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Supporting Information

Synthesis and Evaluation of Gd^{III}-Based Magnetic Resonance Contrast Agents for Molecular Imaging of Prostate-Specific Membrane Antigen**

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Supporting Information

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Experimental Methods

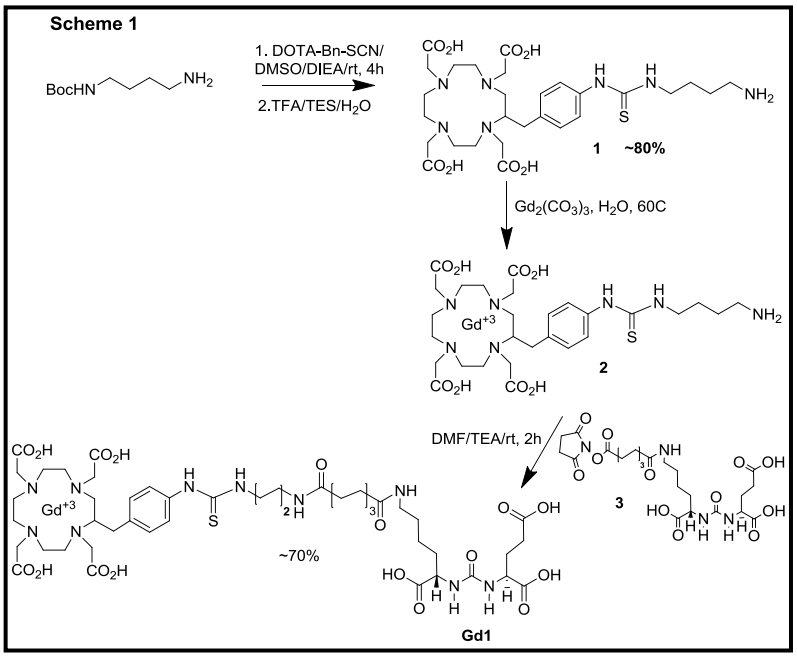
Solvents and chemicals obtained from commercial sources were of analytical grade or better and used without further purification. DOTA-NHS-ester (B-280) and p-SCN-Bn-DOTA (B-205) were purchased from Macrocyclics, Inc. (Dallas, Tx). Gadolinium carbonate, gadolinium acetate, triethylsilane (Et_3SiH), diisopropylethylamine (DIEA) and triethylamine (TEA) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). All other chemicals were purchased from Thermo Fisher Scientific (Pittsburgh, PA) unless otherwise specified. Analytical thin-layer chromatography (TLC) was performed using Aldrich aluminum-backed 0.2 mm silica gel Z19, 329-1 plates and visualized by ultraviolet light (254 nm), I_2 and 1% ninhydrin in EtOH. Flash chromatography was performed using silica gel purchased from Bodman (Aston PA), MP SiliTech 32-63 D 60Å. All experiments were performed in duplicate or triplicate to ensure reproducibility. ^{1}H NMR spectra were recorded on a Bruker Ultrashield™ 400 MHz spectrometer. Chemical shifts (δ) are reported in ppm downfield by reference to proton resonances resulting from incomplete deuteration of the NMR solvent. Low resolution ESI mass spectra were obtained on a Bruker Daltonics Esquire 3000 Plus spectrometer. High resolution mass spectra were obtained by the University of Notre Dame Mass Spectrometry & Proteomics Facility, Notre Dame, IN and from Northwestern University using either ESI or by direct infusion on an Agilent 6210 LC-TOF (ESI, APCI, APPI).

High-performance liquid chromatographic (HPLC) purification of **Gd1, Gd2 and Gd3** was performed using a Phenomenex C_{18} Luna 10 $\times$ 250 mm^2 column on a Waters 600E Delta LC system with a Waters 486 variable wavelength UV/Vis detector, both controlled by Empower software (Waters Corporation, Milford, MA). Compound **1**[2] and **3**[3] were prepared following our previous report. A dual modality monomeric Gd compound with Rhodamine-red-X was prepared following our previous report[2] on PSMA targeted dual modality agent by replacing IRDye800CW dye with Rhodamine-Red™-X. Rhodamine Red™-X, succinimidyl ester was

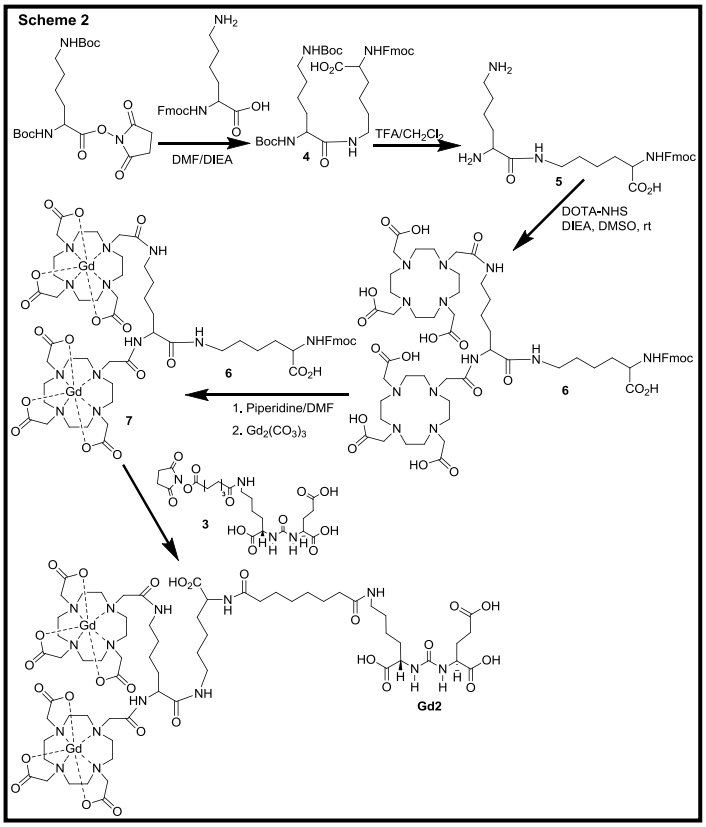
purchased from life technologies. ICP-MS sample preparation and analyses were done following a reported method at Northwestern University.[1]

