Supporting Information

Establishment of the in vivo efficacy of pretargeted radioimmunotherapy utilizing inverse electron demand Diels-Alder click chemistry

Jacob L. Houghton[1,2], Rosemery Membreno[3,4], Dalya Abdel-Atti[1,2], Kristen M. Cunanan[5], Sean Carlin[1], Wolfgang W. Scholz[6], Pat B. Zanzonico[7], Jason S. Lewis[1,2], and Brian M. Zeglis[1,3,4,8]

[1]Department of Radiology, Memorial Sloan Kettering Cancer Center, New York, NY, USA

[2]Program in Molecular Pharmacology, Memorial Sloan Kettering Cancer Center, New York, NY, USA

[3]Department of Chemistry, Hunter College and the Graduate Center of the City University of New York, New York, NY, USA

[4]Ph.D. Program in Chemistry of the Graduate Center of the City University of New York, New York, NY, USA

[5]Department of Epidemiology and Biostatistics , Memorial Sloan Kettering Cancer Center, New York, NY, USA

[6]MabVax Therapeutics, San Diego, CA, USA

[7]Department of Medical Physics, Memorial Sloan Kettering Cancer Center, New York, NY, USA

[8]Department of Radiology, Weill Cornell Medical College, New York, New York, USA

Corresponding Author: *Brian M. Zeglis*; 413 East 69th Street, New York, NY 10021; Phone: 212-896-0443, Fax: 212-896-0484; bz102@hunter.cuny.edu

First author: Jacob L. Houghton; 1275 York Ave, New York, NY 10065; Phone: 646-888-3271, Fax: 646-888-3059; houghtoj@mskcc.org

Supplementary Figures and Tables:

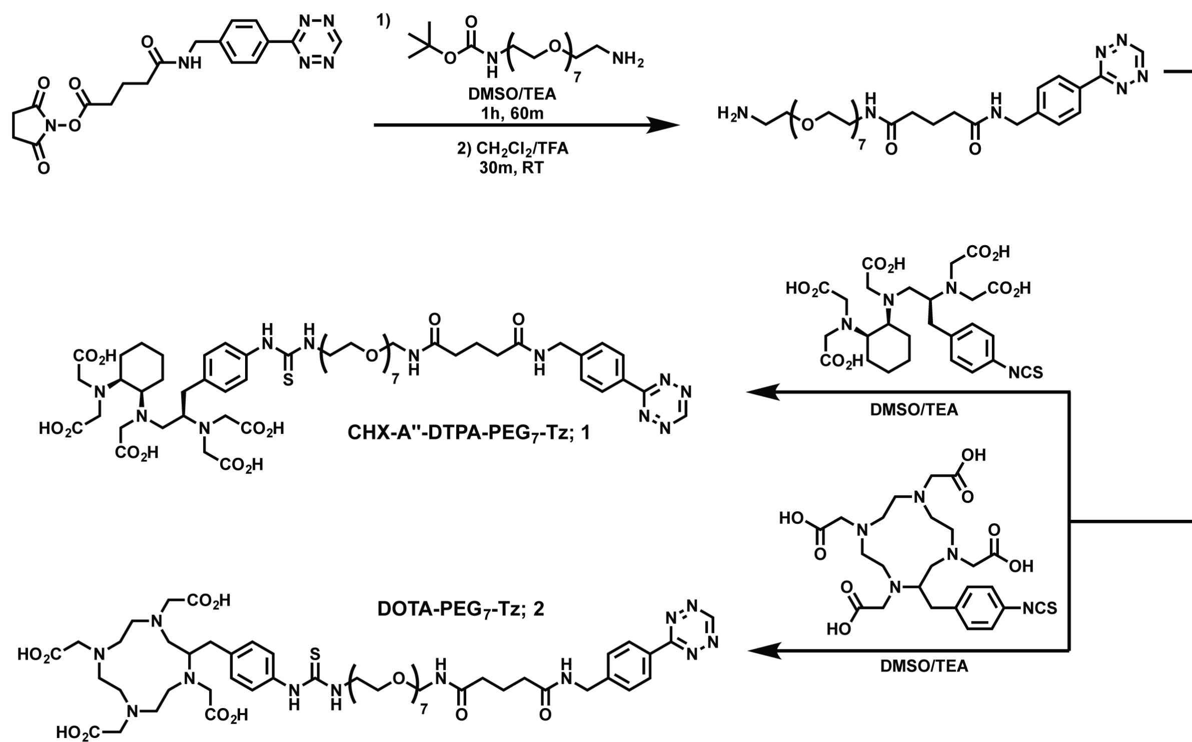
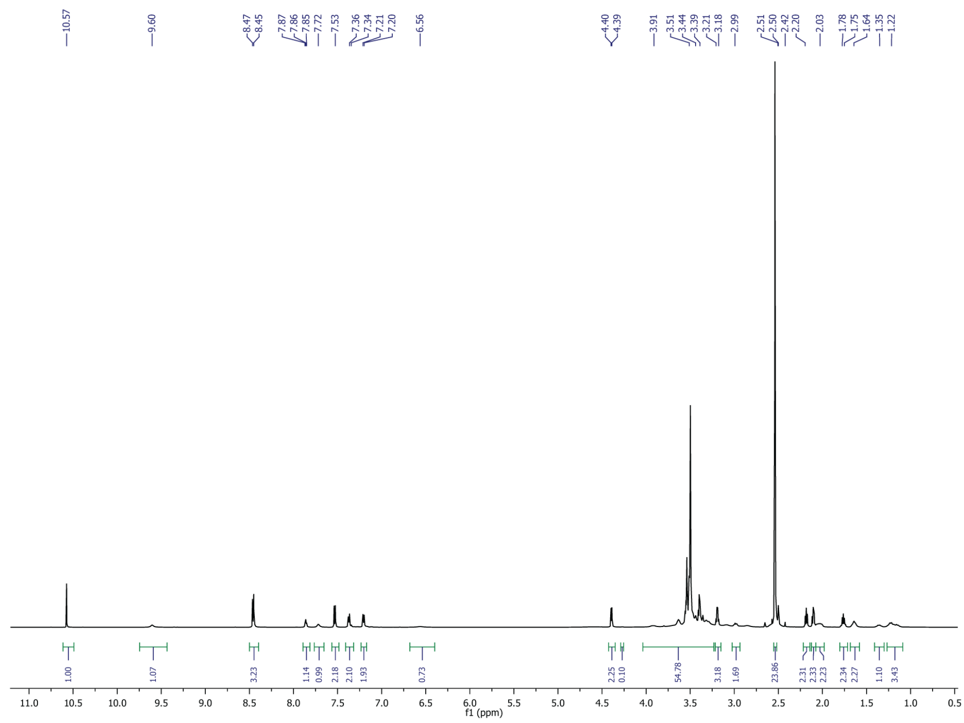


Figure S1. The synthesis of CHX-A″-DTPA-PEG$_7$-Tz (**1**) and DOTA-PEG$_7$-Tz (**2**).



