

⁶⁸Ga-Complex Lipophilicity and the Targeting Property of a Urea-Based PSMA Inhibitor for PET Imaging

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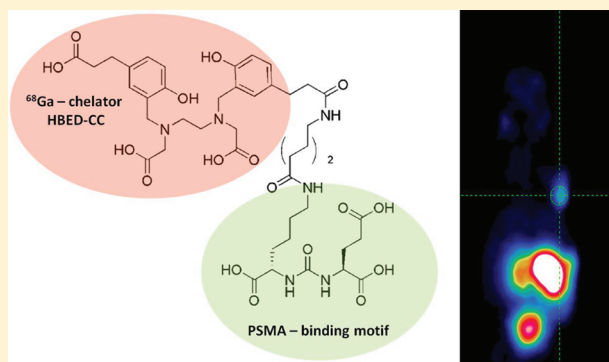
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Supporting Information

ABSTRACT: Urea-based inhibitors of the prostate-specific membrane antigen (PSMA) represent low-molecular-weight peptidomimetics showing the ability to image PSMA-expressing prostate tumors. The highly efficient, acyclic Ga(III) chelator *N,N'*-bis [2-hydroxy-5-(carboxyethyl)benzyl] ethylenediamine-*N,N'*-diacetic acid (HBED-CC) was introduced as a lipophilic side chain into the hydrophilic pharmacophore Glu-NH-CO-NH-Lys which was found favorable to interact with the PSMA “active binding site”. This report describes the syntheses, in vitro binding analyses, and biodistribution data of the radiogallium labeled PSMA inhibitor Glu-NH-CO-NH-Lys(Ahx)-HBED-CC in comparison to the corresponding DOTA conjugate. The binding properties were analyzed using competitive cell binding and enzyme-based assays followed by internalization experiments. Compared to the DOTA-conjugate, the HBED-CC derivative showed reduced unspecific binding and considerable higher specific internalization in LNCaP cells. The ⁶⁸Ga complex of the HBED-CC ligand exhibited higher specificity for PSMA expressing tumor cells resulting in improved in vivo properties. ⁶⁸Ga labeled Glu-NH-CO-NH-Lys(Ahx)-HBED-CC showed fast blood and organ clearances, low liver accumulation, and high specific uptake in PSMA expressing organs and tumor. It could be demonstrated that the PET-imaging property of a urea-based PSMA inhibitor could significantly be improved with HBED-CC.



■ INTRODUCTION

It is still a challenge to select appropriate treatment options for disseminated prostate cancer because of the lack of sensitive imaging agents for diagnosis and therapy monitoring.¹ Prostate-specific membrane antigen (PSMA) is expressed in nearly all prostate cancers with increased expression in poorly differentiated, metastatic, and hormone-refractory carcinomas.² Its expression level is about 1000-fold higher compared to the physiologic levels found in other tissues such as kidney, small intestine, or brain.³ PSMA is primarily restricted to the prostate, it is abundantly expressed at all stages of disease, it is presented on the cell surface, and it is not shed into the circulation.⁴ As a consequence, PSMA can be considered a promising target for specific prostate cancer imaging and therapy.^{5–7}

The radiohalogenated inhibitors of PSMA exhibiting Glu-NH-CO-NH-Lys as a pharmacophore showed the ability to image PSMA-expressing prostate tumor xenografts.^{8,9} More recently, a corresponding DOTA-conjugate was labeled with ⁶⁸Ga which represents an attractive generator-based alternative to cyclotron-based PET radiopharmaceuticals.^{10,11} The readily available ⁶⁸Ga decays with 89% probability by positron emission allowing high-resolution PET images with the option of

accurate quantification. *N,N'*-Bis[2-hydroxy-5-(carboxyethyl)-benzyl]ethylenediamine-*N,N'*-diacetic acid (HBED-CC) was recently proposed as an efficient ⁶⁸Ga chelator with fast complexing kinetics and a high in vitro as well as in vivo complex stability.^{12,13}

Besides the efficient Ga(III) complexing characteristics, HBED-CC was selected because of its lipophilic nature. It was found that the “active binding site” of PSMA is composed of two structural motifs, one representing a lipophilic pocket and the other interacting with urea-based inhibitors.¹⁴ A study comparing a series of linkers located between the urea-based motif and a ^{99m}Tc chelator revealed a clear dependency of binding properties in favor of the more lipophilic compounds.¹⁴ Further studies described the binding site as a pocket interacting with the carboxylic groups and the zinc complexing urea on one hand and with hydrophobic, mostly aromatic groups on the other hand.¹⁵

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Table 1. Analytical and PSMA-Binding Data

Ga-peptide complexes	m/z^a	m/z calculated as $[M+H]^+$	analytical HPLC retention ^b	affinity related IC_{50} [nM] determined in enzyme-based assay ^c	affinity related K_i [nM] determined in cell-based assay ^c
[Ga]7	947.4257	947.4250	3.1 min	7.5 ± 2.2	12.0 ± 2.8
[Ga]8	819.4104	819.4100	2.4 min	19.4 ± 7.1	37.6 ± 14.3
[Ga]12	1284.6368	1284.6364	3.7 min	8.7 ± 3.9	11.1 ± 1.8

^aHigh-resolution mass spectrometry data of the free ligands ($[M+H]^+$). ^bCompounds were labeled with ^{68}Ga ; runs were performed using a linear A–B gradient (0% B to 100% B in 6 min) at a flow rate of 4 mL/min. Solvent A was 0.1% aqueous TFA and solvent B was MeOH. ^cCompounds were complexed with ^{nat}Ga .

This report describes the syntheses of Glu-NH-CO-NH-Lys(Ahx)-HBED-CC (7) and Glu-NH-CO-NH-Lys(Ahx)-DOTA (8) together with a reference PSMA inhibitor previously published by Banerjee et al.¹⁰ After ^{68}Ga complexation, these compounds were evaluated in vitro and in vivo to study the influence of these two chelators and side chain variations.

MATERIALS AND METHODS

Reagents. All commercially available chemicals were of analytical grade and were used without further purification. ^{68}Ga was obtained from a $^{68}\text{Ge}/^{68}\text{Ga}$ generator based on pyrogallol resin support.^{12,16} Protected amino acids were obtained from Novabiochem (Merck, Darmstadt, Germany) or IRIS Biotech (Marktredwitz, Germany).

The preparations were analyzed using reversed-phase high-performance liquid chromatography (RP-HPLC; Chromolith RP-18e, 100×4.6 mm; Merck, Darmstadt, Germany). Runs were performed using a linear A–B gradient (0% B to 100% B in 6 min) at a flow rate of 4 mL/min. Solvent A consisted of 0.1% aqueous TFA and solvent B was 0.1% TFA in CH_3CN or MeOH (in case of determination of radiochemical yield (RCY) or analysis of serum stability).

For all preparative purifications, a Chromolith RP-18e column (100×10 mm; Merck, Darmstadt, Germany) was used with a 6 min gradient starting at 0% raised to 50% and followed by a 1 min increase to 100% B. The flow rate was 6 mL/min. The HPLC system (L6200 A; Merck-Hitachi, Darmstadt, Germany) was equipped with a variable UV and a gamma detector (Bioscan; Washington, USA). UV absorbances were measured at 214 and 254 nm. Mass spectrometry was performed with a MALDI-MS Daltonics Microflex (Bruker Daltonics, Bremen, Germany) system. High-resolution mass spectrometry was performed using a system equipped with a mass spectrometer supporting Orbitrap technology (Exactive, Thermo Fisher Scientific). Full-scan single mass spectra were obtained by scanning $m/z = 200$ – 4000 . NMR data were obtained with Bruker Avance NMR Spectrometers, AV(I)-600 (600 MHz) and AV(III)-400 (400 MHz). The chemical shifts are referenced to solvent signals ($\text{DMSO}-d_6 = 2.50/39.757$ ppm, $\text{DMSO}-d_6 = 39.50$).

Synthesis of Glu-NH-CO-NH-Lys(Ahx)-HBED-CC (7). In a first step, the isocyanate 2 of the glutamyl moiety was generated in situ by adding a mixture of 3 mmol of bis(*tert*-butyl) L-glutamate hydrochloride (Bachem, Switzerland) (1) and 1.5 mL of *N*-ethyl-diisopropylamine (DIPEA) in 200 mL of dry CH_2Cl_2 to a solution of 1 mmol triphosgene in 10 mL of dry CH_2Cl_2 at 0°C over 4 h. After agitation of the reaction mixture for one further hour at 25°C , 0.5 mmol of a resin-immobilized (2-chloro-tritylresin, Merck, Darmstadt) ϵ -allyloxycarbonyl protected lysine was added in one portion (in 4 mL DCM) and reacted for 16 h with gentle agitation leading to

compound 3. The resin was filtered off and the allyloxy-protecting group was removed using 100 mg tetrakis-(triphenyl)palladium(0) (Sigma-Aldrich, Germany) and 400 μL morpholine in 4 mL CH_2Cl_2 for 3 h resulting in compound 4. The following coupling of the aminohexanoic moiety was performed using 2 mmol of the Fmoc-protected 6-amino-hexanoic acid (Sigma-Aldrich, Germany), 1.96 mmol of HBTU (Merck, Darmstadt, Germany), and 2 mmol of *N*-ethyl-diisopropylamine in a final volume of 4 mL DMF. The product 5 was cleaved from the resin by reacting with 4 mL of a 30% 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) in CH_2Cl_2 for two hours at ambient temperature resulting in the *tert*-butyl protected crude product 6 which was purified via RP-HPLC.