Synthesis of new contrast agents

(21S,25S)-8,15,23-trioxo-1-((4-((1,4,7,10-tetrakis(carboxymethyl)-1,4,7,10-tetraazacyclododecan-2-yl)methyl)phenyl)amino)-1-thioxo-2,7,16,22,24-pentaazaheptacosane-21,25,27-tricarboxylic acid, **Gd1**. **Gd1** was prepared in three steps following **Scheme 1** as described below. Compound **1** was prepared following our recent report.[2] A solution of **1** (110 mg, 0.17 mmol in 5 mL distilled water) was added to a solution $Gd_2(CO_3)_3$ (85 mg, 0.17 mmol) and the resulting solution left stirring 60°C for 14 h. The solution was filtered using 0.22 μm filtering disk to remove any unreacted solid and then purified by C_{18} flash chromatography using 90/10 to 80/10 water/acetonitrile (0.1 TFA each) solution. ESI-MS *m/Z*: 795.5 $[M+H]^+$. Compound **2** was then further purified by HPLC. To a solution of compound **2** (50 mg, 63 μmol in 1 mL DMSO) was added DIEA (55μl, 313 μmol) and a solution of compound **3** [3] (70 mg, 95 μmol in 1 mL DMSO) was slowly added and left at rt for 3h. The solution was diluted with water and purified by HPLC. Method: solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile), flow rate 8 mL/min. The elution gradient was 100% A and 0% B for 5 min and 100 % A to 80 % A



and 0% B to 20% B over 5 – 25 min, and 80 %A to 20% A and 20% B to 80% B from 25-30 min. HRESI$^+$-MS: Calcd. for $C_{48}H_{74}GdN_{10}O_{17}S$, 1252.4190, $[M+H]^+$, found: 1252.4230.

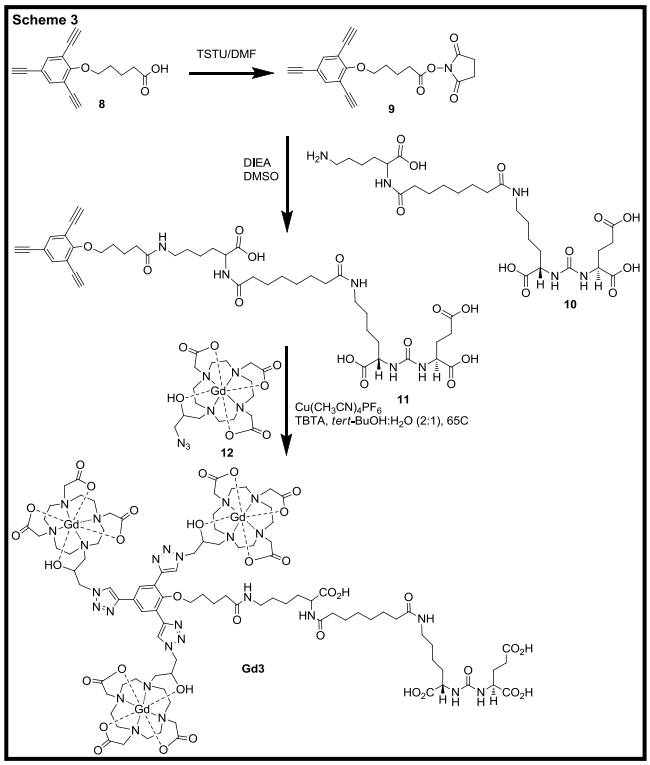


(30S,34S)-2,9,17,24,32-pentaoxo-1-(4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)-8-(2-(4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)acetamido)-3,10,16,25,31,33-hexaazahexatriacontane-11,30,34,36-tetracarboxylic acid, digadolinium(III) salt. Gd2

The compound was prepared by following **Scheme 2**. To a solution of N-Bis-Boc-L-Lysine NHS (3 gm, 6.7 mmol in 10 mL DMF) was added Fmoc-Lys(Boc)-OH (2.49 g, 6.7 mmol in 5 mL DMF) and the solution was kept in a sonication bath at rt for 1 h until a clear solution was obtained. The solution was then stirred for 4h at rt and the solvent was removed under vacuum to obtain **4** in nearly quantitative yield. Compound **4** was further purified by silica gel column using 3/97 MeOH/CH_2Cl_2 as an eluent. ^{1}H NMR ($CDCl_3$) δ: 8.01 (d, 2H), 7.89 (m, 2H), 7.78-7.44 (m, 4H), 6.82(m, 1H), 6.15(m, 1H), 5.58 (m, 1H), 5.01-4.03 (m, 5H), 3.75-3.32(m, 6H), 2.22-1.31 (m, 30H). ESMS *m/Z*: 696 $[M+H]^+$. Compound **4** (2g, 2.9 mmol) was dissolved in 10 mL 1/1 TFA/CH_2Cl_2 solution and left stirring at rt for 2h. After solvent evaporation, the solid residue was washed with 3× 3 mL diethyl ether and dried under vacuum to produce **5** as TFA salt. Compound **5** was obtained in quantitative yield and used without further purification after

lyophilization. ^{1}H NMR (D_2O) δ: 8.01 (d, 2H), 7.89 (m, 2H), 7.78-7.44 (m, 4H), 4.78-4.75 (m, 2H), 4.32 (m, 1H), 4.11-4.09 (m, 1H), 4.01-3.98 (t, 1H), 3.50-3.11 (m, 3H), 3.10-2.99 (m, 2H), 2.01-1.01 (m, 12H). ESMS *m/Z*: 496 $[M+H]^+$. To a solution of DOTA-NHS (100 mg, 0.13 mmol in 0.5 mL DMSO) was added in small portions of **5.2TFA** (32 mg, 0.04 mmol) and DIEA (0.78 mmol, 136 μl) over a period of 45 min at rt. The solution was then stirred for another 2 h and completion of the reaction was monitored using HPLC. After completion of the reaction the reaction was purified by HPLC to obtain **6**. ESMS *m/Z*: 1269 $[M+H]^+$. Compound **6** was treated with 20% piperidine solution in DMF to remove Fmoc group and purified using C_{18} flash chromatography using 90/10 H_2O/CH_3CN (0.1% TFA in each) solution and lyophilized. ESIMS *m/Z*: 1046 $[M+H]^+$. That lyophilized compound **6** (50 mg, 0.047 mmol) was dissolved in distilled water (2 mL) added to solution of $Gd_2(CO_3)_3$ (0.26 mmol in 3 mL water) and stirred at 60°C for 12 h to obtain compound **7**. Compound **7** was purified using C_{18} flash chromatography using a gradient of 90/10 to 80/20 H_2O/CH_3CN (0.1% TFA in each) solution and lyophilized. ESIMS *m/Z*: 1362 $[M+H]^+$. To a solution of **3** [3] (25 mg, 0.004 mmol) in DMSO, was added **7** (40 mg, 0.003 mmol) slowly for 30 min and stirred for about 2h at rt until the reaction was completed. Completion of the reaction was monitored using HPLC. After completion, reaction mixture was subsequently purified by HPLC and product was lyophilized. ESI-MS *m/Z*: 1813.08 $[M+H]^+$, found: 1813.08. Compound was then purified by HPLC. Calcd. for $C_{64}H_{103}Gd_2N_{15}O_{26}$, 1813.5681 $[M]^+$; found 1813.5681 [M+H]. HPLC method: solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile), flow rate 8 mL/min. The elution gradient was 100% A and 0% B for 5 min and 100 % A to 80 % A and 0% B to 20% B over 5 – 25 min, and 80 %A to 20% A and 20% B to 80% B from 25-30 min.