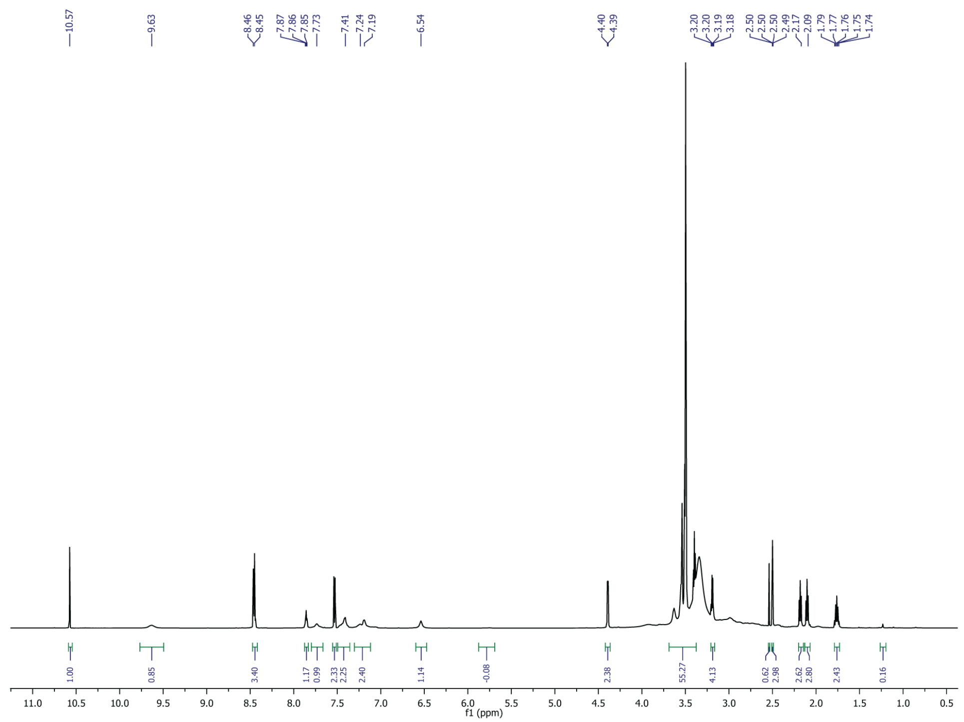


Figure S2. 1H-NMR spectra of CHX-A″-DTPA-PEG$_7$-Tz (top) and DOTA-PEG$_7$-Tz (bottom)

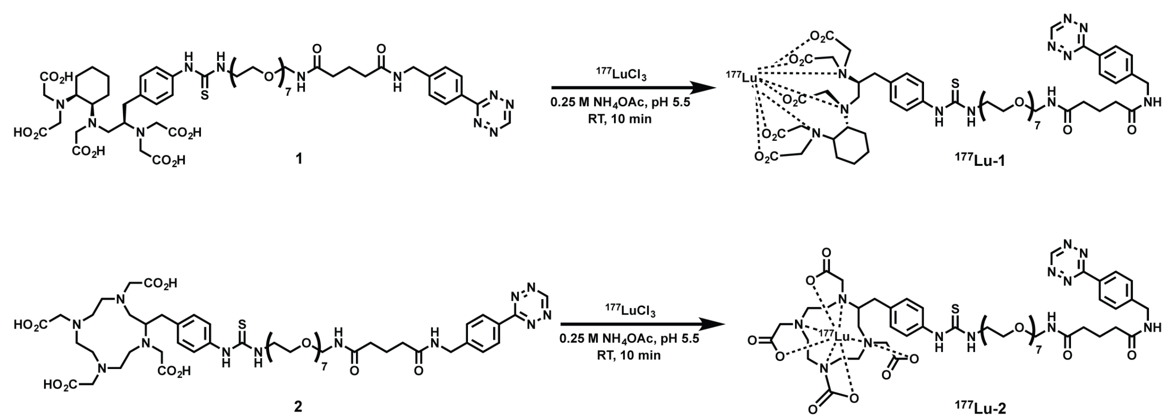


Figure S3. The radiosynthesis of ^{177}Lu- CHX-A″-DTPA-PEG$_7$-Tz (**^{177}Lu-1**) and ^{177}Lu-DOTA-PEG$_7$-Tz (**^{177}Lu-2**).