To conjugate HBED-CC, the purified product 6 was reacted with an equimolar amount of HBED-CC-TFP-ester synthesized as previously described¹² in the presence of 2 equiv of DIPEA in *N,N*-dimethylformamide (DMF; final volume of 1 mL). After HPLC purification, the remaining *tert*-butyl groups were cleaved at room temperature for one hour using 2 mL trifluoroacetic acid to obtain 7 in ~35% yield after purification by HPLC. High-resolution mass spectrometry was used to confirm the identity (Table 1), and purity was analyzed via analytical HPLC at $\lambda = 206$ nm (Supporting Information). Complete and unequivocal NMR ^1H and ^{13}C signal assignments were obtained (Supporting Information).

Synthesis of Glu-NH-CO-NH-Lys(Ahx)-DOTA (8). Precursor 5 was synthesized as mentioned before. After activation with 3.95 equiv of HBTU and DIPEA for 2 h, 4 equiv of tris(*t*-bu)-DOTA (Chematech, Dijon, France) relative to the resin loading were reacted with 5 after removal of the Fmoc-protecting group in a final volume of 3 mL DMF. The product was cleaved from the resin in a 2 mL mixture consisting of trifluoroacetic acid, triisopropylsilane, and water (95:2.5:2.5). The product was purified via semipreparative RP-HPLC resulting in compound 8 (~30% yield). High-resolution mass spectrometry was used to confirm the identity (Table 1) and purity was analyzed via analytical HPLC at $\lambda = 206$ nm (Supporting Information). Complete and unequivocal ^1H and ^{13}C NMR signal assignments were obtained (Supporting Information).

Synthesis of Reference Compound (12). Synthesis of the DOTA conjugate previously published by Banerjee et al.¹⁰ was performed using a modified solid-phase method. Five equivalents of suberic-acid-bis-(*N*-hydroxysuccinimide-ester) (Sigma-Aldrich, Germany) was conjugated to the side chain of the lysine moiety of compound 4 in the presence of 5 equiv of triethylamine in 3 mL DMF. After 2 h, the resin was washed with DMF. In the presence of five equiv of triethylamine, five equiv of Fmoc-Lys-oAll was coupled to the immobilized NHS-ester of intermediate 9 in a final volume of 3 mL DMF for 16 h resulting in 10. The remaining conjugations of two phenylalanine building blocks and tris(*t*-bu)-DOTA were performed

according to standard Fmoc-protocols resulting in **11**. Cleavage from resin was performed using a 3 mL mixture of trifluoroacetic acid, triisopropylsilane, and water (95:2.5:2.5). The product was purified via RP-HPLC to obtain **12** in ~8% yield. High-resolution mass spectrometry was used to confirm the identity (Table 1), and purity was analyzed via analytical HPLC at $\lambda = 206$ nm (Supporting Information).

⁶⁸Ga-Labeling. Typically, 0.1–1 nmol of Glu-NH-CO-NH-Lys(Ahx)-HBED-CC (**7**, in 0.1 M HEPES buffer pH 7.5) or 1 nmol of the DOTA conjugates (**8/12**, in 0.1 M HEPES buffer pH 7.5) were added in a volume of 100 μ L to a mixture of 10 μ L 2.1 M HEPES solution and 10 μ L [⁶⁸Ga]Ga³⁺ eluate (50–100 MBq). The pH of the labeling solution was adjusted to 4.2. Depending on the chelator, the reaction mixture was incubated either at ambient temperature or at 80 °C for 2 min. The radiochemical yield (RCY) was determined using RP-HPLC.

⁶⁷Ga-Labeling. ⁶⁷Ga was purchased from MDS Nordion (Fleurus, Belgium) as [⁶⁷Ga]GaCl₃ in 0.1 N HCl. Typically, 0.1 nmol of Glu-NH-CO-NH-Lys(Ahx)-HBED-CC (**7**) was added in a volume of 100 μ L to a mixture of 10 μ L 2.1 M HEPES solution, 2 μ L 1 N HCl, and 10 μ L [⁶⁷Ga]GaCl₃ (~100 MBq) in 0.1 N HCl resulting in a solution with a pH of 4.2. The reaction mixture was incubated for 2 min at ambient temperature. The RCY was determined using RP-HPLC.

^{nat}Ga-Complexes. A 10 $\times$ molar excess of Ga(III)-nitrate (Sigma Aldrich, Germany) in 0.1 N HCl (10 μ L) was reacted with the compounds **7**, **8**, or **12** (1 mM in 0.1 M HEPES buffer pH 7.5, 40 μ L) in a mixture of 10 μ L 2.1 M HEPES solution and 2 μ L 1 N HCl for 2 min at 80 °C.

Radiochemical Stability. The radiochemical stability of the ⁶⁸Ga-labeled compounds **7**, **8**, and **12** was determined by incubating in both PBS and human serum for 2 h at 37 °C. An equal volume of MeCN was added to the samples to precipitate serum proteins. Subsequently, the samples were centrifuged for 5 min at 13 000 rpm. An aliquot of the supernatant and the PBS sample was analyzed by RP-HPLC. In addition, serum samples of compound [⁶⁸Ga]**7** were run on a Superdex 200 GL 5/150 gel filtration column (GE Healthcare, Munich, Germany) to analyze protein binding. To analyze the complex stability against human transferrin, a 400 μ L aliquot of [⁶⁸Ga]**7** was added to 250 μ g apo-transferrin in PBS at pH 7 and incubated at 37 °C (water bath) for 2 h. The complex stability was determined using a Superdex 200 GL 5/150 short column with PBS pH 7 as eluent.

Naaladase Assay. Recombinant human PSMA (rhPSMA, R&D systems, Wiesbaden, Germany) was diluted in assay buffer (50 mM HEPES, 0.1 M NaCl, pH 7.5) to 0.4 μ g/mL. The substrate Ac-Asp-Glu (Sigma, Taufkirchen, Germany, 40 μ M final concentration) was mixed with [^{nat}Ga]**7**, [^{nat}Ga]**8**, or [^{nat}Ga]**12** at concentrations ranging from 0.05 nM to 1000 nM in a final volume of 125 μ L assay buffer. The mixtures were combined with 125 μ L of the rhPSMA solution (0.4 μ g/mL) and incubated for one hour at 37 °C. The reaction was stopped by heating at 95 °C for 5 min. 250 μ L of a 15 mM solution of *ortho*-phthalaldehyde (Sigma, Taufkirchen, Germany) was added to all vials and incubated for 10 min at ambient temperature. Finally, 200 μ L aliquots of the reaction solutions were loaded onto a F16 Black Maxisorp Plate (Nunc, Langenselbold, Germany) and read at excitation and emission wavelengths of 330 and 450 nm, respectively, using a microplate reader (DTX-880, Beckman Coulter, Krefeld, Germany). The data were analyzed by a one-site total binding

regression algorithm of *GraphPad Prism* (GraphPad Software, California, USA).

Cell Binding Studies. Cell binding studies were performed using PSMA⁺ LNCaP cells (metastatic lesion of human prostatic adenocarcinoma, ATCC CRL-1740) and PSMA[−] PC-3 cells (bone metastasis of a grade IV prostatic adenocarcinoma, ATCC CRL-1435) cultured in DMEM medium supplemented with 10% fetal calf serum and 2 mmol/L L-glutamine (all from Invitrogen). During cell culture, cells were grown at 37 °C in an incubator with humidified air, equilibrated with 5% CO₂. The cells were harvested using trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA; 0.25% trypsin, 0.02% EDTA, all from Invitrogen).