(3S,7S)-5,13,20,28-tetraoxo-32-(2,4,6-tris(1-(2-hydroxy-3-(4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)propyl)-1H-1,2,3-triazol-4-yl)phenoxy)-4,6,12,21,27-pentaazadotriacontane-1,3,7,22-tetracarboxylic acid, trigadolinium(III) salt. Gd3



Gd3 is prepared by using multistep synthesis as shown in **Scheme 3.** Compound **8** was prepared following previous report [4]

2,5-dioxopyrrolidin-1-yl 5-(2,4,6-triethynylphenoxy)pentanoate, 9. To a solution of **8** (300 mg, 1.13 mmol in 5 mL DMF) was added TSTU (440 mg, 1.47 mmol) and TEA (541 μl , 3.39 mmol) and the resulting solution was left stirring at room temp for 4 h until the reaction was completed monitored by TLC. The solvent was removed under high vacuum and the residue was dissolved in CH_2Cl_2 and purified by silica gel column using 40/60 to 50/50 EtOAc/hexane solution as eluent. Fractions containing the product were combined together and evaporated to obtain the desired product as colorless solid. Yield ~310 mg. NMR ($CDCl_3$): δ 7.56 (s, 2H), 4.26 (t, 2H), 3.39 (s, 2H), 3.04 (s, 1H), 2.78 (s, 4H) 2.48 (t, 2H), 2.01-1.80 (m, 4H). ESI-MS: 364 $[M+H]^+$,

(3S,7S)-26-amino-5,13,20-trioxo-4,6,12,21-tetraazahexacosane-1,3,7,22-tetracarboxylic acid 2,2,2-trifluoroacetic acid salt, 10. Compound **10** was prepared following our previous report.[5] Briefly, to a solution of *tert*-Bu protected **3** (100 mg 0.135 mmol dissolved in 1.35 ml DMF), H-Lys(Boc)(O-t-Bu) (59.5 mg, 0.175 mmol) was added followed by DIEA (70.7 μl, 0.135)

and the clear solution was stirred overnight at rt. The solution was then concentrated under vacuum to a clear oily residue. The residue was dissolved in a 2:1 MeCN/water (6 ml) and lyophilized to obtain a clear foamy product. Product was used without further purification. Yield : 117 mg , 0.126 mmol , 93 %. ESI-MS: 928 $[M+H]^+$. The compound was dissolved in an ice-cold solution of 2 ml TFA/CH_2Cl_2 followed by dropwise addition of TES (278 µl, 1.7 mmol). The clear solution was kept stirring for 5h, concentrated under vacuum. The residue was dissolved in 5 mL water and purified using reverse phase flash chromatography. Product was eluted using 80/20 water/CH_3CN (0.1 % TFA in each).

(3S,7S)-5,13,20,28-tetraoxo-32-(2,4,6-triethynylphenoxy)-4,6,12,21,27-pentaazadotriacontane-1,3,7,22-tetracarboxylic acid, 11. 2,5-dioxopyrrolidin-1-yl 5-(2,4,6-triethynylphenoxy)pentanoate (132 mg, 0.362 mmol) was added in one portion to a solution containing (3S,7S)-26-amino-5,13,20-trioxo-4,6,12,21-tetraazahexacosane-1,3,7,22-tetracarboxylic acid 2,2,2-trifluoroacetic acid salt (260 mg, 0.362 mmole), triethylamine (0.202 mL, 1.44 mmol) and DMF (3.62 mL). The mixture was stirred at room temperature for 4 h and concentrated to a tan residue. The residue was dissolved in 1:1 acetonitrile:water (3 mL) and purified using C_{18} reverse phase flash chromatography with a step gradient consisting of 100% water, 0.1% TFA, then 80/20 water/acetonitrile, (0.1% TFA in each), then 60/40 water/acetonitrile (0.1% TFA in each). Each gradient step consisted of approximately 144 mL solvent volume. The flow rate was 40 mL/min. Fractions containing the desired product were concentrated to a residue and lyophilized to give (3S,7S)-5,13,20,28-tetraoxo-32-(2,4,6-triethynylphenoxy)-4,6,12,21,27-pentaazadotriacontane-1,3,7,22-tetracarboxylic acid, **11**, as a white solid. 169 mg, 54% yield. ESI-MS calcd for $C_{43}H_{57}N_5O_{13}$ $[M+H]^+$ 852.4, found 851.9. ^1H NMR (400 MHz, DMSO-d_6) 12.12 (bs, 4H) 8.01 (d, 1H), 7.76 (m, 2 H), 7.57 (s,2H), 6.33 (m, 2H), 4.47 (s, 2H), 4.28 (s,1H), 4.09 - 4.15 (m, 4H), 3.00 (m, 4H), 2.21-2.27 (m, 2H), 2.10 (m, 4H), 2.02 (t, 2H), 1.89- 1.94 (m, 1H), 1.22 – 1.69 (m, 24H). ^{13}C NMR (100 MHz, DMSO-d_6) δ175.0,

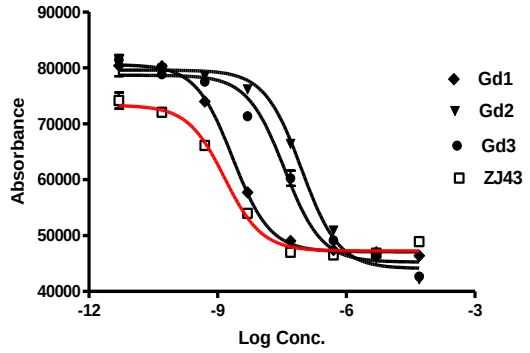
174.6, 174.3, 174.1, 172.8, 172.3, 172.1, 162.1, 158.9, 158.5, 157.7, 137.6, 118.0, 117.5, 86.6, 82.0, 81.5, 78.6, 74.1, 52.7, 52.1, 38.7, 38.6, 35.8, 35.5, 32.2, 31.1, 30.3, 29.7, 29.3, 29.2, 28.9, 28.8, 27.9, 25.7, 25.6, 23.3, 23.0, 22.1.

