DCM – dichloromethane

Dde - N-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl);

DIPEA - N,N-diisopropylethylamine

DMF dimethylformamide

DMSO - dimethyl sulfoxide

Fmoc - fluorenylmethyloxycarbonyl

HATU - (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate)

Lys - Lysine

PBS- Phosphate-buffered saline

PMPA – 2-(phosphonomethyl)pentane-1,5-dioic acid

PSMA – Prostate specific membrane antigen

TEA - triethanolamine

TFA - trifluoroacetic acid

THP - tris(hydroxypyridinone)

TIPS – triisopropylsilane

REFERENCES

1. Kampmeier F, Williams JD, Maher J, Mullen GE, Blower PJ. Design and preclinical evaluation of a ^{99m}Tc-labelled diabody of mAb J591 for SPECT imaging of prostate-specific membrane antigen (PSMA). *EJNMMI Res.* 2014;4:13.
2. Zhou T, Neubert H, Liu DY, et al. Iron binding dendrimers: a novel approach for the treatment of haemochromatosis. *J Med Chem.* 2006;49:4171-4182.
3. bin Othman MF, Mitry NR, Lewington VJ, Blower PJ, Terry SY. Re-assessing gallium-67 as a therapeutic radionuclide. *Nucl Med Biol.* 2017;46:12-18.
4. Ščasnár V, Lier JE. The use of SEP-PAK S1 cartridges for the preparation of gallium chloride from the citrate solution. *Eur J Nucl Med Mol Imaging.* 1993;20:273-273.



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