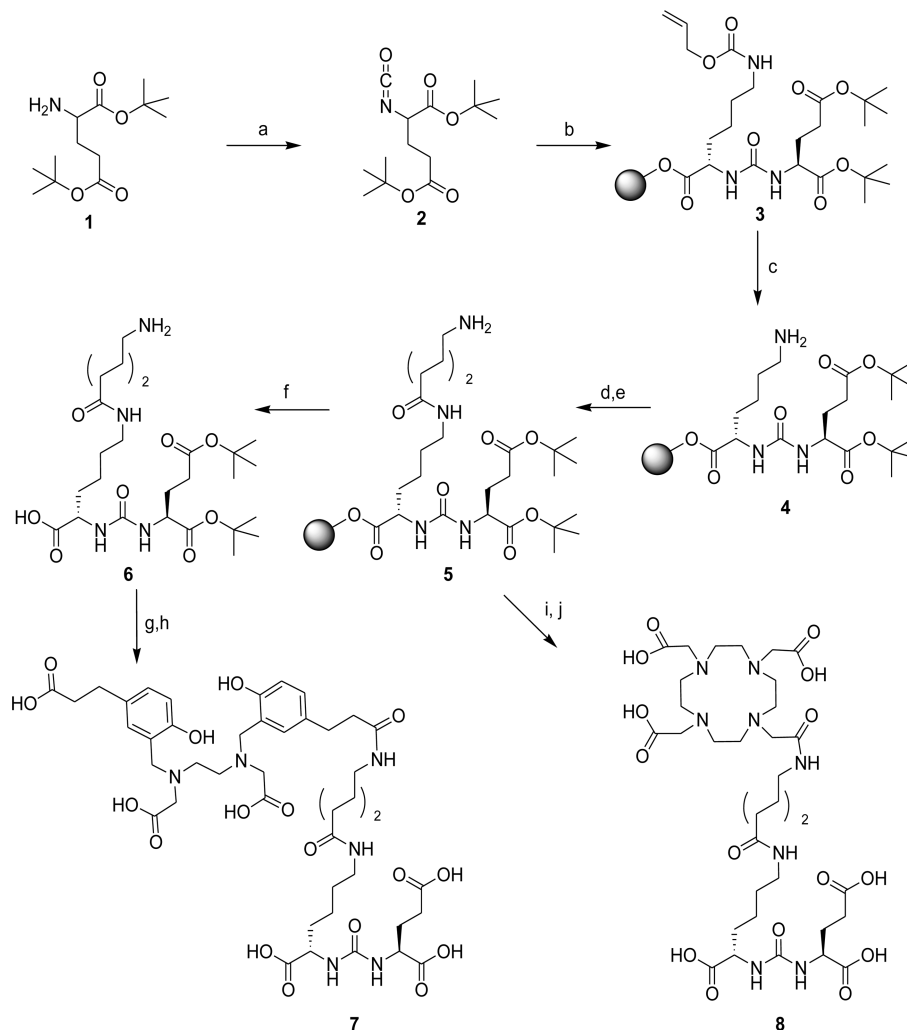
In order to determine the binding affinity, a competitive cell binding assay was performed. LNCaP cells (10⁵ per well) were incubated with a 0.2 nM solution of [⁶⁷Ga]**7** in the presence of 12 different concentrations of [^{nat}Ga]**7**, [^{nat}Ga]**8**, or [^{nat}Ga]**12** (0–5000 nM, 200 μ L/well). After incubation at ambient temperature for 1 h with gentle agitation, the binding buffer was removed using a multiscreen vacuum manifold (Millipore, Billerica, MA). After washing twice with 100 μ L and once with 200 μ L ice-cold binding buffer, the cell-bound radioactivity was measured with a gamma counter (Packard Cobra II, GMI, Minnesota, USA). The 50% inhibitory concentration (IC₅₀) values were calculated by fitting the data using a nonlinear regression algorithm (GraphPad Software). Experiments were performed at least three times including quadruplicate sample measurements.

Determination of Binding Specificity and Internalization. Internalization experiments were performed as previously described.¹⁷ Briefly, 10⁵ LNCaP or PC-3 cells were seeded in poly(L-lysine)-coated 24-well cell culture plates 24 h before incubation. After washing with PBS, the cells were incubated with the radiolabeled compounds [⁶⁸Ga]**7**, [⁶⁸Ga]**8**, and [⁶⁸Ga]**12** (25 nM final concentration) for 45 min at 37 °C and at 4 °C, respectively. To determine specific cell uptake, cells were blocked with 2-(phosphonomethyl)-pentanedioic acid (PMPA, Axxora, Loerrach, Germany) to a final concentration of 100 μ M. Cellular uptake was terminated by washing 4 times with 1 mL of ice-cold PBS. To remove surface-bound radioactivity, cells were incubated twice with 0.5 mL glycine-HCl in PBS (50 mM, pH 2.8) for 5 min at room temperature. The cells were washed with 1 mL of ice-cold PBS and lysed using 0.5 mL of 0.3 M NaOH. The surface-bound and internalized fractions were measured in a gamma counter.

Specificity of binding was additionally analyzed in a concentration-dependent cell uptake experiment. Solutions of [⁶⁸Ga]**7**, [⁶⁸Ga]**8**, or [⁶⁸Ga]**12** at final concentrations of 2.5, 25, and 250 nM were added to 7 $\times$ 10⁵ cells suspended in 50 μ L OPTIMEM medium (Gibco, Auckland, New Zealand). After 45 min at 37 °C, the samples were briefly vortexed and a 10 μ L aliquot was transferred to a 400 μ L microfuge tube (Roth, Germany) containing 350 μ L of a 75:25 mixture of silicon oil, density 1.05 (Sigma Aldrich, Germany), and mineral oil, density 0.872 (Acros, Nidderau, Germany). Separation of cells from the medium was performed by centrifugation at 12 000 rpm for 2 min. After freezing the tubes using liquid nitrogen, the bottom tips containing the cell pellet were cut off. The cell pellets and the supernatants were separately counted in a gamma counter.

Biodistribution and PET Imaging. Five $\times$ 10⁶ cells of either LNCaP or PC-3 in 50% Matrigel (Becton Dickinson, Heidelberg, Germany) were subcutaneously inoculated into the right trunk of male 7- to 8-week-old BALB/c nu/nu mice

Scheme 1. Syntheses of Glu-NH-CO-NH-Lys(Ahx)-HBED-CC (7) and Glu-NH-CO-NH-Lys(Ahx)-DOTA (8)



(a) Triphosgene, DIPEA, CH_2Cl_2 , 0°C ; (b) H-Lys(Alloc)-2CT-Resin, CH_2Cl_2 ; (c) $\text{Pd}[\text{P}(\text{C}_6\text{H}_5)_3]_4$, morpholine, CH_2Cl_2 ; (d) Fmoc-6-Ahx-OH, HBTU, DIPEA, DMF; (e) 20% piperidine, DMF; (f) hexafluoroisopropanol/ CH_2Cl_2 ; (g) HBED-CC-TFP ester, DIPEA, DMF; (h) TFA; (i) tris(*t*-bu)DOTA, HBTU, DIPEA; (j) TFA.

(Charles River Laboratories). The tumors were allowed to grow for 3 to 4 weeks until approximately 1 cm^3 in size.

The ^{68}Ga -radiolabeled compounds of 7, 8, and 12 were injected via tail vein (1–2 MBq per mouse; 0.1–0.2 nmol). At 1 h after injection, the animals were sacrificed. Organs of interest were dissected, blotted dry, and weighed. The radioactivity was measured with a gamma counter and calculated as % ID/g.

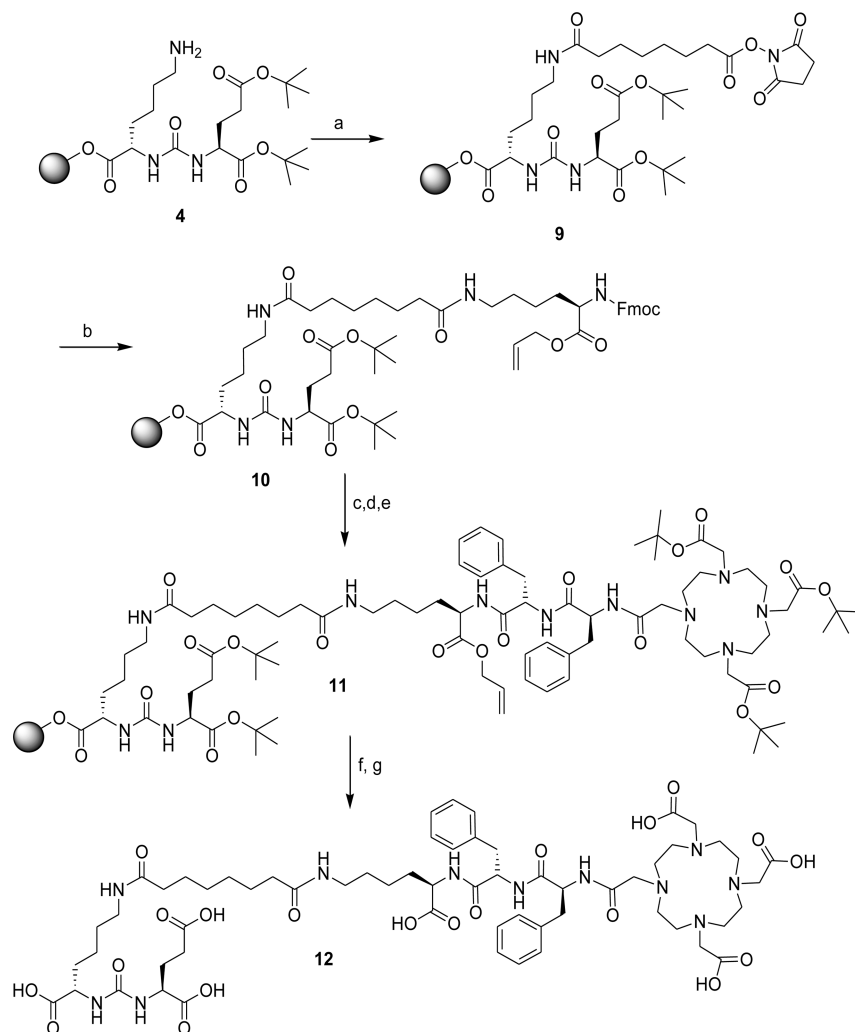
For the microPET studies, 10–25 MBq of compounds ^{68}Ga 7 and ^{68}Ga 8 in a volume of 0.15 mL (~ 0.5 nmol) were injected via a lateral tail vein into mice bearing LNCaP tumor xenografts. The anesthetized animals (2% sevoflurane, Abbott, Wiesbaden, Germany) were placed in prone position into the Inveon small animal PET scanner (Siemens, Knoxville, Tenn, USA) to perform a 50 min dynamic microPET scan starting at 1 min post injection followed by a 20 min static scan.

Statistical Aspects. All experiments were performed at least in triplicate. Quantitative data were expressed as mean $\pm$ SD. If applicable, means were compared using Student's *t*-test. *P* values of <0.05 were considered statistically significant.