Gd3. To a mixture containing **11** (12 mg, 0.14 mmol), compound **12**[4] (28 mg, 0.046 mmol) and *t*-butanol (0.1 mL) was added water (0.05 mL) followed by TBTA (0.15 mg, 0.3 μmol) and tetrakis(acetonitrile)copper(I)hexafluorophosphate (0.11 mg, 0.3 μmol). The mixture was stirred at 65^{o}C for 18 h. The reaction mixture was dissolved in 2.5 mL of 0.1% sodium bicarbonate and filtered. The solution thus obtained was purified on HPLC using a Phenomenex, Luna, 10 micron, 10 x250 mm column and a gradient consisting of 0-95% acetonitrile:water over 20 minutes. The desired product eluted at 6.1-7.1 minutes. The fractions containing **Gd3** were combined, concentrated and lyophilized to afford a white solid. 13 mg, 34% yield. ESI-MS calcd for $C_{94}H_{141}Gd_3N_{26}O_{34}$ $[M-H]^-$ 2650.7, found 2648.9.

Biological Studies

NAALADase Assay

PSMA inhibitory activity of **Gd1-Gd3** were determined using a fluorescence-based assay according to a previously reported procedure.[2] Briefly, lysates of LNCaP cell extracts (25 μL) were incubated with the inhibitor (12.5 μL) in the presence of 4 μM N-acetylaspartylglutamate (NAAG) (12.5 μL) for 120 min. The amount of the released glutamate by NAAG hydrolysis was measured by incubating with a working solution (50



IC_{50} values of Gd1, Gd2 and Gd3

μL) of the Amplex Red Glutamic Acid Kit (Molecular Probes Inc., Eugene, OR) for 60 min. Fluorescence was measured with a VICTOR3V multilabel plate reader (Perkin Elmer Inc., Waltham, MA) with excitation at 530 nm and emission at 560 nm. Inhibition curves were determined using semi-log plots and IC_{50} values were determined at the concentration at which enzyme activity was inhibited by 50%. Assays were performed in triplicate. Enzyme inhibitory constants (Ki values) were generated using the Cheng-Prusoff conversion.[6] Data analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California).

^{177}Lu-**1**. To a solution of $^{177}LuCl_3$ (purchased from PerkinElmer) (185-370 MBq in 0.1 M 20 μL nitric acid) to was added approximately 20 - 30 μg of **1** in 20-30 μL 0.2 M NaOAc and neutralized to pH ~5.5 - 6 by adding 15 μL of 0.2 M NaOAc followed by brief vortexing of the mixture, which was subsequently incubated for 60 min at 95°C. The reaction mixture was diluted with 1 mL water. Complexation was monitored by injecting aliquots of 10 - 15 μL of the solution onto the HPLC. The radiolabeled product ^{177}Lu-**1** was obtained in ~ 90-97% radiochemical yield with radiochemical purity > 98%, as measured by ITLC (Gelman ITLC strips, 10mM EDTA). A radioactive peak was obtained R_t, ~ 13.9-14.8 min, for the desired product as mixture isomeric compounds and the R_t for the free ligand was 15.8 min. The acidic eluate was neutralized with 50 μL of 1 M sodium carbonate solution and the volume of the eluate was reduced under vacuum to dryness. The solid residue was diluted with saline to the desired radioactivity concentration for biodistribution studies.

Cell Culture and Animal Models

Sublines of the androgen independent PC3 human prostate cancer xenograft originally derived from an advanced androgen independent bone metastasis were used. These sublines have been modified to express high (PC3 PIP) and low (PC3 flu) PSMA levels and were generously provided by Dr. Warren Heston (Cleveland Clinic). Both PSMA-expressing (PC3 PIP) and non-expressing (PC3 flu) prostate cancer cell lines were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Invitrogen) and 1% Pen-Strep (Biofluids, Camarillo, CA) as previously described.[7] All cell cultures were maintained at 5% carbon dioxide (CO_2), at 37°C in a humidified incubator. Animal studies were carried out in full compliance with the regulations of the Johns Hopkins Animal Care and Use Committee. Six to eight week old male, non-obese diabetic (NOD)/severe-combined immunodeficient (SCID) mice (Charles River Laboratories, Wilmington, MA) were implanted subcutaneously with PSMA+ PC3 PIP and PSMA– PC3 flu cells (2 x 10^6 in 100 μL of Matrigel) at the lower right and left flanks, respectively. Mice were imaged when the xenografts reached 5 to 7 mm in diameter.

Cell surface binding assay

A monolayer of PSMA+ PC3 PIP and PSMA– PC3 flu (~10x10^6 cells per cell line) in 15 cm cell culture plates were treated with 10 mL of pre-blocking buffer (PBS buffer with BSA) at 4°C to minimize non-specific binding interactions. The pre-blocking buffer was removed and the cells were then exposed to 10 mL of 50 μM solution of either of **Gd1, Gd2** and **Gd3** for 4h at 37°C. A control experiment was done without any contrast agent added. Cells were then washed twice with cold PBS buffer and then they were removed from the plates with enzyme-free cell dissociation buffer (GIBCO Inc) for 5 min. Cells were transferred to 10 mL plastic vials and centrifuged for 5 min at 1000 rpm at 4°C. Cell pellets were transferred to a multi-well plate for

imaging as shown in **Figure 2** (main text). For blocking study, cells were pretreated with 1 mM ZJ43 for 30 min at 37°C before incubation with contrast agent.

For internalization assays, cells were detached using nonenzymatic buffer, and aliquots of 1 million cells per tube were incubated with 50 μM **Gd1**, **Gd2** and **Gd3** for 4 h at 37°C in the phosphate buffered saline binding buffer. Assuming minimal receptor endocytosis at 4°C, the internalization assay was performed only with cells incubated at 37°C. The medium was removed after 4 h and cells were washed once with binding buffer followed by a mild acidic buffer (50 mM glycine, 150 mM NaCl (*p*H 3.0)) at 4°C for 5 min. Then the acidic buffer was collected, and cells were washed twice with binding buffer. Pooled washes (containing cell surface–bound **Gd3**) and cell pellets (containing internalized **Gd3**) were analyzed for Gd concentration by using ICP-MS. The values were converted into percentage of incubated dose (% ID) per million cells.

Cell viability via MTS assay.

PSMA– PC3 PIP and PSMA– PC3 flu cells were seeded in 96-well plates at a density of 10,000 cells per well. After incubation overnight (37°C, 5% CO_2), the medium in each well was aspirated off and loaded with 100 μL of fresh medium containing **Gd1** - **Gd3** at different Gd^{3+} concentrations. After 24 hours incubation, the medium containing contrast agents in each well was aspirated off and replaced with 100 μL of fresh medium and 10 μL of MTS reagent (3- [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt). The cells were incubated for 24 hours. Then, 100 μL detergent reagent was added and left at room temperature in the dark for 2 hours. The absorbance at 570 nm was measured using a microplate reader (BioTek Instruments, Inc).