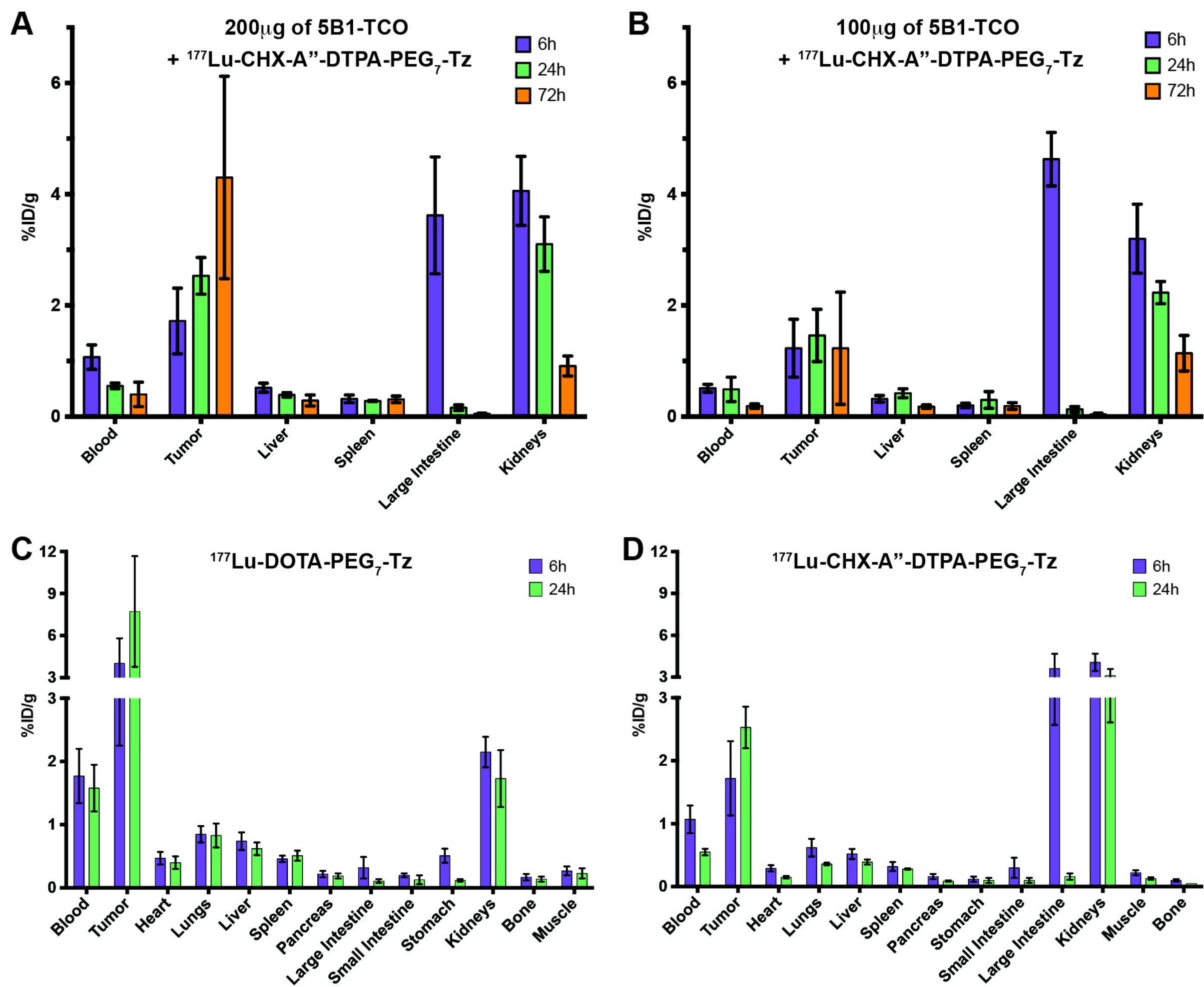


Figure S4. Bar graphs showing data from pilot biodistribution studies of mice with subcutaneous BxPC3 xenografts that were injected with 200 μg 5B1-TCO followed by ^{177}Lu-Tz-PEG$_7$-CHX-A″-DTPA (**A** and **D**), 100 μg 5B1-TCO followed by ^{177}Lu- CHX-A″-DTPA-PEG$_7$-Tz (**B**), or 200 μg 5B1-TCO followed by ^{177}Lu-DOTA-PEG$_7$-Tz (**C**). In all cases, injection of radioligand was performed 72h after injection of 5B1-TCO.

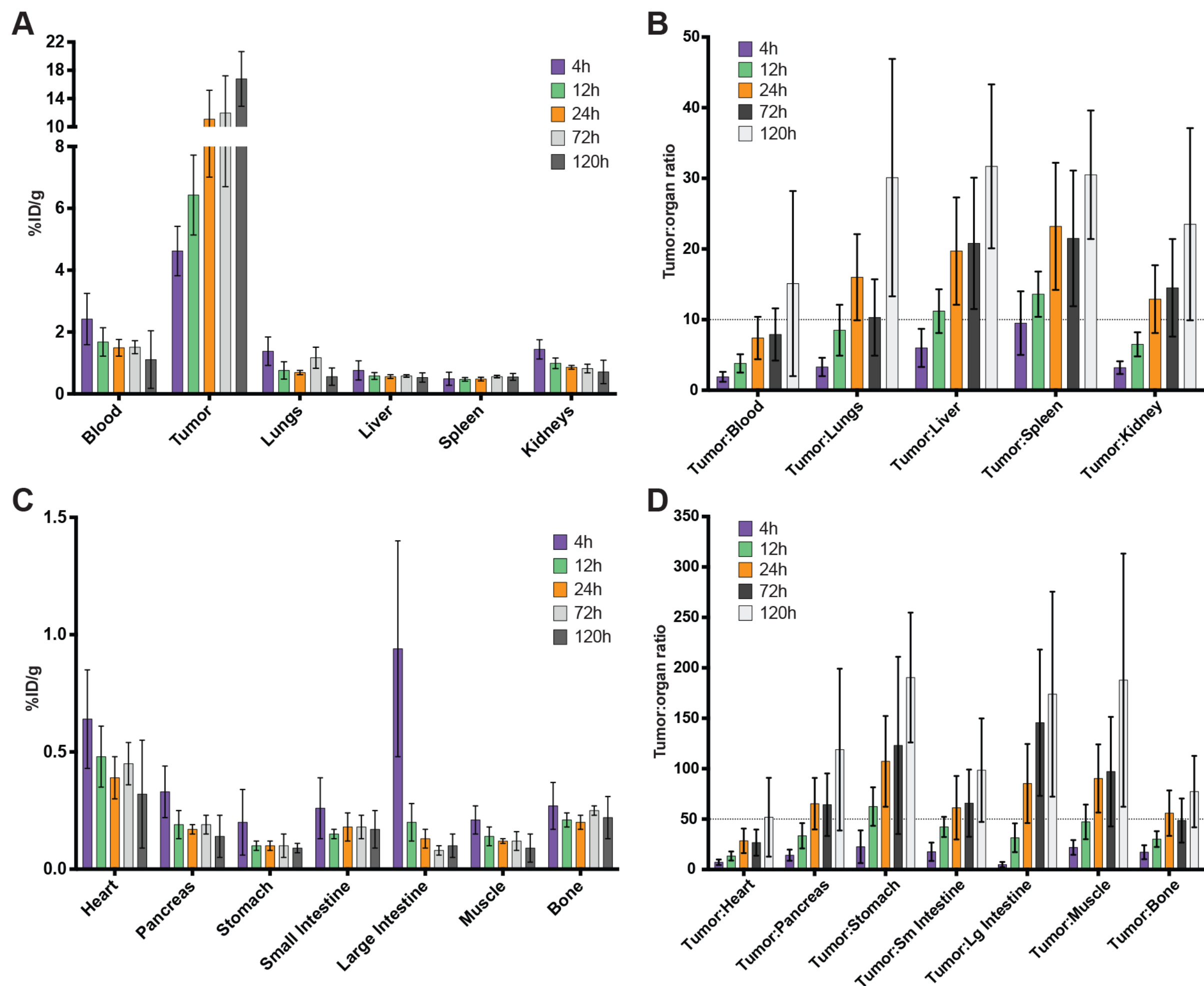


Figure S5. Data from large scale biodistribution studies of mice with subcutaneous BxPC3 xenografts that were injected with 200 μg 5B1-TCO followed by ^{177}Lu-DOTA-PEG$_7$-Tz 72h later. The %ID/g (**A** and **C**) and the tumor-to-tissue uptake ratios (**B** and **D**) are shown for all organs collected.

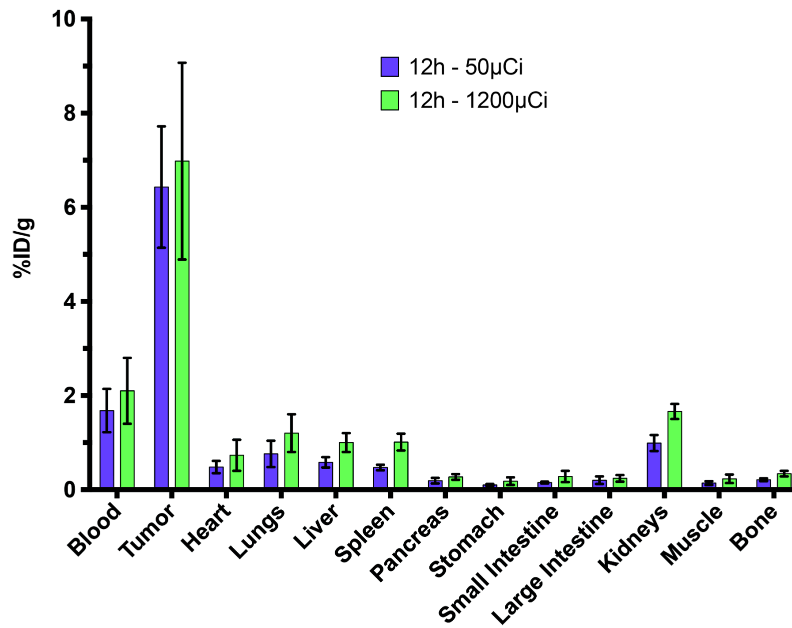


Figure S6. Biodistribution results comparing injection of high specific activity (1200 μCi) and low specific activity (1200 μCi) radioligand 12h post injection of the radiotracer. The results indicate that the amount of activity does not significantly impact the distribution when the mass is balanced with cold Lu–labeled compound.