RESULTS

Chemistry. The synthesis of the resin-bound intermediate 5 was performed using solid-phase chemistry as outlined in Scheme 1. To couple the respective chelators, compound 5 was either cleaved from resin and reacted with an equimolar amount of HBED-CC-TFP-ester resulting in 7 or coupled with HBTU activated tris(*t*-bu)DOTA resulting in the DOTA-conjugate 8. The preparation of the reference compound 12 previously published by Banerjee et al.¹⁰ was performed using a modified solid-phase method as outlined in Scheme 2. Briefly, disuccinimidyl suberate was conjugated to the ϵ -amino group of the lysine group of 4. After Fmoc-Lys-OAll was coupled to the resulting immobilized NHS-ester, the conjugation of two phenylalanine building blocks and tris(*t*-bu)-DOTA were performed according to standard Fmoc-protocols. Analytical data of compounds 7, 8, and 12 are summarized in Table 1.

Radiolabeling and Stability. Radiolabeling with ^{68}Ga was performed at pH 4.2 by incubating the conjugates 7, 8, or 12 in a mixture consisting of 50–100 MBq ^{68}Ga 3+ and HEPES. Radiochemical yields were determined by RP-HPLC analysis of the reaction mixtures. The retention times obtained for each compound are shown in Table 1. An amount of 0.1 nmol of the

Scheme 2. Modified Solid-Phase Synthesis of the DOTA Conjugate Previously Published by Banerjee et al.¹⁰


(a) Disuccinimidyl suberate (DSS), TEA, DMF; (b) Fmoc-Lys-oAll, TEA, DMF; (c) 20% piperidine, DMF; (d) Fmoc-Phe-OH, HBTU, DIPEA, DMF; (e) tris(*t*-bu)DOTA, HBTU, DMF; (f) Pd[P(C₆H₅)₃]₄, morpholine, CH₂Cl₂; (g) TFA, triisopropylsilane, H₂O (95/2.5/2.5).

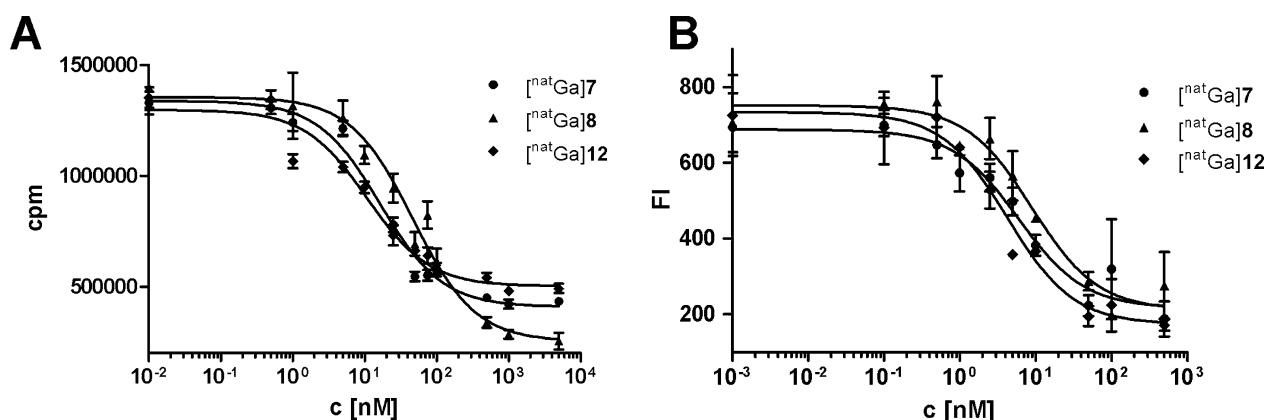


Figure 1. Determination of binding affinity of compounds [natGa]7, [natGa]8, and [natGa]12 by competitive titration on LNCaP cells (A) and purified receptor using an enzyme-based assay (B). Data are expressed as mean $\pm$ SD ($n = 4$). cpm = counts per minute; FI = fluorescence intensity.

HBED-CC conjugate 7 in a final concentration of 1.7 μ M led to a radiochemical yield of more than 99% in less than 1 min at room temperature. As a consequence, specific activities in the range 500–1000 GBq/ μ mol were obtained. In order to achieve comparable high radiochemical yields with the DOTA-

conjugates 8 and 12, the compounds were incubated for 2 min at 80 $^{\circ}$ C using 1 nmol precursor.

Incubation of the ⁶⁸Ga-labeled compounds 7, 8, and 12 in human serum for 2 h at 37 $^{\circ}$ C resulted in no detectable changes in the radiograms. Stability of the ⁶⁸Ga-labeled HBED-CC-

conjugate 7 could additionally be demonstrated by transferrin challenging and incubation in human serum. No activity was exchanged in presence of 250 μ g transferrin or transferred to other serum proteins.

Determination of PSMA Affinity. The variants were competitively analyzed in terms of their binding capacity by performing an enzyme-based assay on rhPSMA (Naaladase-Assay) and a binding assay on LNCaP cells with ^{68}Ga -labeled 7 (RCY > 99%; specific activity ~ 1000 GBq/ μmol) as radioligand. The affinity related IC_{50} and the calculated K_i values of both assays were determined by analyzing the respective binding curves (Figure 1) with *GraphPad Prism*. The data are summarized in Table 1. Compared to the DOTA conjugate [^{68}Ga]8, the HBED-CC conjugate [^{68}Ga]7 and the more lipophilic reference compound [^{68}Ga]12 showed slightly better affinities to both the purified extracellular receptor rhPSMA and LNCaP cells.

Specific Cell Binding and Internalization. Comparative cell uptake and internalization experiments with LNCaP (PSMA positive) and PC-3 (PSMA negative) cells revealed unspecific cell uptake of [^{68}Ga]8 and [^{68}Ga]12 in both cell lines which could not be blocked with excess PMPA (Figure 2A). In contrast, the HBED-CC conjugate ([^{68}Ga]7) showed almost no uptake in PSMA-negative PC-3 cells or in LNCaP cells blocked with PMPA. Furthermore, [^{68}Ga]7 significantly revealed higher specific internalization and cell surface accumulation as compared to the DOTA conjugates (Figure 2B). In another cell uptake experiment, three different concentrations of the radiolabeled compounds (2.5 nM, 25 nM, and 250 nM) were incubated with either LNCaP (Figure 3A) or PC-3 cells (Figure 3B). In accordance with the results received from the internalization experiments, [^{68}Ga]7 showed a considerably higher uptake in LNCaP cells. Furthermore, the unspecific uptake in PC-3 cells was lower as compared to the DOTA derivatives [^{68}Ga]8 and [^{68}Ga]12.

Biodistribution Studies. The organ and tumor uptake values of the complexes [^{68}Ga]7, [^{68}Ga]8, and [^{68}Ga]12 differed in accordance with the aforementioned *in vitro* data (Figure 4). The HBED-CC conjugated compound [^{68}Ga]7 was cleared rapidly from the circulation and PSMA negative tissue. Remarkably, the liver activity of [^{68}Ga]7 amounted to only $0.87 \pm 0.05\%$ ID/g as early as one hour after injection. The high kidney, spleen, and lung uptake of $139.4 \pm 21.4\%$ ID/g, $17.90 \pm 2.87\%$ ID/g, and $2.49 \pm 0.27\%$ ID/g was nearly completely blocked to $4.02 \pm 1.14\%$ ID/g, $1.54 \pm 0.33\%$ ID/g, and $0.64 \pm 0.32\%$ ID/g, respectively, after the coinjection of 2 mg/kg 2-PMPA (Figure 4B). The tumor uptake amounted to $7.70 \pm 1.45\%$ ID/g on LNCaP and $1.30 \pm 0.12\%$ ID/g on PC-3.

The DOTA complex [^{68}Ga]8 showed a completely different uptake pattern. The liver uptake of the DOTA-conjugate was enhanced by a factor of 5.7, the tumor uptake was reduced by factor 2.6, and the kidney uptake was surprisingly lowered to background values. The uptake values of PSMA-negative PC-3 tumors were low for all compounds ($1.30 \pm 0.13\%$ ID/g ([^{68}Ga]7), $0.60 \pm 0.06\%$ ID/g ([^{68}Ga]8), and $0.58 \pm 0.07\%$ ID/g ([^{68}Ga]12), respectively) (Figure 4C).