MRI Imaging in solution, in cellulo and *in vivo*

All MRI experiments were performed on a 9.4T Bruker horizontal bore scanner, equipped with a 30 mm radiofrequency coil (Bruker Biospin GmbH). The Paravision 5.1.0 software was used for all image acquisitions. Both T_1-weighted and T_2-weighted images were acquired using a spin echo pulse sequence. T_1-weighted sequence: rapid acquisition with refocused echoes (RARE); echo time (TE) = 7 ms; effective echo time (TE) = 7; RARE factor = 8; repetition times (TR) = 500, 1000, 2000, 6000 ms; number of averages (NA) = 4; field of view (FOV) = 35 × 35 mm; matrix size (MS) = 128 x 128 pixels; and slice thickness = 0.7 mm. T_2-weighted sequence: rapid acquisition with refocused echoes (RARE); echo time (TE) = 7 ms; effective echo times (TE) = 7, 35, 63, 91; RARE factor = 4; repetition time (TR) = 4000 ms; number of averages (NA) = 5; field of view (FOV) = 35 × 35 mm; matrix size (MS) = 128 × 128 pixels; and slice thickness = 1 mm. Quantitative pixel-by-pixel reconstruction of T_1 and T_2 maps was performed using an in-house program written in IDL software (Exelis Visual Information Solutions). Final image analyses were performed using the NIH ImageJ program.

Statistical analyses

Data is presented as the mean ± standard deviation for atleast four independent experiments or as stated in the text (technical replicates). A t-test (two-tailed, unequal variance) was used to compare the data as needed. The results were considered statistically significant at $P < 0.05$.

Receptor concentration of PSMA in PSMA+ PC3 PIP Cell

The receptor concentration of PSMA per cell in PSMA+ PC3 PIP cell line, $[PSMA]_{cell}$, was calculated using the formula shown below by assuming that the cell morphology is close to spherical.

$$\text{PC3 cell volume} = \frac{4}{3}\pi r_{cell}^3 = \sim 2.57\ pL$$

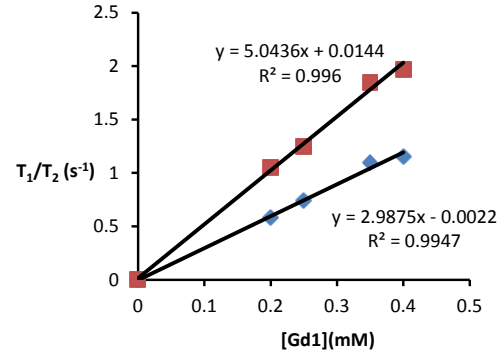
$$[PSMA]_{cell} = \frac{\text{N.R.C.}/N_A}{\frac{4}{3}\pi r_{cell}^3} = \sim 3.16\ \mu\text{M}$$

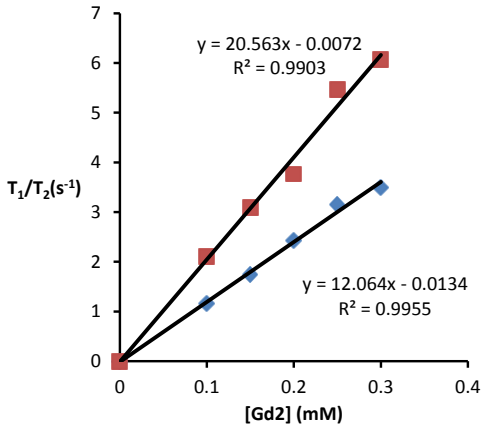
Where

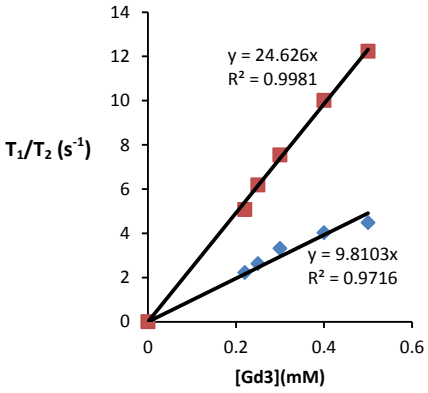
Number of Receptor per cell (N.R.C.)[8] = 4.9 x 10^6

N_A = Avogadro's number = 6.022 × 10^{14} $nmol^{-1}$

r_{cell} = cell radius = 8.75 µm (determined using confocal microscopy by us and also see ref. [9])







Plot of $1/T_1$ (blue) or $1/T_2$ (red) vs either of **[Gd1]**, **[Gd2]** and **[Gd3]** respectively to determine realxivities r_1 or r_2 of the agents.

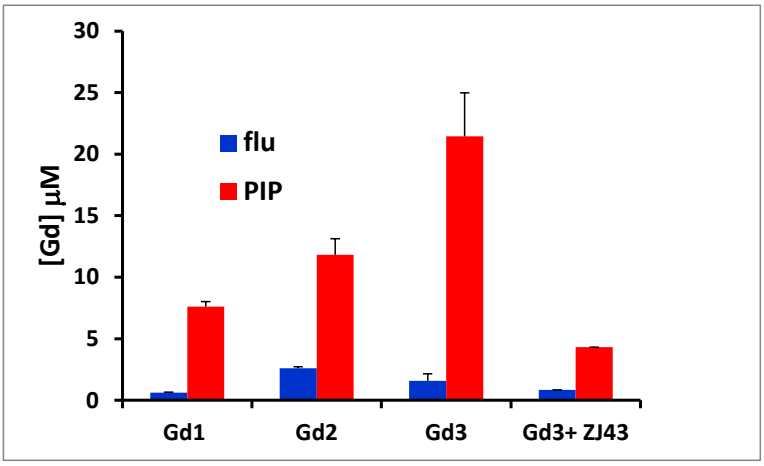


Figure S1. Concentration of Gd in cell pellets used for MR imaging studies (from ICP-MS).