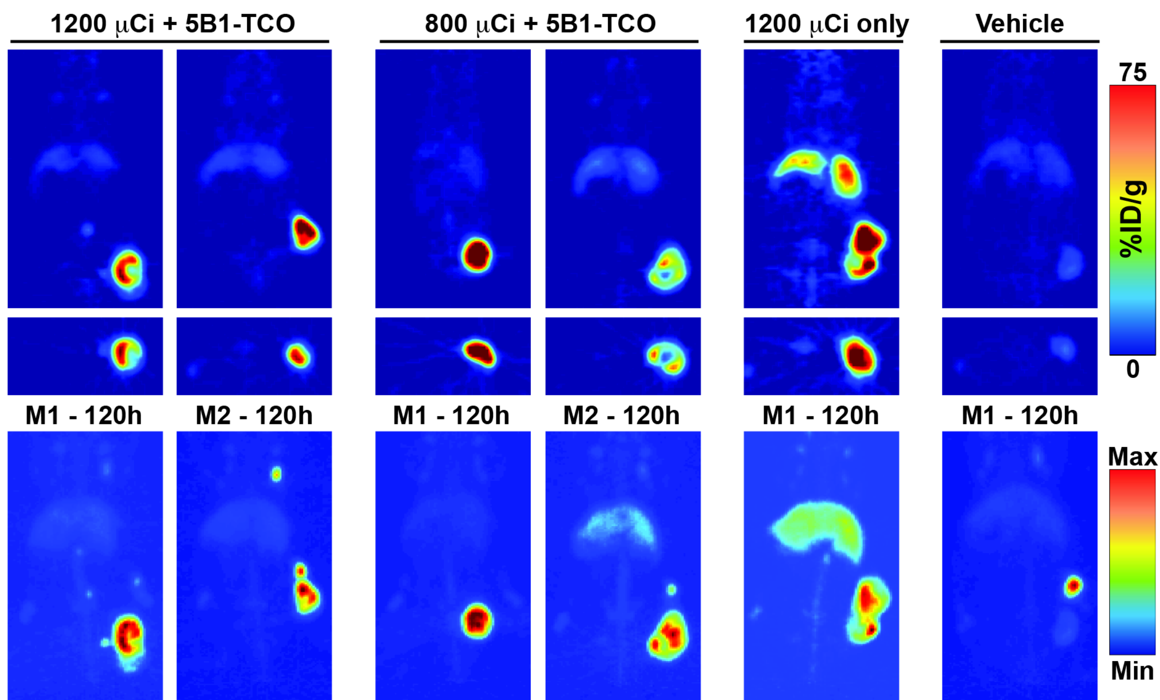


Figure S7. Tomographic slices (top panels) and maximum intensity projections (bottom panels) of PET images acquired 120h post injection of ^{89}Zr-5B1 following the conclusion of the therapy study. Pictured are two mice each from the therapy cohorts receiving 800 or 1200 μCi of ^{177}Lu-DOTA-PEG$_7$-Tz mice and one from each control cohort.

Time (h)	^{177}Lu-DOTA-PEG$_7$-Tz		^{177}Lu-CHX-A″-DTPA-PEG$_7$-Tz	
	PBS	**Serum**	**PBS**	**Serum**
1	94.1 ± 0.7	97.7 ± 0.4	94.9 ± 0.8	97.5 ± 0.8
4	91.8 ± 3.0	96.2 ± 0.8	92.9 ± 3.9	93.9 ± 3.4
24	89.1 ± 5.9	95.2 ± 0.8	86.6 ± 9.7	90.4 ± 1.9
48	80.2 ± 8.0	93.6 ± 0.9	81.8 ± 5.4	85.5 ± 3.0

Table S1. Percent of ^{177}Lu-Tz-PEG$_7$-DOTA and ^{177}Lu-Tz-PEG$_7$-CHX-A″-DTPA intact after incubation in PBS (pH 7.4) or human serum at 37 °C.

Organ	4h	24h	48h	72h	120h
Blood	2.42 ± 0.83	1.68 ± 0.46	1.49 ± 0.27	1.51 ± 0.21	1.11 ± 0.93
Tumor	4.62 ± 0.80	6.43 ± 1.29	11.09 ± 4.08	11.96 ± 5.26	16.78 ± 3.87
Heart	0.64 ± 0.21	0.48 ± 0.13	0.39 ± 0.09	0.45 ± 0.09	0.32 ± 0.23
Lungs	1.38 ± 0.46	0.76 ± 0.28	0.69 ± 0.07	1.17 ± 0.34	0.56 ± 0.28
Liver	0.76 ± 0.31	0.58 ± 0.11	0.56 ± 0.06	0.58 ± 0.04	0.53 ± 0.15
Spleen	0.49 ± 0.21	0.47 ± 0.06	0.48 ± 0.06	0.56 ± 0.04	0.55 ± 0.11
Pancreas	0.33 ± 0.11	0.19 ± 0.06	0.17 ± 0.02	0.19 ± 0.04	0.14 ± 0.09
Stomach	0.20 ± 0.	0.10 ± 0.02	0.10 ± 0.02	0.10 ± 0.05	0.09 ± 0.02
Sm Intestine	0.26 ± 0.14	0.15 ± 0.02	0.18 ± 0.06	0.18 ± 0.05	0.17 ± 0.08
Lg Intestine	0.94 ± 0.13	0.20 ± 0.08	0.13 ± 0.04	0.08 ± 0.02	0.10 ± 0.05
Kidney	1.44 ± 0.31	0.99 ± 0.17	0.86 ± 0.06	0.82 ± 0.14	0.71 ± 0.38
Muscle	0.21 ± 0.06	0.14 ± 0.04	0.12 ± 0.01	0.12 ± 0.04	0.09 ± 0.06
Bone	0.27 ± 0.10	0.21 ± 0.03	0.20 ± 0.03	0.25 ± 0.02	0.22 ± 0.09

Table S2. Biodistribution data is shown from mice BxPC3 xenografts that were injected with 5B1-TCO (200 μg) followed by ^{177}Lu-DOTA-PEG$_7$-Tz 72 h later.

Cohort	ln(2) / β[1]	Significance[2] H_0: $\beta = 0$	Significant difference from control[2,3] H_0: $\beta_j = \beta_c$	Significant difference from 400 µCi H_0: $\beta_j = \beta_{400}$
5B1 only	+34 (21,47)	< 0.0001	1	-
1200 µCi – No 5B1	+28 (22,35)	< 0.0001	-	-
400 µCi	+122 (-79,323)	0.24	0.12	-
800 µCi	+8214 (-6.8e5, 7.1e5)	0.98	*	0.91
1200 µCi	- 41 (-48, -34)	< 0.0001	<0.0001	<0.0001

Table S3. Results of the statistical analysis of in vivo PRIT. [1]For positive β, the estimated doubling time (days) of the log tumor volume (95% CI); for negative β, the estimated halving time (days) of the log tumor volume (95% CI). [2]P-value from Likelihood Ratio Test. [3]Significant difference from the "1200 µCi - No 5B1" group. *Results not reported- model failed to converge.

