Reagents and general procedures. Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich and used without additional purification. Ultra-pure water (>18.2 $M\Omega cm^{-1}$) and dry, molecular biology grade dimethyl sulfoxide (DMSO), and *N*,*N*-dimethylformamide (DMF) were used. ^{64}Cu, which was produced by the $^{64}Ni(p,n)^{64}Cu$ reaction on a CS-15 cyclotron (Cyclotron Corp.), was purchased from the Washington University School of Medicine. All activity measurements were performed using a Capintec CRC-15R dose calibrator (Capintec). Instant thin-layer chromatography (ITLC) for radioITLC 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.). Radioactive tissue samples were measured using an Automatic Wizard^2 γ-counter (Perkin Elmer) calibrated for ^{64}Cu. The 5B1 mAb was produced at MabVax Therapeutics as previously described. (1) All experiments involving laboratory animals were performed in accordance with the Memorial Sloan Kettering Institutional Animal Care and Use Committee.

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; 2): Tz-NHS (**1**; 10 mg; 0.025 mmol; 398.4 g/mol), *O*-(2-aminoethyl)-*O*'-[2-(bocamino)ethyl]hexaethylene glycol (15 mg; 0.032 mmol; 1.3 equiv.; 468.6 g/mol), and 10 μL triethylamine (7.3 mg; 0.072 mmol; 101.2 g/mol) were dissolved in 400 μL and agitated at 300 rpm for 30 minutes at room temperature. After this incubation, the reaction mixture was purified via C_{18} HPLC employing a gradient of 5:95 CH_3CN:H_2O to 95:5 CH_3CN:H_2O (both with 0.1% trifluoroacetic acid) over 30 min (retention time = 18.2 min). The HPLC eluent was then lyophilized to yield the product as a prink solid (MW = 751.9 g/mol; 16 mg; 0.021 mmol; 85% yield). ^1H 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$_2$; 3): Tz-PEG$_7$-NHBoc (**2**; 10 mg; 0.014 mmol; 717.5 g/mol) was taken up in 500 μL of a 1:1 mixture of dichloromethane (CH_2Cl_2) and trifluoroacetic acid (TFA) and subsequently agitated at 300 rpm for 30 minutes at room temperature. After this incubation, the CH_2Cl_2:TFA solvent was removed via rotary evaporation, and the product was purified via C_{18} HPLC employing a gradient of 5:95 CH_3CN:H_2O to 95:5 CH_3CN:H_2O (both with 0.1% trifluoroacetic acid) over 30 min (retention time = 12.5 min). The HPLC mobile phase was then removed via lyophilization to yield the product as a pink

solid (MW = 651.7; 9 mg; 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: $\varepsilon_{525} = 535\ M^{-1}cm^{-1}$.

Synthesis of 2,2',2''-(2-(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)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid (Tz-PEG$_7$-NOTA; 4): Tz-PEG$_7$-NH$_2$ (**3**; 5 mg; 0.008 mmol; 651.8 g/mol), *p*-NCS-Bn-NOTA (10 mg, 0.022 mmol; 2.75 equiv.; 450.5 g/mol), and triethylamine (10 μL; 7.3 mg; 0.072 mmol; 101.2 g/mol) were dissolved in 400 μL DMSO, and the solution was agitated at 300 rpm for 30 minutes at room temperature. After the incubation, the reaction was purified via C_{18} HPLC employing a gradient of 5:95 $CH_3CN:H_2O$ to 95:5 $CH_3CN:H_2O$ (both with 0.1% trifluoroacetic acid) over 30 min (retention time = 15.5 min). The HPLC mobile phase was then removed via lyophilization to yield the product as a pink solid (MW = 1102.2; 6 mg; 0.005 mmol; 68% yield). ^{1}H NMR (500 MHz, DMSO), δ, ppm: 10.51 (s, 1H), 9.50 (bs, 1H), 8.40 (m, 3H), 7.79 (m, 1H), 7.62 (m, 1H), 7.47 (d, 2H), 7.35 (d, 2H), 7.03 (d, 2H), 4.43 (d, 2H), 4.00-3.20 (m, 50H), 3.12 (q, 2H), 2.96 (bs, 2H), 2.11 (t, 2H), 2.03 (t, 2H), 1.70 (q, 2H). ESI-MS(-): *m/z* (%) = 1100.6 $[M-H]^-$; 549.9 $[M-2H]^{2-}$ HRMS (ESI): *m/z* calcd. for $C_{50}H_{76}N_{11}O_{15}S$: 1102.5243; found: 1102.5253. UV-Vis: $\varepsilon_{525} = 540\ M^{-1}cm^{-1}$.