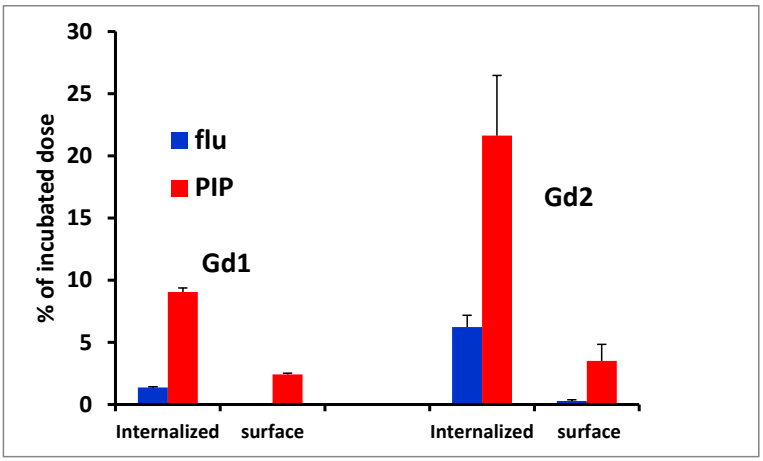
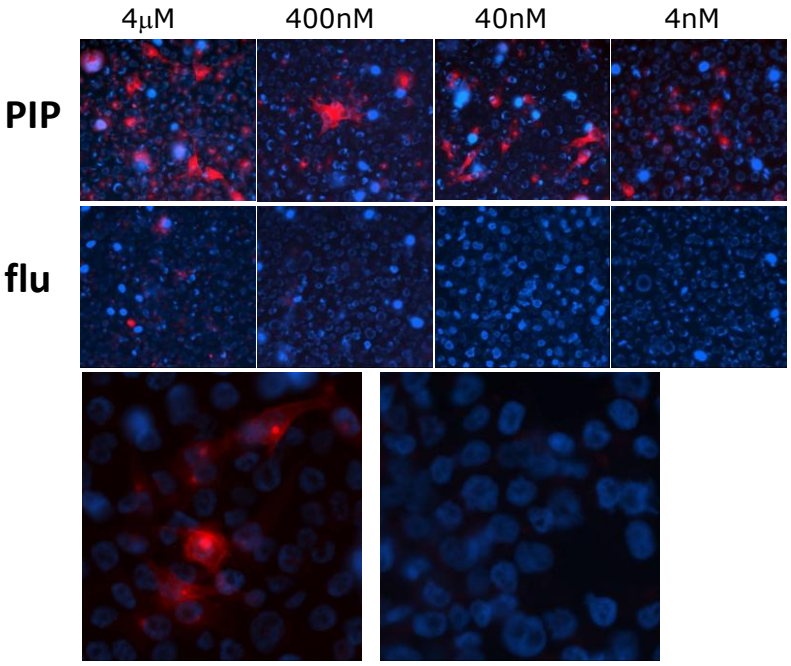


Figure S2. Percent of incubated dose (%ID) internalized and cell surface bound for **Gd1** and **Gd2.**



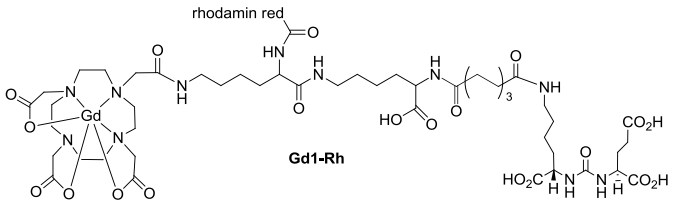


Figure S3. Top. Cellular uptake and internalization of **Gd1-Rh** by fluorescence imaging. PSMA+ PC3 PIP and PSMA– PC3 flu cells were incubated with a serially diluted solution of **Gd1-Rh** (4μM -4nM) for 30 min at 37°C followed by removal of excess contrast agents with cold PBS. **Middle**. Enlarged view of PC3 PIP (left) and PC3 flu right) at 4 nM concentration of the contrast agent. Rhodamine fluorescence is shown in red, and nuclei counter stained with DAPI are displayed in blue. **Bottom**. Structure of **Gd1-Rh.**

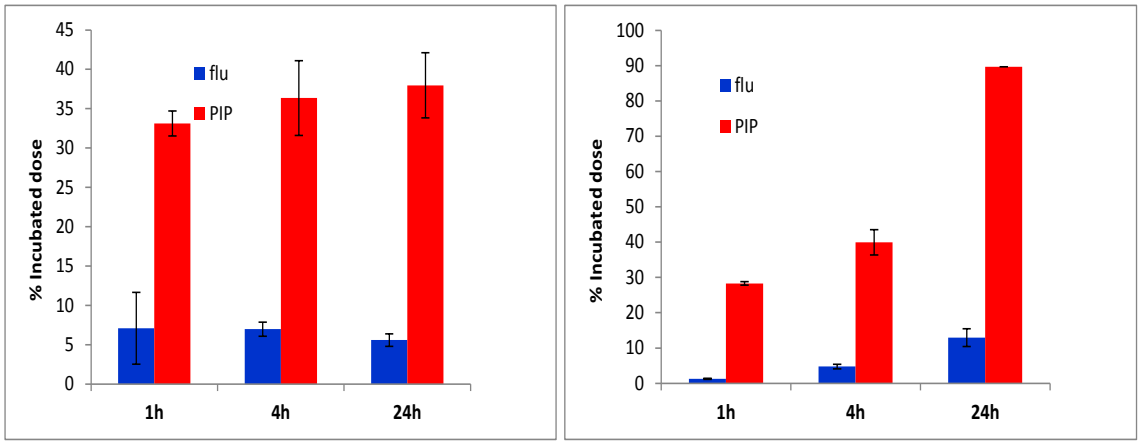


Figure S4. %ID of **Gd3** cell surface bound (left) and internalized (right) at 1, 4 and 24 h**.**

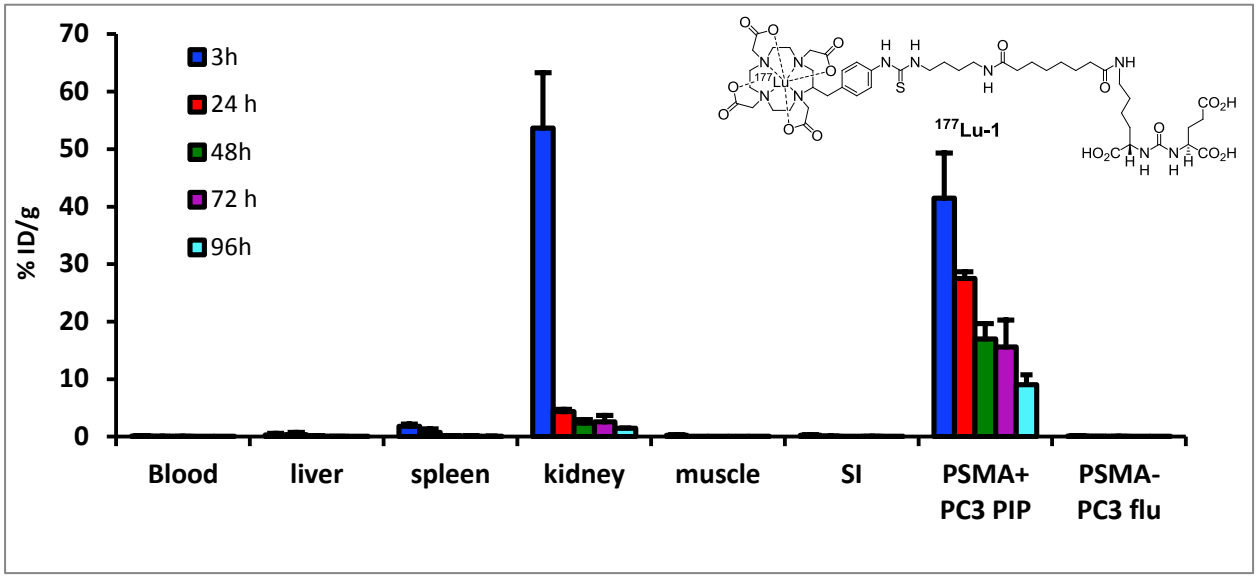


Figure S5. Tissue biodistribution of **^{177}Lu-1** in male SCID-NOD mice bearing PSMA+ PC3 PIP and PSMA- PC3 flu tumor xenografts at 3, 24, 48 72 and 96 h. (number of animals = 4)

Table 1. Tissue Biodistribution of **Gd1** and **Gd3** (nmol/g) at 48 h post-injection.