Methods and Materials:

Unless otherwise noted, all chemicals were acquired from Sigma-Aldrich (St. Louis, MO) and were used without further purification. All water used was ultrapure (>18.2 MΩcm^{-1} at 25 °C), all DMSO was of molecular biology grade (>99.9%), and all other solvents were of the highest grade commercially available. Acetonitrile (CH_3CN) and dimethylformamide (DMF) were purchased from Acros Organics (Waltham, MA) as extra dry over molecular sieves. Amine-reactive *trans*-cyclooctene [(E)-cyclooct-4-enyl 2,5-dioxo-1-pyrrolidinyl carbonate; TCO-NHS)] and amine reactive tetrazine (2,6-dioxo-1-pyrrolidinyl 5-[4-(1,2,4,5-tetrazin-3-yl)benzylamino]-5-oxopentanoate; Tz-NHS) were purchased from Sigma-Aldrich (St. Louis, MO). p-SCN-Bn-DOTA and p-SCN-Bn-CHX-A"-DTPA chelators were purchased from Macrocyclics, Inc. (Dallas, TX). ^{177}Lu was procured from PerkinElmer (PerkinElmer Life and Analytical Sciences, Wellesley, MA, effective specific activity of 29.27 Ci/mg) as $^{177}LuCl_3$ in 0.05 M HCl. ^{89}Zr was produced at Memorial Sloan Kettering Cancer Center using an EBCO TR19/9 variable-beam energy cyclotron (Ebco Industries Inc., British Columbia, Canada) via the ^{89}Y(p,n)^{89}Zr reaction. ^{89}Zr was purified in accordance with previously reported methods to create ^{89}Zr with a specific activity of 5.3 – 13.4 mCi/μg (195 – 497 MBq/μg).(1) The 5B1 mAb was produced at MabVax Therapeutics as previously described.(2) Amine-reactive AlexaFluor 680 (AF680-NHS) was purchased from ThermoFisher Scientific (Waltham, MA). All experiments using laboratory animals were performed in accordance with a protocol approved by the Memorial Sloan Kettering Institutional Animal Care and Use Committee.

Instrumentation:

All instruments were calibrated and maintained in accordance with standard quality-control procedures. UV-Vis measurements were taken on a Thermo Scientific NanoDrop 2000 Spectrophotometer. NMR spectroscopy was performed on a Bruker 600 MHz NMR with TopSpin 2.1 software for spectrum analysis. Low-resolution mass spectrometry was performed using a Waters liquid chromatography–mass spectrometer (LC–MS) consisting of a Waters ZQ quadrupole spectrometer equipped with an ESCI electrospray/chemical ionization ion source and a Waters 2695 HPLC system (Waters, Milford, MA). A Capintec CRC-15R Dose Calibrator (Capintec, Ramsey, NJ) was used for all activity measurements. Accurate measurements of activity concentrations were performed on a Perkin Elmer (Waltham, MA) Automatic Wizard^2

Gamma Counter. All experimental samples were counted for at least 1 min. Instant thin-layer chromatography (iTLC) for radio-iTLC experiments was performed on strips of glass-fiber, silica-impregnated paper (PallCorp), read on a Bioscan AR-2000 radioTLC plate reader, and analyzed using Winscan Radio-TLC software (Bioscan Inc.).

HPLC: All HPLC purifications (Buffer A: 0.1% TFA in water, Buffer B: 0.1% TFA in CH_3CN) were performed on a Shimadzu UFLC HPLC system equipped with a DGU-20A degasser, a SPD-M20A UV detector, a LC-20AP pump system, a CBM-20A communication BUS module, and a FRC-10A fraction collector using a C_{18} reversed phase XTerra® Preparative MS OBD™ column (10 µm, 19.2 mm × 250 mm) or a C_{18} reversed phase semi-Prep Phenomenex® Jupiter column (5 µm, 10 mm × 250 mm). Quality controls of synthesized compounds were performed using a C_{18} reversed phase Atlantis® T3 column (5 µm, 4.6 mm × 250 mm). All radio-HPLC analysis and purification experiments were performed using a Shimadzu HPLC equipped with a C_{18} reversed phase column (Phenomenex Luna analytical 4.6 x 250 mm), 2 LC-10AT pumps, a SPD-M10AVP photodiode array detector, a Bioscan Flow Counts radioactivity detector, and a gradient of 5:95 CH_3CN:H_2O (both with 0.1% TFA) to 95:5 CH_3CN:H_2O over 20 min.

Synthesis and Characterization

*Synthesis of tert-butyl (1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-3,7-dioxo-11,14,17,20,23,26,29-heptaoxa-2,8-diazahentriacontan-31-yl)carbamate (**Tz-PEG$_7$-NHBoc; 1**)*

Tz-NHS (10 mg; 0.025 mmol; 398.4 g/mol) was dissolved in 400 µL DMSO and added to 15 mg *O*-(2-aminoethyl)-*O*'-[2-(bocamino)ethyl]hexaethylene glycol (0.032 mmol; 1.3 equiv.; 468.6 g/mol). To this solution, 10 µL triethylamine (7.3 mg; 0.072 mmol; 101.2 g/mol) was then added, and the solution was placed on an agitating thermomixer at 300 rpm at room temperature. After 30 minutes, the reaction was purified via preparative C_{18} HPLC using a gradient of 5:95 CH_3CN:H_2O (both with 0.1% TFA) to 95:5 CH_3CN:H_2O over 30 min (t_R = 18.2 min). Lyophilization of the HPLC eluent yielded the purified product as 16 mg of a bright pink powder (MW = 751.9 g/mol; 0.021 mmol; 85% yield). ^{1}H NMR (500 MHz, DMSO), δ, ppm: 10.52 (s, 1H), 8.50 (m, 3H), 7.82 (t, 1H), 7.46 (d, 2H), 6.69 (t, 1H), 4.33 (d, 2H), 3.42 (m, 22H), 3.33 (t, 2H), 3.31 (t, 2H), 3.12 (q, 2H), 2.99 (q, 2H), 2.12 (t, 2H), 2.03 (t, 2H), 2.12 (t, 2H), 1.70 (q, 2H), 1.29 (s, 9H). ESI-MS(+): *m/z* (%) = 753.1 $[M+H]^+$ HRMS (ESI): *m/z* calcd. for $C_{35}H_{57}N_7O_{11}Na$: 774.4005; found: 774.4014. UV-Vis: $\varepsilon_{525} = 530\ M^{-1}cm^{-1}$.