PET-Imaging. The tumor-targeting efficiency of [^{68}Ga]7 and [^{68}Ga]8 was evaluated by 50 min dynamic microPET scans followed by a 20 min static scan. The differences observed in biodistribution could have also been visualized by PET imaging. The complexes [^{68}Ga]7 and [^{68}Ga]8 were injected intravenously into LNCaP tumor-bearing mice, and microPET scans were carried out for up to 70 min p.i. A coronal PET slice after

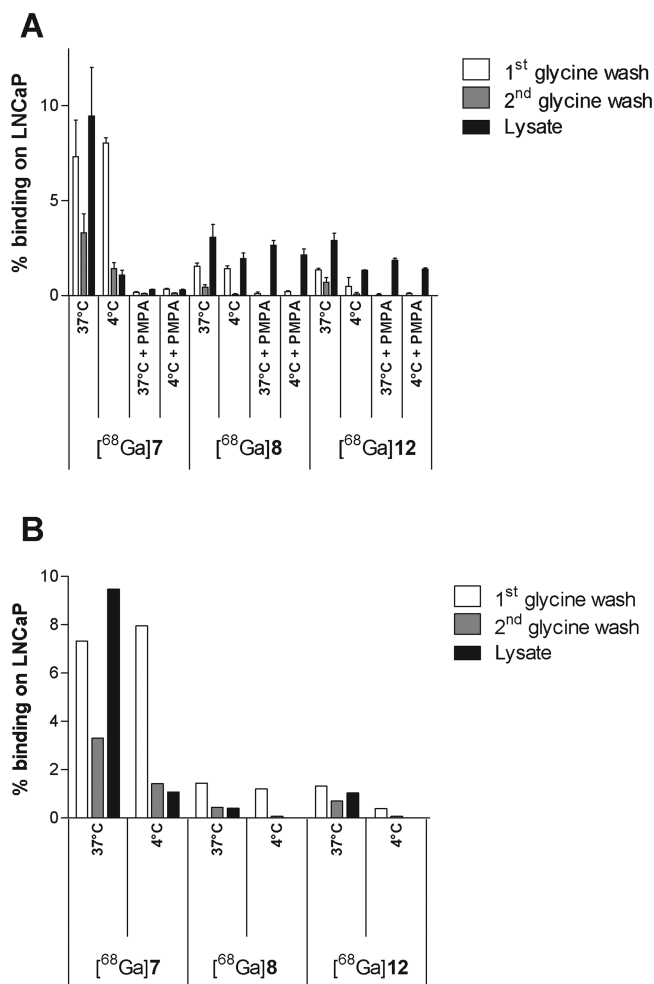


Figure 2. Cell binding and internalization of compounds [^{68}Ga]7, [^{68}Ga]8, and [^{68}Ga]12 (A). Specific cell uptake was evaluated by blockade with 100 μM PMPA. Specificity of cell uptake (B) was calculated by subtracting the respective signals resulting from PMPA-blockage. Values are expressed as % of applied radioactivity bound to 10^6 cells. Data are expressed as mean $\pm$ SD ($n = 3$).

injection of [^{68}Ga]7 is shown in Figure 5a. The DOTA complex [^{68}Ga]8 failed to image the tumor proven by the time activity curves over tumor and muscle (Figure 5B).

Comparison of Biological Properties of L- and D-Forms of [^{68}Ga]7. The ^{68}Ga complex of D-Glu-NH-CO-NH-Lys(Ahx)-HBED-CC was synthesized and compared with the corresponding L-isomer. Time-activity curves obtained from dynamic PET measurements revealed D-[^{68}Ga]7 to be cleared from the kidneys into the urinary bladder while L-[^{68}Ga]7 is retained in this organ (Figure 6). This finding is supported by the $\sim 10^3$ times lower PSMA affinity of D-[^{68}Ga]7 (data not shown).

DISCUSSION

PSMA is strongly expressed on prostate cancer cells¹⁸ and therefore represents a promising target for the development of imaging agents for tumor staging and followup. Among the currently used radioisotopes, ^{68}Ga exhibits several favorable properties as there are the availability and the facile labeling technology by means of complexation.¹¹ A series of chelators are available for the complexation of ^{68}Ga which are conjugated to targeting carrier molecules. Besides some reported stabilizing

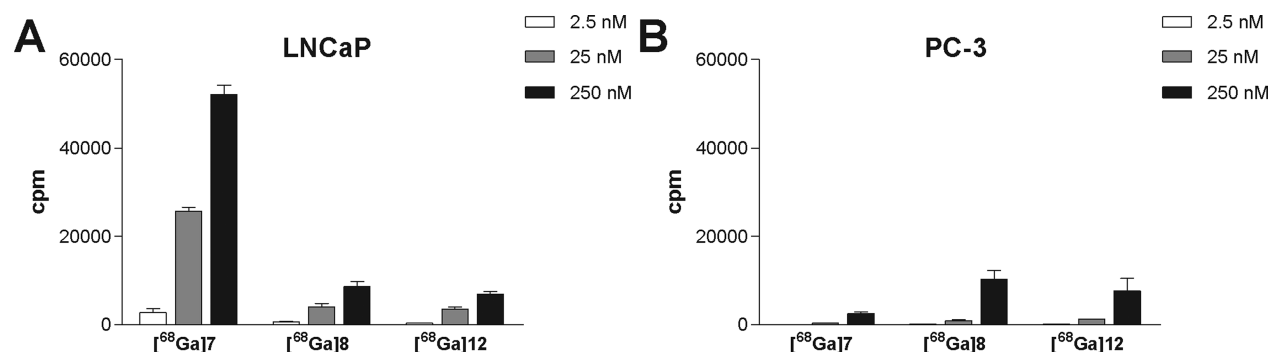


Figure 3. Binding of ^{68}Ga 7, ^{68}Ga 8, and ^{68}Ga 12 to LNCaP (PSMA⁺) cells (A) and PC-3 (PSMA⁻) cells (B) depending on ^{68}Ga -complex concentration. Values were normalized with respect to added radioactivity of each compound. Data are expressed as mean $\pm$ SD ($n = 3$).

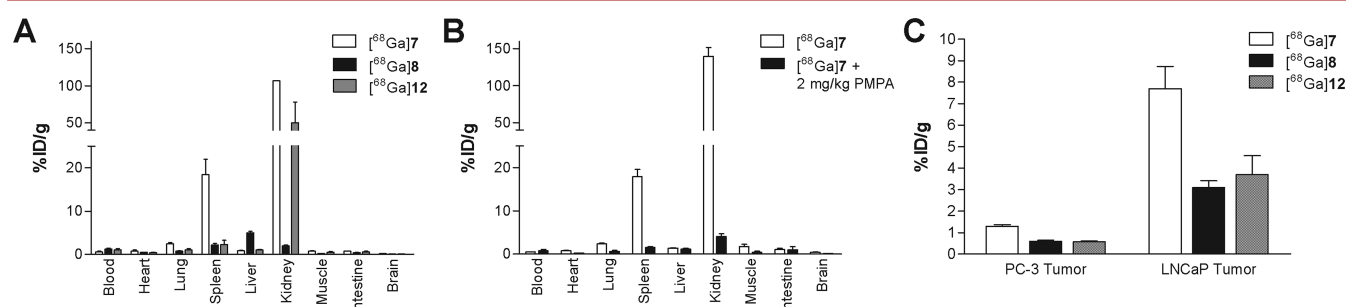


Figure 4. Organ distribution expressed as % ID/g tissue one hour post injection. (A) Comparison of the compounds ^{68}Ga 7 (PSMA-binding motif coupled to HBED-CC), ^{68}Ga 8 (PSMA-binding motif coupled to DOTA), and ^{68}Ga 12. (B) PSMA-blocking by coadministration of 2 mg/kg body weight PMPA indicating PSMA-specific uptake in kidney and spleen. (C) Uptake in PC-3 tumors and LNCaP tumors 1 h after injection. Data are expressed as mean $\pm$ SD ($n = 3$).

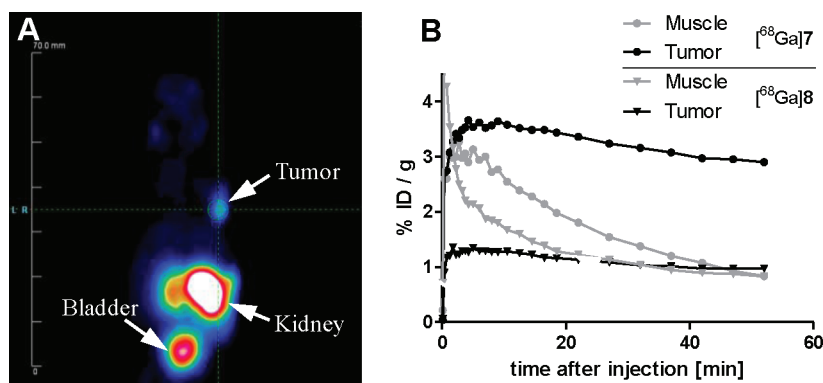


Figure 5. Whole-body coronal microPET image of an athymic male nude mice bearing LNCaP tumor xenografts. The tumor-targeting efficacy of ^{68}Ga 7 was demonstrated by dynamic microPET scan (B) followed by a static scan (A). The static scan up to 1 h post injection of ^{68}Ga 7 is shown in (A). Approximately 15 MBq/mouse was injected. Graph B shows the respective time-activity curves in muscle and tumor for ^{68}Ga 7 and ^{68}Ga 8.

effects or pretargeting approaches,^{17,19} the function of the chelator in radiopharmaceuticals is usually restricted to serving as a complexing agent without influencing the receptor binding. However, the size-demanding radiometal complexes often exert their influence on the targeting molecule by changing lipophilicity or charge. In particular, the pharmacological property of small molecules can strongly be influenced or abolished with respect to its target binding properties.