Tissues	**Gd1**	**Gd1+ZJ43**	**Gd3**
muscle	0.095	0.03	0.12
heart	0.08	0.07	0.10
liver	0.35	0.36	0.34
spleen	0.73	0.31	0.46
kidney	2.98	3.43	4.93
PSMA+ PC3 PIP	2.95	1.0	2.61
PSMA- PC3 flu	0.27	0.26	0.24

For **Gd1**, dose was 0.1 mmol/kg, for **Gd3,** dose was 0.06 mmol/kg (n=1), analyzed by ICP-MS. ZJ43 (100 mg/kg) was injected 30 min before the contrast agent administration.

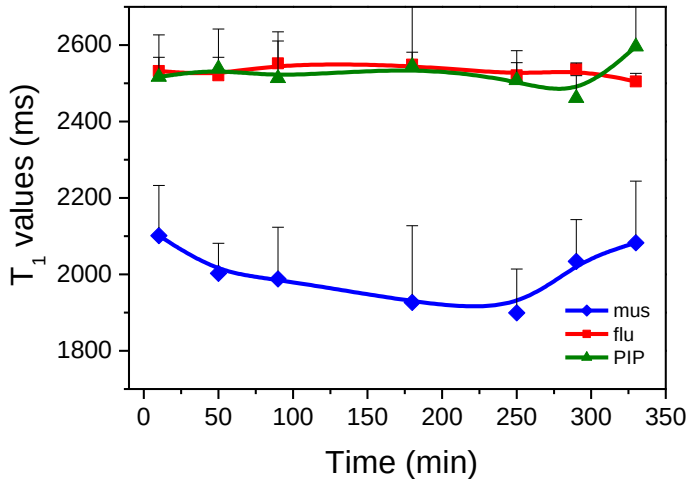
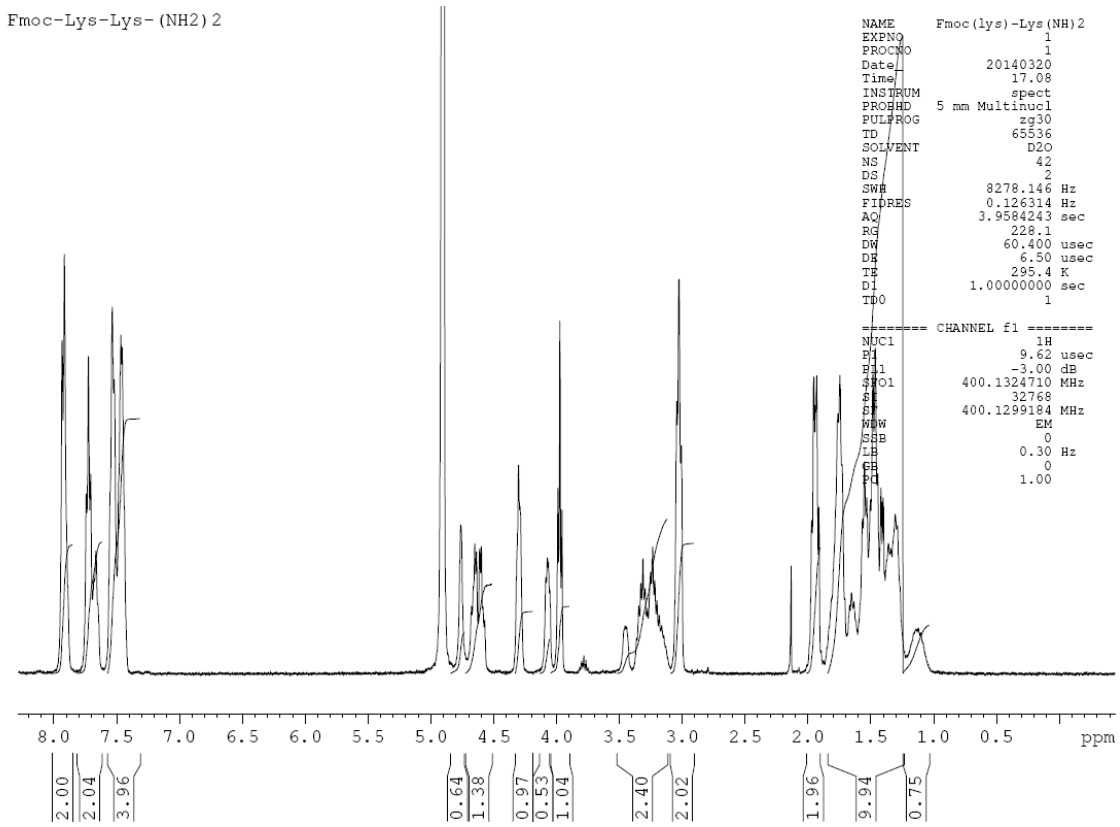
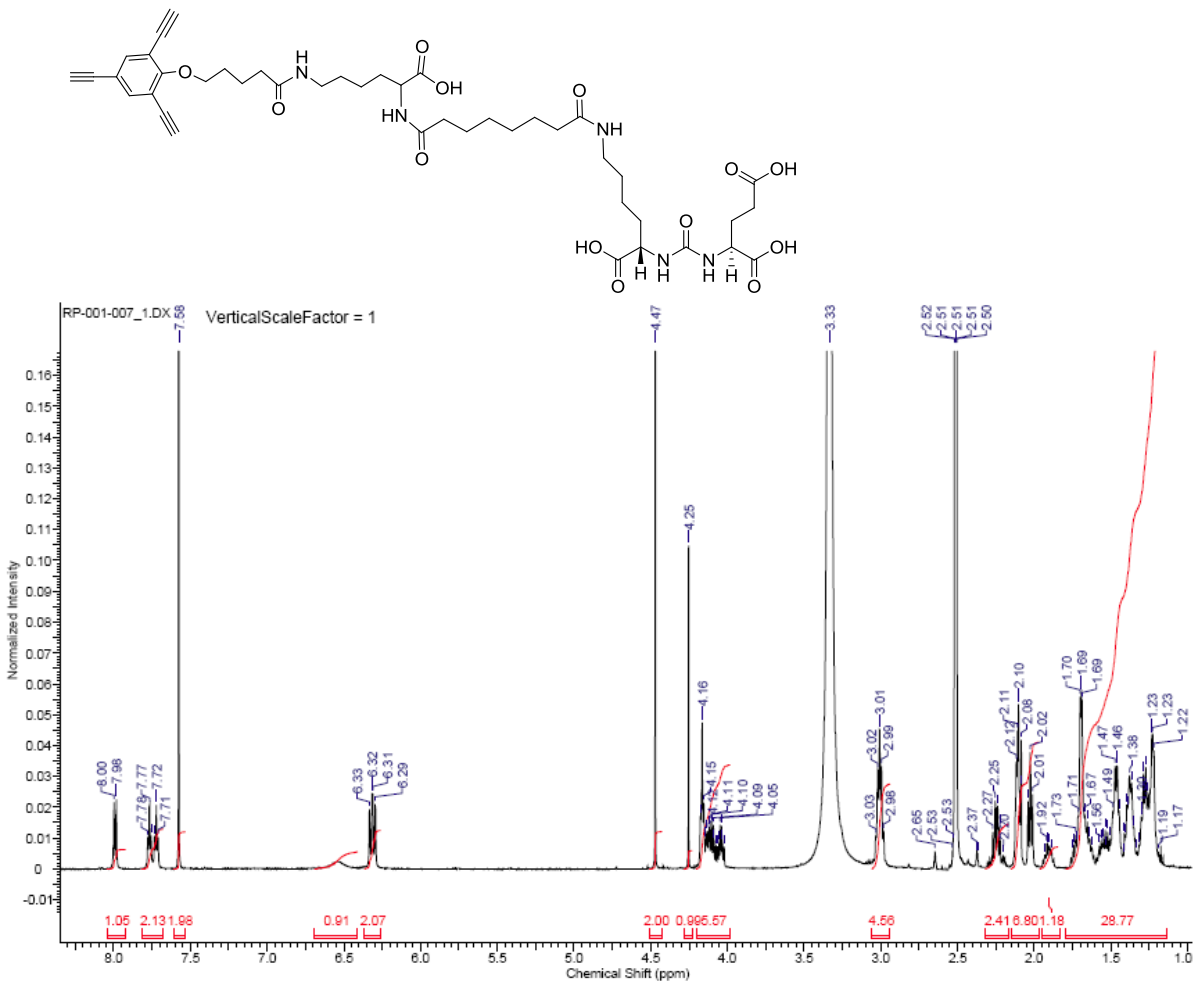