*Synthesis of N^1-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-N^5-(23-amino-3,6,9,12,15,18,21-heptaoxatricosyl)glutaramide (**Tz-PEG$_7$-NH$_2$; 2**):*

Tz-PEG$_7$-NHBoc (**1,** 10 mg; 0.014 mmol; 717.5 g/mol) was dissolved in 400 μL of 1:1 CH_2Cl_2:TFA and placed on an agitating thermomixer at 300 rpm for 30 minutes at room temperature. After 30 minutes, the solvent was removed via rotary evaporation, the residue was taken back up in H_2O, and the reaction was purified via preparative C_{18} HPLC using a gradient of 5:95 CH_3CN:H_2O (both with 0.1% TFA) to 95:5 CH_3CN:H_2O over 30 min (t_R = 12.5 min). Lyophilization of the HPLC eluent yielded the purified product as 9 mg of a bright pink powder (MW = 651.7; 0.013 mmol; 95% yield). ^{1}H NMR (500 MHz, DMSO), δ, ppm: 10.58 (s, 1H), 8.46 (m, 2H), 7.87 (t, 1H), 7.75 (d, 2H), 7.52 (d, 1H), 4.40 (d, 2H), 3.60-3.50 (m, 26H), 3.40 (t, 2H), 3.32 (bs, 2H), 3.20 (q, 2H), 2.99 (bs, 2H), 2.19 (t, 2H), 2.12 (t, 2H), 1.79 (q, 2H). ESI-MS(+): *m/z* (%) = 652.9 $[M+H]^+$ HRMS (ESI): *m/z* calcd. for $C_{30}H_{50}N_7O_9$: 652.3670; found: 652.3676. UV-Vis: ε_{525} = 535 $M^{-1}cm^{-1}$.'

*Synthesis of 2,2',2'',2'''-(2-(4-(3-(1-([3,3'-bi(1,2,4,5-tetrazin)]-6-yl)-3,7-dioxo-11,14,17,20,23,26,29-heptaoxa-2,8-diazahentriacontan-31-yl)thioureido)benzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid (**DOTA-PEG$_7$-Tz ; 3**):*

Tz-PEG$_7$-NH$_2$ (**2,** 11.5 mg; 0.0176 mmol; 652.4 g/mol) was dissolved in 400 μL DMSO and added to 14.8 mg p-SCN-BN-DOTA (0.0022 mmol; 1.2 equiv.; 688.0 g/mol). 20 μL triethylamine (14.8 mg; 0.15 mmol; 101.2 g/mol) was then added to this solution, and the solution was placed on an agitating thermomixer at 300 rpm for 60 minutes at room temperature. After 60 minutes, the reaction was purified via preparative C_{18} HPLC using a gradient of 5:95 CH_3CN:H_2O (both with 0.1% TFA) to 95:5 CH_3CN:H_2O over 30 min (t_R = 20.7 min). Lyophilization of the HPLC eluent yielded the purified product as 15.4 mg of a bright pink powder (MW = 1203.4; 0.0128 mmol; 72.7% yield). ^{1}H NMR (500 MHz, DMSO), δ, ppm: 10.57 (s, 1H), 9.63 (bs, 1H), 8.45 (m, 3H), 7.86 (m, 1H), 7.73 (bs, 1H), 7.54 (d, 2H), 7.41 (m, 2H), 7.19 (m, 2H), 6.54 (bs, 1H), 4.40 (d, 2H), 4.00-3.20 (m, 55H), 3.20 (q, 4H), 2.54 (s, 1H), 2.18 (t, 3H), 2.10 (t, 3H), 1.76 (q, 2H). ESI-MS(-): *m/z* (%) = 1203.0 $[M-H]^-$; 601.8 $[M-2H]^{2-}$ HRMS (ESI): *m/z* calcd. for $C_{50}H_{76}N_{11}O_{15}S$: 1202.56; found: 1203.5741.

Synthesis of 2,2'-(((1S,2R)-2-(((R)-3-(4-(3-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-3,7-dioxo-11,14,17,20,23,26,29-heptaoxa-2,8-diazahentriacontan-31-yl)thioureido)phenyl)-2-

(bis(carboxymethyl)amino)propyl)(carboxymethyl)amino)cyclohexyl)azanediyl)diacetic acid ***(CHX-A″-DTPA-PEG*** $_7$***-Tz ; 4)***:

Tz-PEG$_7$-NH$_2$ (**2,** 11.5 mg; 0.0176mmol; 652.4 g/mol) was dissolved in 400 μL DMSO and added to 15.8 mg p-NCS-Bn-CHX-A″-DTPA (0.022 mmol; 1.3 equiv.; 704.0 g/mol). 22 μL triethylamine (15.8 mg; 0.156 mmol; 101.2 g/mol) was then added to this solution, and the solution was placed on an agitating thermomixer at 300 rpm for 30 minutes at room temperature. After 30 minutes, the reaction was purified via preparative C$_{18}$ HPLC using a gradient of 5:95 CH_3CN:H_2O (both with 0.1% TFA) to 95:5 CH_3CN:H_2O over 30 min (t_R = 17.6 min). Lyophilization of the HPLC eluent yielded the purified product as 19 mg of a bright pink powder (MW = 1246.4; 0.015 mmol; 87% yield). ^{1}H NMR (600 MHz, DMSO), δ, ppm: 10.57 (s, 1H), 9.60 (bs, 1H), 8.45 (m, 3H), 7.86 (m, 1H), 7.72 (m, 1H), 7.53 (d, 2H), 7.34 (d, 2H), 7.21 (d, 2H), 6.56 (bs, 1H), 4.39 (d, 2H), 4.00-3.25 (m, 55H), 3.20 (q, 2H), 2.99 (m, 2H), 2.18 (t, 2H), 2.10 (t, 2H), 2.03 (m, 2H), 1.76 (q, 2H). ESI-MS(-): *m/z* (%) = 1246.1 $[M-H]^-$; 623.4 $[M-2H]^{2-}$ HRMS (ESI): *m/z* calcd. for $C_{50}H_{76}N_{11}O_{15}S$: 1245.56; found:1246.5661.

Synthesis of 3-(2-((1E,3E)-5-((Z)-3-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-3,7,33-trioxo-11,14,17,20,23,26,29-heptaoxa-2,8,32-triazaoctatriacontan-38-yl)-3-methyl-5-sulfo-1-(3-sulfopropyl)indolin-2-ylidene)penta-1,3-dien-1-yl)-5-bromo-3,3-dimethyl-3H-pyrrolo[2,3-b]pyridin-7-ium-7-yl)propane-1-sulfonate ***(Tz-PEG*** $_7$***-AF680; 5)***:

Tz-PEG$_7$-NH$_2$ (1 mg; 0.0015 mmol; 651.8 g/mol) was dissolved in 400 μL DMSO and added to 2 mg AF680-NHS (.0021 mmol; 1.4 equiv.; 955.9 g/mol). 10 μL triethylamine (7.3 mg; 0.072 mmol; 101.2 g/mol) was then added to this solution, and the solution was placed on an agitating thermomixer at 300 rpm for 30 minutes at room temperature. After 30 minutes, the reaction was purified via preparative C$_{18}$ HPLC using a gradient of 5:95 CH_3CN:H_2O (both with 0.1% TFA) to 95:5 CH_3CN:H_2O over 30 min (t_R = 11.2 min). Lyophilization of the HPLC eluent yielded the purified product as a 2 mg of a deep blue powder (MW 1492.6; 0.0013 mmol; 79% yield).