An interesting study comparing various linkers located between the PSMA binding group 2-[3-(1,3-dicarboxypropyl)-ureido]pentanedioic acid (DUPA) and a $^{99\text{m}}\text{Tc}$ complex showed that the lipophilicity of the linker significantly correlates with improved binding properties.¹⁴ Crystal structure investigations also indicated that, besides the electrostatic

interactions of urea and carboxylic groups at the active, Zn(II)-containing center of PSMA, there are lipophilic interactions resulting from a hydrophobic pocket located next to the active site.^{8,20} A further study substantiates the PSMA active site as a pocket with multiple interactions as well. The pharmacophore was proposed to present three carboxylic groups able to interact with the respective side chains of PSMA, an oxygen as part of zinc complexation in the active center and an aromatic structure able to interact with a hydrophobic part of the binding pocket composed of tyrosines.¹⁵ This work suggests that these interactions are additive in terms of functional efficiency. Only inhibitors which interact with all parts of the binding site displayed a tight binding mode and a high rate of internal-

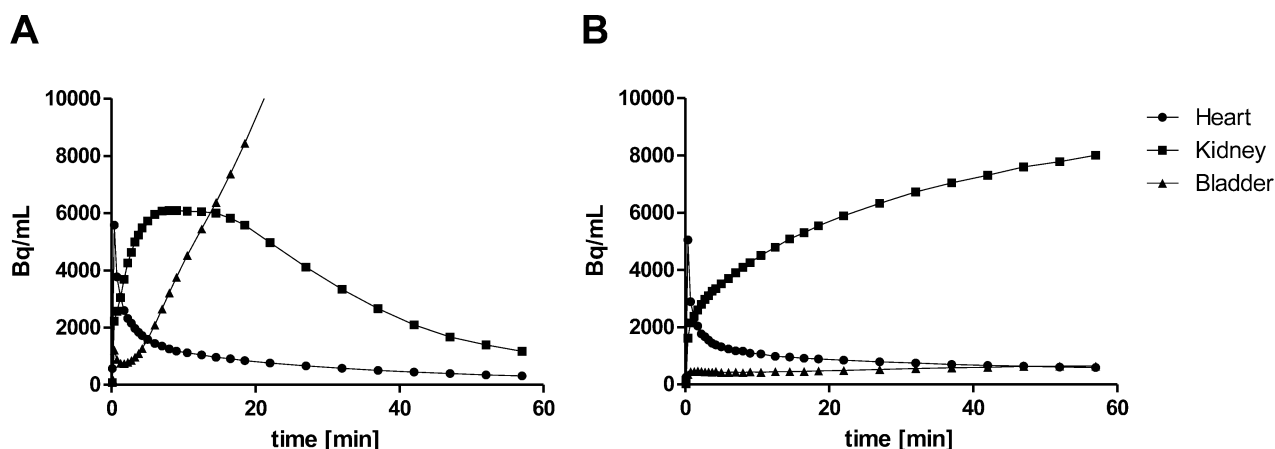


Figure 6. Time–activity curves taken from dynamic PET measurements and expressed as Bq/mL organ normalized to injected dose. The D-form of ^{68}Ga 7 (A) shows $\sim 10^3$ reduced binding capacity to PSMA as compared to the L-form (B). As a consequence, the L-form accumulates in kidneys whereas the D-isomer is rapidly cleared from kidneys into the bladder.

ization. Molecules lacking one of these interactions showed other modes of action and weaker binding.¹⁵

These results led to the development of the amphiphilic, PSMA-specific tracer ^{68}Ga 7 consisting of the urea-based pharmacophore and the ^{68}Ga -HBED-CC complex able to interact with the hydrophobic binding pocket. Two ^{68}Ga -DOTA complexes, the hydrophilic ^{68}Ga 8 and the amphiphilic ^{68}Ga 12, were compared with ^{68}Ga 7 in terms of PSMA-binding characteristics and internalization.

Compared to the DOTA complexes ^{68}Ga 8 and ^{68}Ga 12, the HBED-CC complex ^{68}Ga 7 showed a significantly enhanced PSMA-specific internalization in LNCaP cells (Figure 2B). In another cell uptake experiment, where three different concentrations of the radiolabeled compounds were given to either LNCaP (Figure 3A) or PC-3 cells (Figure 3B), ^{68}Ga 7 showed a considerably higher uptake in LNCaP cells, while the unspecific uptake in PC-3 cells was significantly lower as compared to the DOTA derivatives ^{68}Ga 8 and ^{68}Ga 12. The binding data are characteristic for specific and unspecific binding because the values rise in an exponential manner on PSMA⁺ LNCaP cells (Figure 3A) and linearly on PSMA[−] PC-3 cells (Figure 3B).

Despite clear differences regarding unspecific cell uptake, the obtained binding affinity data of all complexes are nearly identical. This might be explained by the fact that, in contrast to the internalized fraction, the cell surface bound radioactivity of the DOTA complexes ^{68}Ga 8 and ^{68}Ga 12 could successfully be blocked with PMPA indicating that there is negligible unspecific binding on cell surface proteins. As a consequence, their unspecific cell uptake had only moderate effects on the binding affinity, since competitively determined K_D values were derived from cell surface interactions.

Since it has been reported that the lack of only one interaction or its exact position can have considerable influence on the mode of binding and not obligatory on binding affinity,¹⁵ the obvious effects on internalization and cell uptake, while observing nearly constant K_i values after the replacement with a more hydrophilic chelate, can be considered consistent with such findings. The extent of conformational changes of PSMA induced by inhibitor binding determines the rate of internalization as a motif in the cytoplasmic tail seems to mediate PSMA internalization.^{15,21} On the basis of our understanding, these extracellular and intracellular processes

depend on the existence of exact interactions and to a lesser extent on binding affinity. As a consequence, hydrophobic interactions of HBED-CC seem to be responsible for the improved binding properties of ^{68}Ga 7. However, since the DOTA derivative ^{68}Ga 12 showed even more lipophilicity (HPLC retention in Table 1), the optimal position of the aromatic structure is probably more important than the lipophilicity of the linker region in general.

Interestingly, the unspecific binding observed in vitro had no particular effect on the tumor uptake in vivo. Since the concentration has a strong influence on unspecific binding, the extent of unspecific uptake in vivo is low, as only small amounts of the tracer reach the tumor cell surfaces. Furthermore, unspecific binding potentially occurs throughout the whole organism, so that the amounts of active substance in the PSMA-positive as well as -negative tumors are reduced, leading to lower tumor uptakes and tumor-to-background ratios. This might explain why unspecific binding of the DOTA-conjugates could not be demonstrated in vivo.

However, clear differences between the HBED-CC complex and the DOTA derivative were observed in organ distribution and tumor uptake. The lower liver value and the higher tumor uptake was in favor of ^{68}Ga 7. The extremely low kidney uptake of ^{68}Ga 8 cannot be explained but is potentially a binding kinetics related effect in the kidney parenchyma. In contrast, the ^{68}Ga 7 showed high kidney and spleen uptake which was almost completely blocked by coinjection of the PSMA inhibitor PMPA, indicating that the uptake in these organs was mediated by specific binding to PSMA known to be overexpressed in murine kidney and spleen.^{18,22–24} With reference to clinical application, PSMA expression is lower in human kidneys.³

Both organ distribution and PET imaging indicate improved characteristics of the HBED-CC conjugate. Remarkably, the absolute uptake in dissected tumors was higher as compared to PET imaging. This finding can be attributed to the fact that PET imaging was performed using another batch of mice. The tumor values are generally reduced in the PET images, 7.7 to $\sim 3.5\%$ ID/g for ^{68}Ga 7 and 3 to $\sim 1\%$ ID/g for ^{68}Ga 8, which prevented sufficient tumor-to-background contrast. Explanations might be the batch of mice with a potentially different PSMA expression level or the higher amount of injected compound in PET possibly leading to a competitive effect.

However, since the conditions within each experiment were identical for both compounds, we conclude that the tumor-to-background contrast of [^{68}Ga]7 is higher leading to better image quality even in the case of potentially reduced receptor expression.

To substantiate the notion that the reduced kidney accumulation of [^{68}Ga]7 after PMPA blockade is PSMA-specific, the D-form of [^{68}Ga]7 was included in our experiments. The PET study in Figure 6 showed that the D-form is cleared rapidly from the kidney while [^{68}Ga]7 is accumulating with little bladder excretion. This result adds to the specific interaction of the title complex with PSMA.

The recently summarized¹⁵ structural interactions necessary to form a PSMA binding radiopharmaceutical were obviously met by the HBED-CC complex [^{68}Ga]7, which proved to be superior in comparison to the respective ^{68}Ga -DOTA complex [^{68}Ga]8 in all aspects of the described interactions. Besides the favorable functional properties, this compound can efficiently be obtained with fast Ga(III)-complexing kinetics resulting in high radiochemical yields and excellent specific radioactivities. The possibility of an efficient room-temperature labeling procedure in combination with the advantages of ^{68}Ga as a PET radionuclide may allow the development of a kit-formulated radiopharmaceutical for the targeted imaging of prostate cancer.