Figure S6. In vivo time-dependent changes inT_1 values of the tumor (n = 1) before and after injection of a 1X PBS

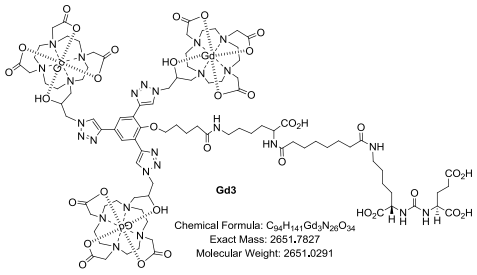


^{1}H of compound **5** in DMSO-d_6 at room temperature

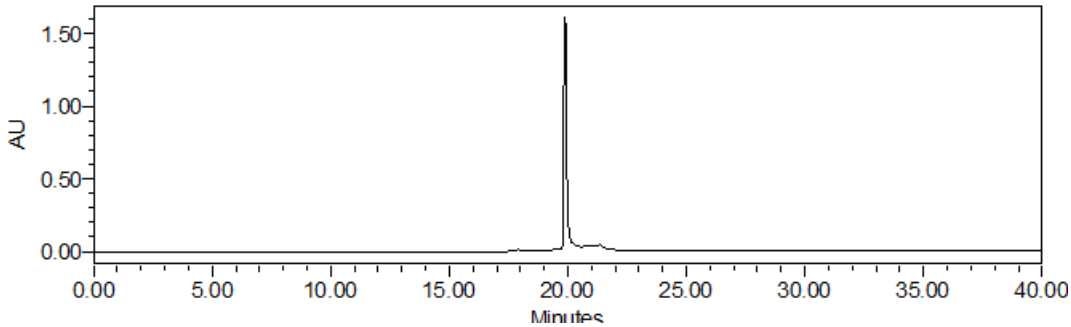


^{1}H of compound **11** (before click reaction with Gd-DOTA-azide) in DMSO-d_6 at room temperature

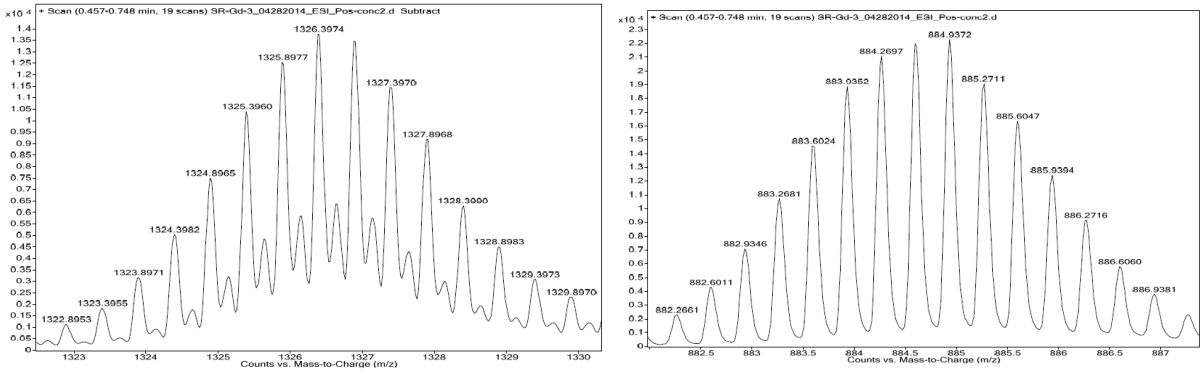
Characterization of Gd3



HPLC method. C_{18}, 10mm*250mm, flow rate: 8 ml/min		
time	CH_3CN (0.1% TEAA)	H_2O (0.1% TEAA)
0 min	0	100
5	0	100
25	30	70
30 min	100	0



HPLC chromatogram of **Gd3**; uv peak at λ= 250 nm



ESI-HRMS of **Gd3** showing $[M+3H]^{+3}$(*M/Z* found: 884.9372, calculated: 884.93606); (right) and $[M+2H]^{+2}$ (left) $[M+2H]^{+2}$(*M/Z* found: 1326.3974, calculated: 1326.3999)

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