TCO conjugation: 5B1 (4.25 mg, 5.6 mg/mL) in PBS buffer (pH 6.0) was adjusted to pH 8.5 using sodium bicarbonate solution (200 mM). Thirty molar equivalents of TCO-NHS (25.0 mg/mL, 94 mM) in DMF were added. The reaction was incubated at 37°C for 90 min before purification with a PD-10 desalting column (GE Healthcare) into sterile 0.9% saline solution for injection.

Radiolabeling

Preparation of ^{177}Lu-DOTA-PEG$_7$-Tz: A solution of DOTA-PEG$_7$-Tz (5−25 µg; 4.1−20.7 nmol) in NH_4OAc buffer (0.25 M, pH 5.5, 200 µL) was first prepared. Then, the desired amount of $^{177}LuCl_3$ in 0.1M HCl (1500−7500 µCi, 55.5-277.5 MBq) was added to the reaction mixture, and the solution was placed on an agitating thermomixer at 300 rpm for 10 min at 37 °C. After this incubation, the progress of the ^{177}Lu-DOTA-PEG$_7$-Tz radiolabeling was determined by iTLC, which revealed quantitative labeling of >98% radionuclidic purity, thus no further purification was deemed necessary. The final specific activity of ^{177}Lu-DOTA-PEG$_7$-Tz was 387.1 ± 129 µCi/µg (466.1 ± 155 mCi/µmol; 17.2 ± 5.7 GBq/µmol; n=4).

Preparation of ^{177}Lu-CHX-A″-DTPA-PEG$_7$-Tz: A solution of CHX-A″-DTPA-PEG$_7$-Tz (13 µg; 0.78 nmol) in NH_4OAc buffer (0.25 M, pH 5.5, 200 µL) was first prepared. Then, the desired amount of $^{177}LuCl_3$ in 0.1M HCl (4000 µCi, 148 MBq) was added to the reaction mixture, and the solution was placed on an agitating thermomixer at 300 rpm for 10 min at 37°C. After this incubation, the progress of the ^{177}Lu-CHX-A″-DTPA-PEG$_7$-Tz radiolabeling was determined by iTLC which revealed quantitative labeling of >99% radionuclidic purity, thus no further purification was deemed necessary. The final specific activity of ^{177}Lu-CHX-A″-DTPA-PEG$_7$-Tz was 307.6 µCi/µg (383.3 mCi/µmol; 14.2 GBq/µmol; n=1).

Functional Characterization

Determination of the TCO occupancy of 5B1-TCO: A solution of *5B1*-TCO (100 µg; 0.66 nmol) in 900 µL PBS (pH 7.4) was first prepared (0.74 µM). Next, 100 µL of a 1 mM solution of Tz-PEG$_7$-AF680 in DMSO was added to create a reaction volume of 1000 µL and concentrations of 0.66 µM 5B1-TCO and 100 µM Tz-PEG$_7$-AF680 (a ~150 fold excess of Tz). This solution was placed on an agitating thermomixer at 300 rpm at room temperature. After 180 minutes of incubation, the resulting fluorophore-labeled immunoconjugate was purified using size-exclusion chromatography (Sephadex G-25 M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with 2 mL of PBS, pH 7.4) and concentrated by using centrifugal filtration units with a 50,000 molecular weight cut off (Amicon^{TM} Ultra 4, Millipore Corp., Billerica, MA). The degree of labeling (DOL) was determined by UV-Vis analysis. Absorbance measurements were taken at 280 nm and 680 nm, and the DOL was calculated using the following formulas:

$$A_{mAb} = A_{280} - A_{max}(CF)$$

$$DOL = [A_{max} * MW_{mAb}]/[[mAb] * \varepsilon_{AF680}]$$

where the correction factor (CF) for AF680 was given as 0.05 by the supplier, $MW_{5B1} = 150{,}000$, $\varepsilon_{AF680} = 184{,}000$, and $\varepsilon_{280,\ mAb} = 225{,}000$. Given the rapid and quantitative nature of the IEDDA reaction, the degree of labeling of AF680 was assumed to be the degree of labeling of TCO.

Determination of Partition Coefficient: ^{177}Lu-DOTA-PEG$_7$-Tz, or ^{177}Lu-CHX-A″-DTPA-PEG$_7$-Tz (10 µCi) was added to a mixture of 0.5 mL PBS (pH 7.4) and 0.5 mL 1-octanol. The resulting mixture was then vortexed thoroughly for 1 minute and subsequently centrifuged at 1,000 rpm for 10 min. 100 µL of each layer (PBS and 1-octanol) was then collected, and the amount of radioactivity in each sample was counted on a gamma counter calibrated for ^{177}Lu. The partition coefficient (logD) was calculated using the formula:

$$\text{Log D} = \log_{10}[(\text{counts}_{octanol})/(\text{counts}_{PBS}]$$

All experiments were performed in triplicate.

PBS and Serum Stability of ^{177}Lu-DOTA-PEG$_7$-Tz and ^{177}Lu-CHX-A″-DTPA-PEG$_7$-Tz: ^{177}Lu-DOTA-PEG$_7$-Tz or ^{177}Lu-CHX-A″-DTPA-PEG$_7$-Tz (1000 µCi) were incubated on an agitating thermomixer (300 rpm) at 37 °C in 500 µL of either PBS (pH 7.4) or human serum. At each prescribed time-point, 100 µL of the solution was removed and placed into a 1.7 mL microcentrifuge tube. For the PBS samples, the solution was directly analyzed via iTLC. For the serum samples, 100 µL cold CH_3CN was added to precipitate dissolved proteins, and the resultant mixture was vortexed and centrifuged at 10,000 rpm for 10 min. After this, the clear supernatant was then analyzed via iTLC. The residual protein from the centrifuge spins was checked for radioactivity, and only minimal residual activity remained. The fraction of intact ^{177}Lu-DOTA-PEG$_7$-Tz or ^{177}Lu-CHX-A″-DTPA-PEG$_7$-Tz was determined by integrating the peak appearing at the baseline corresponding to the remaining intact compound. All experiments were performed in triplicate.

Cell culture and xenografting

BxPC3 cells were grown in RPMI medium modified to contain 4.5 g/L glucose, 1.5g/L sodium bicarbonate and supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 100 IU penicillin, 100 μg/mL streptomycin, 10mM HEPES and 10 cc/L non-essential amino acids. All media was purchased from the Media Preparation Facility at Memorial Sloan Kettering Cancer Center.

Subcutaneous Xenograft Models: Female athymic homozygous nude mice, strain Crl:NU(NCr)-Foxn1^{nu} (Charles River Laboratories, Wilmington, MA) age between 6-8 weeks were xenografted subcutaneously with 5x10^{6} cells, suspended in 150 μL of a solution containing a 1:1 mixture of Matrigel (Becton Dickinson, Bedford, MA) and cell culture medium. BxPC3 tumors were grown for 21-28 days post-implantation prior to imaging or biodistribution.