■ ASSOCIATED CONTENT

■ Supporting Information

Analytical HPLC of the purified compounds and NMR data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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Supporting Information

The ⁶⁸Ga-complex lipophilicity and the targeting property of a urea based PSMA inhibitor for PET imaging

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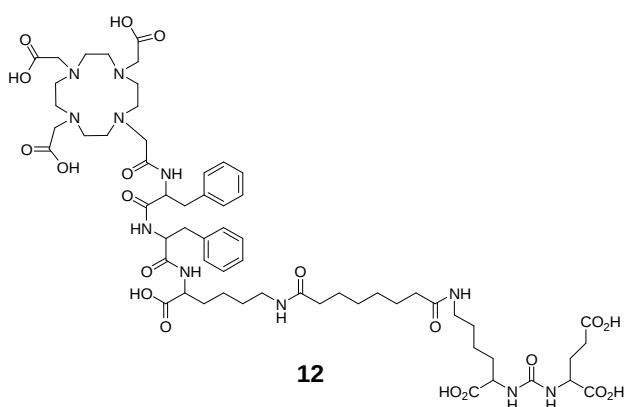
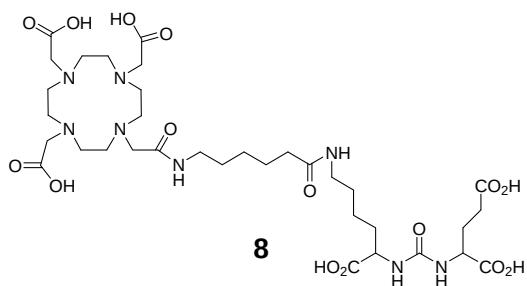
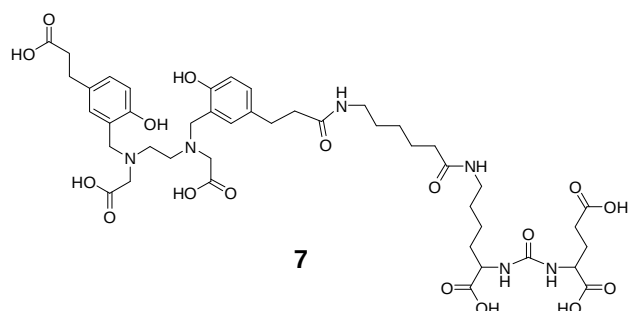
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Table of Content	Page / file
Analytical HPLC for 7,8 and 12	2
NMR data for 7 and 8	3



2

8 in DMSO-d6, NMR 03122 @ 400.13 MHz for 3H

Measurements	Temperature	Time
1D COSY-45	303 K	3.00 h
1D 13C	303 K	12 h
2D COSY-45	303 K	2h, 2h
2D 1H-ROESY Overhauser, 300 ms	303 K	4h, 8 h, 2h
1H/13C-HMBC (8 Hz cor.)	303 K	7.9 h, 13.5 h

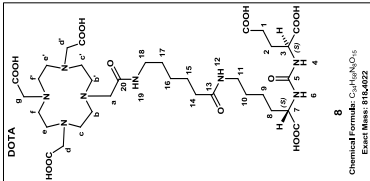
For both compounds, variables and unassigned 1H and 13C signal assignments were obtained through the 2D experiments.

The DOTA derivative 8 was measured at 400 MHz and 303K to achieve fast exchange averaging of DOTA conformations and resolution of all chemical shifts.

Where possible chem. shifts were taken from 1D spectra, when necessary from 2D spectra. 3H coupling constants were estimated by simple 1st-order analysis of multiplets.

Instruments used: Bruker Avance NMR Spectrometers, AVXII-600 (600 MHz) and AVXII-400 (400 MHz), 5 mm sample tubes

Chem. Shifts are referenced to solvent signals **DMSO-d6** = 2.50 / 39.77 ppm, **DMSO-d6** = 39.50 at all temperatures.