PET imaging

Following an injection of ^{89}Zr-DFO-5B1 via the lateral tail vein, static scans were recorded 120 hours post administration with a minimum of 12 million coincident events (8-25 m total scan time). Images were recorded on a microPET Focus scanner (Concorde Microsystems). An energy window of 350-700 keV and a coincidence timing window of 6 ns were used. Data were sorted into 2-dimensional histograms by Fourier rebinning, and transverse images were reconstructed by filtered back-projection (FBP) into a 128 × 128 × 63 (0.72 × 0.72 × 1.3 mm) matrix. The imaging data was then normalized to correct for non-uniformity of response of the PET, dead-time count losses, positron branching ratio, and physical decay of the isotope to the time of injection- but no attenuation, scatter, or partial-volume averaging correction was applied. Activity concentrations (percentage of dose per gram of tissue [%ID/g]) were determined by conversion of the counting rates from the reconstructed (filtered back-projection) images. Maximum intensity projection (MIP) images were generated from 3-dimensional ordered subset expectation maximization reconstruction (3DOSEM). All of the resulting images were analyzed using ASIPro VM™ software.

Biodistribution

The acute biodistributions of ^{177}Lu-DOTA-PEG$_7$-Tz and ^{177}Lu-CHX-A″-DTPA-PEG$_7$-Tz were determined using a BxPC3 subcutaneous xenograft (right flank, ~200mm^3) model in athymic, nude mice. Mice were randomized into groups and warmed gently using a heat lamp prior to

being injected with the radioligand (40 - 50μCi) via the lateral tail vein at 72h after the administration of 5B1-TCO (100μg or 200μg in 200μL of 0.9% saline). At the appropriate time post injection, mice (n = 5) were euthanized via asphyxiation using CO_2(g) and 13 tissues including the tumor were collected, dried in open air for 5 minutes, and placed into pre-weighed tubes. The mass of each organ was determined and then each sample was counted using a Wizard[2] automatic gamma counter calibrated for ^{177}Lu. A calibration curve generated from standards of known activity was used to convert counts into activity. The counts from each sample were decay- and background corrected from the time of injection, and the activity in each sample was then converted to %ID/g by normalization to the total activity injected into the respective animal.

Dosimetry

The biodistribution data for the ^{177}Lu-DOTA-PEG$_7$-Tz pretargeting system described in this investigation was expressed as normal-organ mean standard uptake values (SUVs) versus time post-administration. Assuming, in first order, that SUVs are independent of body mass and thus the same among species, the mean SUV in mouse organ i, SUV_i, was converted to the fraction of the injected dose in each human organ I, FID_I, using the following formula:

$$FID_I = SUV_i \bullet \frac{MassofHumanOrgan_I}{MassofHumanTotalBody}$$

and the organ and total-body masses of the 70-kg Standard Man anatomic model. These data were decay corrected and fit to exponential time-activity functions. The residence time in a human organ i (t_i, in μCi-h/μCi) was calculated by integrating the time-activity function in organ i, replacing the biological clearance constant, $(l_b)_x$ for each component, x, of the fitted exponential function with the corresponding effective clearance constant, $(l_e)_x$ [$(l_e)_x = (l_b)_x + l_p$, where l_p is the physical decay constant of the radionuclide. The resulting organ residence times were entered into the OLINDA computer program to yield the mean organ absorbed doses and effective dose in rad/mCi and rem/mCi, respectively, which were then converted to cGy/MBq.(3)

Therapy study

Mice bearing established BxPC3 subcutaneous tumors (mean tumor volume 150 ± 50 mm^3) were randomly sorted into 5 groups of 8 mice per group. In order to see the therapeutic window of the

pretargeted methodology, the treatment groups received injections of the radioligand loaded with 400, 800, or 1200 μCi (14.8, 29.6, 44.4MBq) via the lateral tail vein 72 h after administration of 5B1-TCO (200 μg in 200 μL of 0.9% saline). The remaining two cohorts served as control groups, receiving either saline or 1200 μCi (44.4MBq) of ^{177}Lu-DOTA-PEG$_7$-Tz. Neither control group was administered 5B1-TCO. All mice were weighed, and the tumor burden was assessed by caliper measurement every 3-4 days until day 17, and then every 7 days thereafter until the conclusion of the experiment. Mice were sacrificed if the tumor grew to >1.0 cm^3 or if the mouse lost >20% of its body mass during the course of the study. The remaining mice were sacrificed 7 days after completion of the treatment study for histological evaluation by the MSKCC Laboratory of Comparative Pathology.

Ex vivo analysis

Following PET imaging, tumors were excised and embedded in optimal-cutting-temperature mounting medium (OCT, Sakura Finetek) and frozen on dry ice. Series of 10 μm frozen sections were then cut and mounted on glass slides. To determine radiotracer distribution, digital autoradiography was performed by placing tissue sections in a film cassette against a phosphor imaging plate (Fujifilm BAS-MS2325; Fuji Photo Film) at −20°C for an appropriate exposure period. Phosphor imaging plates were read at a pixel resolution of 25 μm with a Typhoon 7000 IP plate reader (GE Healthcare). Contiguous frozen sections were then used for staining and microscopy.

Immunofluorescence staining and imaging was performed essentially as previously described.(4) For detection of CA19.9, sections were fixed in 4% paraformaldehyde, and subsequently incubated with 5B1 antibody overnight at 4°C, followed by secondary detection using goat anti-human Alexa568 (Molecular Probes, 2ug/ml). Masson's Trichrome and H&E staining were performed using standard methods. Whole mount fluorescence images were acquired using a BX60 fluorescence microscope (Olympus America, Inc.) equipped with a motorized stage (Prior Scientific Instruments Ltd.) and DP80 camera (Olympus). Montage images were obtained by acquiring multiple fields at × 100 magnifications, followed by alignment using cellSens Dimension software v1.13 (Olympus). Fluorescence, bright-field and autoradiographic images were co-registered using Adobe Photoshop (CS6) as previously described.(5)

References:

1. Holland JP, Sheh Y, Lewis JS. Standardized methods for the production of high specific-activity zirconium-89. Nucl Med Biol. 2009;36:729-39.
2. Viola-Villegas NT, Rice SL, Carlin S, Wu X, Evans MJ, Sevak KK, et al. Applying PET to broaden the diagnostic utility of the clinically validated CA19.9 serum biomarker for oncology. J Nucl Med. 2013;54:1876-82.
3. Stabin MG, Sparks RB, Crowe E. OLINDA/EXM: the second-generation personal computer software for internal dose assessment in nuclear medicine. Journal of Nuclear Medicine. 2005;46:1023-7.
4. Oehler C, O'Donoghue JA, Russell J, Zanzonico P, Lorenzen S, Ling CC, et al. 18F-fluromisonidazole PET imaging as a biomarker for the response to 5, 6-dimethylxanthenone-4-acetic acid in colorectal xenograft tumors. Journal of Nuclear Medicine. 2011;52:437-44.
5. Carlin S, Zhang H, Reese M, Ramos NN, Chen Q, Ricketts S-A. A comparison of the imaging characteristics and microregional distribution of 4 hypoxia PET tracers. Journal of Nuclear Medicine. 2014;55:515-21.