Compound 8, 348K in DMSO-d6

13C Shift	1H Shift	multispectrum	3JH-H couplings at 348K
29.722	2.232	ddd	ddd (1a,1b): 5.2 (1a), 5.2 (1b)
27.387	1.759	ddd	ddd (2a,2b): 5.4 (2a), 5.4 (2b)
19.448	0.660	ddd	ddd (3): 5.3 (2b,3)
51.607	6.273	ddd	ddd (4): 7.6 (3,4)
157.026	6.231	ddd	ddd (5): 7.6 (6,7)
53.159	1.672	ddd	ddd (6a,6b): 5.7 (6a), 5.7 (6b)
31.607	1.532	ddd	ddd (7): 13.4 (1a,8b): 5.6 (7)
29.304	1.672	ddd	ddd (8): 5.6 (7,8)
29.310	1.414	ddd	ddd (9): 5.6 (8,9)
38.062	3.023	ddd	ddd (10): 5.6 (9,10)
171.665	7.504	ddd	ddd (11): 5.6 (11,12)
35.043	2.058	ddd	ddd (12): 7.4 (14,15)
24.565	1.510	ddd	ddd (13): 7.5 (15,16)
24.565	1.510	ddd	ddd (14): 5.6 (16,17)
24.565	1.510	ddd	ddd (15): 5.6 (17,18)
38.374	3.105	ddd	ddd (16): 5.3 (18,19), 8.6 (17,18)
168.279	8.120	ddd	ddd (17): 5.6 (19,20)
55.023	3.677	ddd	ddd (18): 5.6 (20,21)
49.155	3.051	ddd	ddd (19): 5.6 (21,22)
53.686	3.010	ddd	ddd (20): 5.6 (22,23)
59.696	3.105	ddd	ddd (21): 5.6 (23,24)
54.046	3.754	ddd	ddd (22): 5.6 (24,25)
173.131	C	ddd	ddd (23): 5.6 (25,26)
173.469	C	ddd	ddd (24): 5.6 (26,27)
173.862	C	ddd	ddd (25): 5.6 (27,28)
174.040	C	ddd	ddd (26): 5.6 (28,29)
174.220	C	ddd	ddd (27): 5.6 (29,30)
174.400	C	ddd	ddd (28): 5.6 (30,31)
174.580	C	ddd	ddd (29): 5.6 (31,32)
174.760	C	ddd	ddd (30): 5.6 (32,33)
174.940	C	ddd	ddd (31): 5.6 (33,34)
175.120	C	ddd	ddd (32): 5.6 (34,35)
175.300	C	ddd	ddd (33): 5.6 (35,36)
175.480	C	ddd	ddd (34): 5.6 (36,37)
175.660	C	ddd	ddd (35): 5.6 (37,38)
175.840	C	ddd	ddd (36): 5.6 (38,39)
176.020	C	ddd	ddd (37): 5.6 (39,40)
176.200	C	ddd	ddd (38): 5.6 (40,41)
176.380	C	ddd	ddd (39): 5.6 (41,42)
176.560	C	ddd	ddd (40): 5.6 (42,43)
176.740	C	ddd	ddd (41): 5.6 (43,44)
176.920	C	ddd	ddd (42): 5.6 (44,45)
177.100	C	ddd	ddd (43): 5.6 (45,46)
177.280	C	ddd	ddd (44): 5.6 (46,47)
177.460	C	ddd	ddd (45): 5.6 (47,48)
177.640	C	ddd	ddd (46): 5.6 (48,49)
177.820	C	ddd	ddd (47): 5.6 (49,50)
178.000	C	ddd	ddd (48): 5.6 (50,51)
178.180	C	ddd	ddd (49): 5.6 (51,52)
178.360	C	ddd	ddd (50): 5.6 (52,53)
178.540	C	ddd	ddd (51): 5.6 (53,54)
178.720	C	ddd	ddd (52): 5.6 (54,55)
178.900	C	ddd	ddd (53): 5.6 (55,56)
179.080	C	ddd	ddd (54): 5.6 (56,57)
179.260	C	ddd	ddd (55): 5.6 (57,58)
179.440	C	ddd	ddd (56): 5.6 (58,59)
179.620	C	ddd	ddd (57): 5.6 (59,60)
179.800	C	ddd	ddd (58): 5.6 (60,61)
180.000	C	ddd	ddd (59): 5.6 (61,62)
180.200	C	ddd	ddd (60): 5.6 (62,63)
180.400	C	ddd	ddd (61): 5.6 (63,64)
180.600	C	ddd	ddd (62): 5.6 (64,65)
180.800	C	ddd	ddd (63): 5.6 (65,66)
181.000	C	ddd	ddd (64): 5.6 (66,67)
181.200	C	ddd	ddd (65): 5.6 (67,68)
181.400	C	ddd	ddd (66): 5.6 (68,69)
181.600	C	ddd	ddd (67): 5.6 (69,70)
181.800	C	ddd	ddd (68): 5.6 (70,71)
182.000	C	ddd	ddd (69): 5.6 (71,72)
182.200	C	ddd	ddd (70): 5.6 (72,73)
182.400	C	ddd	ddd (71): 5.6 (73,74)
182.600	C	ddd	ddd (72): 5.6 (74,75)
182.800	C	ddd	ddd (73): 5.6 (75,76)
183.000	C	ddd	ddd (74): 5.6 (76,77)
183.200	C	ddd	ddd (75): 5.6 (77,78)
183.400	C	ddd	ddd (76): 5.6 (78,79)
183.600	C	ddd	ddd (77): 5.6 (79,80)
183.800	C	ddd	ddd (78): 5.6 (80,81)
184.000	C	ddd	ddd (79): 5.6 (81,82)
184.200	C	ddd	ddd (80): 5.6 (82,83)
184.400	C	ddd	ddd (81): 5.6 (83,84)
184.600	C	ddd	ddd (82): 5.6 (84,85)
184.800	C	ddd	ddd (83): 5.6 (85,86)
185.000	C	ddd	ddd (84): 5.6 (86,87)
185.200	C	ddd	ddd (85): 5.6 (87,88)
185.400	C	ddd	ddd (86): 5.6 (88,89)
185.600	C	ddd	ddd (87): 5.6 (89,90)
185.800	C	ddd	ddd (88): 5.6 (90,91)
186.000	C	ddd	ddd (89): 5.6 (91,92)
186.200	C	ddd	ddd (90): 5.6 (92,93)
186.400	C	ddd	ddd (91): 5.6 (93,94)
186.600	C	ddd	ddd (92): 5.6 (94,95)
186.800	C	ddd	ddd (93): 5.6 (95,96)
187.000	C	ddd	ddd (94): 5.6 (96,97)
187.200	C	ddd	ddd (95): 5.6 (97,98)
187.400	C	ddd	ddd (96): 5.6 (98,99)
187.600	C	ddd	ddd (97): 5.6 (99,100)
187.800	C	ddd	ddd (98): 5.6 (100,101)
188.000	C	ddd	ddd (99): 5.6 (101,102)
188.200	C	ddd	ddd (100): 5.6 (102,103)
188.400	C	ddd	ddd (101): 5.6 (103,104)
188.600	C	ddd	ddd (102): 5.6 (104,105)
188.800	C	ddd	ddd (103): 5.6 (105,106)
189.000	C	ddd	ddd (104): 5.6 (106,107)
189.200	C	ddd	ddd (105): 5.6 (107,108)
189.400	C	ddd	ddd (106): 5.6 (108,109)
189.600	C	ddd	ddd (107): 5.6 (109,110)
189.800	C	ddd	ddd (108): 5.6 (110,111)
190.000	C	ddd	ddd (109): 5.6 (111,112)
190.200	C	ddd	ddd (110): 5.6 (112,113)
190.400	C	ddd	ddd (111): 5.6 (113,114)
190.600	C	ddd	ddd (112): 5.6 (114,115)
190.800	C	ddd	ddd (113): 5.6 (115,116)
191.000	C	ddd	ddd (114): 5.6 (116,117)
191.200	C	ddd	ddd (115): 5.6 (117,118)
191.400	C	ddd	ddd (116): 5.6 (118,119)
191.600	C	ddd	ddd (117): 5.6 (119,120)
191.800	C	ddd	ddd (118): 5.6 (120,121)
192.000	C	ddd	ddd (119): 5.6 (121,122)
192.200	C	ddd	ddd (120): 5.6 (122,123)
192.400	C	ddd	ddd (121): 5.6 (123,124)
192.600	C	ddd	ddd (122): 5.6 (124,125)
192.800	C	ddd	ddd (123): 5.6 (125,126)
193.000	C	ddd	ddd (124): 5.6 (126,127)
193.200	C	ddd	ddd (125): 5.6 (127,128)
193.400	C	ddd	ddd (126): 5.6 (128,129)
193.600	C	ddd	ddd (127): 5.6 (129,130)
193.800	C	ddd	ddd (128): 5.6 (130,131)
194.000	C	ddd	ddd (129): 5.6 (131,132)
194.200	C	ddd	ddd (130): 5.6 (132,133)
194.400	C	ddd	ddd (131): 5.6 (133,134)
194.600	C	ddd	ddd (132): 5.6 (134,135)
194.800	C	ddd	ddd (133): 5.6 (135,136)
195.000	C	ddd	ddd (134): 5.6 (136,137)
195.200	C	ddd	ddd (135): 5.6 (137,138)
195.400	C	ddd	ddd (136): 5.6 (138,139)
195.600	C	ddd	ddd (137): 5.6 (139,140)
195.800	C	ddd	ddd (138): 5.6 (140,141)
196.000	C	ddd	ddd (139): 5.6 (141,142)
196.200	C	ddd	ddd (140): 5.6 (142,143)
196.400	C	ddd	ddd (141): 5.6 (143,144)
196.600	C	ddd	ddd (142): 5.6 (144,145)
196.800	C	ddd	ddd (143): 5.6 (145,146)
197.000	C	ddd	ddd (144): 5.6 (146,147)
197.200	C	ddd	ddd (145): 5.6 (147,148)
197.400	C	ddd	ddd (146): 5.6 (148,149)
197.600	C	ddd	ddd (147): 5.6 (149,150)
197.800	C	ddd	ddd (148): 5.6 (150,151)
198.000	C	ddd	ddd (149): 5.6 (151,152)
198.200	C	ddd	ddd (150): 5.6 (152,153)
198.400	C	ddd	ddd (151): 5.6 (153,154)
198.600	C	ddd	ddd (152): 5.6 (154,155)
198.800	C	ddd	ddd (153): 5.6 (155,156)
199.000	C	ddd	ddd (154): 5.6 (156,157)
199.200	C	ddd	ddd (155): 5.6 (157,158)
199.400	C	ddd	ddd (156): 5.6 (158,159)
199.600	C	ddd	ddd (157): 5.6 (159,160)
199.800	C	ddd	ddd (158): 5.6 (160,161)
200.000	C	ddd	ddd (159): 5.6 (161,162)
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200.400	C	ddd	ddd (161): 5.6 (163,164)
200.600	C	ddd	ddd (162): 5.6 (164,165)
200.800	C	ddd	ddd (163): 5.6 (165,166)
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202.200	C	ddd	ddd (170): 5.6 (172,173)
202.400	C	ddd	ddd (171): 5.6 (173,174)
202.600	C	ddd	ddd (172): 5.6 (174,175)
202.800	C	ddd	ddd (173): 5.6 (175,176)
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203.200	C	ddd	ddd (175): 5.6 (177,178)
203.400	C	ddd	ddd (176): 5.6 (178,179)
203.600	C	ddd	ddd (177): 5.6 (179,180)
203.800	C	ddd	ddd (178): 5.6 (180,181)
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204.200	C	ddd	ddd (180): 5.6 (182,183)
204.400	C	ddd	ddd (181): 5.6 (183,184)
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204.800	C	ddd	ddd (183): 5.6 (185,186)
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205.200	C	ddd	ddd (185): 5.6 (187,188)
205.400	C	ddd	ddd (186): 5.6 (188,189)
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206.200	C	ddd	ddd (190): 5.6 (192,193)
206.400	C	ddd	ddd (191): 5.6 (193,194)
206.600	C	ddd	ddd (192): 5.6 (194,195)
206.800	C	ddd	ddd (193): 5.6 (195,196)
207.000	C	ddd	ddd (194): 5.6 (196,197)
207.200	C	ddd	ddd (195): 5.6 (197,198)
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207.600	C	ddd	ddd (197): 5.6 (199,200)
207.800	C	ddd	ddd (198): 5.6 (200,201)
208.000	C	ddd	ddd (199): 5.6 (201,202)
208.200	C	ddd	ddd (200): 5.6 (202,203)
208.400	C	ddd	ddd (201): 5.6 (203,204)
208.600	C	ddd	ddd (202): 5.6 (204,205)
208.800	C	ddd	ddd (203): 5.6 (205,206)
209.000	C	ddd	ddd (204): 5.6 (206,207)
209.200	C	ddd	ddd (205): 5.6 (207,208)
209.400	C	ddd	ddd (206): 5.6 (208,209)
209.600	C	ddd	ddd (207): 5.6 (209,210)
209.800	C	ddd	ddd (208): 5.6 (210,211)
210.000	C	ddd	ddd (209): 5.6 (211,212)
210.200	C	ddd	ddd (210): 5.6 (212,213)
210.400	C	ddd	ddd (211): 5.6 (213,214)
210.600	C	ddd	ddd (212): 5.6 (214,215)
210.800	C	ddd	ddd (213): 5.6 (215,216)
211.000	C	ddd	ddd (214): 5.6 (216,217)
211.200	C	ddd	ddd (215): 5.6 (217,218)
211.400	C	ddd	ddd (216): 5.6 (218,219)
211.600	C	ddd	ddd (217): 5.6 (219,220)
211.800	C	ddd	ddd (218): 5.6 (220,221)
212.000	C	ddd	ddd (219): 5.6 (221,222)
212.200	C	ddd	ddd (220): 5.6 (222,223)
212.400	C	ddd	ddd (221): 5.6 (223,224)
212.600	C	ddd	ddd (222): 5.6 (224,225)
212.800	C	ddd	ddd (223): 5.6 (225,226)
213.000	C	ddd	ddd (224): 5.6 (226,227)
213.200	C	ddd	ddd (225): 5.6 (227,228)
213.400	C	ddd	ddd (226): 5.6 (228,229)
213.600	C	ddd	ddd (227): 5.6 (229,230)
213.800	C	ddd	ddd (228): 5.6 (230,231)
214.000	C	ddd	ddd (229): 5.6 (231,232)
214.200	C	ddd	ddd (230): 5.6 (232,233)
214.400	C	ddd	ddd (231): 5.6 (233,234)
214.600	C	ddd	ddd (232): 5.6 (234,235)
214.800	C	ddd	ddd (233): 5.6 (235,236)
215.000	C	ddd	ddd (234): 5.6 (236,237)
215.200	C	ddd	ddd (235): 5.6 (237,238)
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215.600	C	ddd	ddd (237): 5.6 (239,240)
215.800	C	ddd	ddd (238): 5.6 (240,241)
216.000	C	ddd	ddd (239): 5.6 (241,242)
216.200	C	ddd	ddd (240): 5.6 (242,243)
216.400	C	ddd	ddd (241): 5.6 (243,244)
216.600	C	ddd	ddd (242): 5.6 (244,245)
216.800	C	ddd	ddd (243): 5.6 (245,246)
217.000	C	ddd	ddd (244): 5.6 (246,247)
217.200	C	ddd	ddd (245): 5.6 (247,248)
217.400	C	ddd	ddd (246): 5.6 (248,249)
217.600	C	ddd	ddd (247): 5.6 (249,250)
217.800	C	ddd	ddd (248): 5.6 (250,251)
218.000	C	ddd	ddd (249): 5.6 (251,252)
218.200	C	ddd	ddd (250): 5.6 (252,253)
218.40			