Radiosynthesis of ^{64}Cu-NOTA-PEG$_7$-Tz (^{64}Cu-4): Radiolabeling (Fig. S2) was achieved by adding $^{64}CuCl_2$ (1500-7500 μCi in 0.1 M HCl) to a solution of NOTA-PEG$_7$-Tz (**4**; 5-25 μg; 4.5-22.6 nmol) in NH_4OAc buffer (0.2 M, pH 5.5, 200 μL), and the resultant solution was agitated at 300 rpm for 30 minutes at room temperature. After this incubation, the radioligand was purified via C_{18} HPLC employing a gradient of 5:95 $CH_3CN:H_2O$ to 95:5 $CH_3CN:H_2O$ (both with 0.1% trifluoroacetic acid) over 15 min (retention time = 9.7 min).

Dosimetry: The biodistribution data for the ^{64}Cu-NOTA-PEG$_7$-Tz pretargeting system described in this investigation as well as the biodistribution data for the previously reported ^{89}Zr-DFO-5B1 (*1*) radioimmunoconjugate were expressed as normal-organ mean standard uptake values (SUVs) versus time post-administration (Table 3). 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 (corrected for radioactive decay) were fit to exponential time-activity functions. The cumulated activity, or residence time, in 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.(*2*)

Cell culture.

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. Capan2 cells were grown in Dulbecco's modified essential medium (DMEM) modified to contain 4.5g/L glucose and supplemented with 20% (vol/vol) heat-inactivated fetal calf serum, 100 IU penicillin and 100μg/mL streptomycin. All media was purchased from the Media Preparation Facility at Memorial Sloan Kettering Cancer Center.

Murine xenograft models of PDAC.

Subcutaneous Xenograft Models: BxPC3 - 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 150μl 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.

Orthotopic Xenograft Model: For orthotopic pancreas xenografts, athymic homozygous nude mice, strain Crl:NU(NCr)-Foxn1^{nu} (Charles River Laboratories) age 6-8 weeks were used. Mice were anesthetized with 1-2% isoflurane gas in medical air at a rate of 2 L/minute and surgery was performed on a heated platform to help maintain body temperature. Bupivicaine, a local anesthetic agent, was injected intra-

dermally in the area surrounding the incision line. Skin was prepped for surgery using alternating scrubs of povidone-iodine and 70% ethanol. A longitudinal incision (0.5-1 cm in length) was made in the skin and the peritoneum, allowing for the spleen and pancreas to be exteriorized. Capan2 cells ($2x10^6$ cells in 30μl containing 1:1 cell media and Matrigel) were slowly injected into the parenchyma of the pancreas. The spleen and pancreas were returned to the peritoneal cavity, the peritoneal wall was closed using 4-0 Vicryl sutures and the skin was closed using sterile wound clips. Buprenorphine was administered prior to recovery and dosage repeated post-surgery as needed every 4-6 hours. Tumors reached optimal size within 14-21 days post-implantation.

In vivo imaging

PET imaging: Female, athymic nude mice with subcutaneous BxPC3 xenografts on the right flank were injected via the lateral tail vein with ^{64}Cu-NOTA-PEG$_7$-Tz or ^{64}Cu-NOTA-Tz at 48 h, 72 h, or 120 h post administration of 5B1-TCO (200μg in 200μL of 0.9% saline). Static scans were recorded at the various time points 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 re-binning, and transverse images were reconstructed by filtered back-projection (FBP) into a 128 × 128 × 63 (0.72 × 0.72 × 1.3 mm) matrix. The image data were normalized to correct for non-uniformity of response of the PET, dead-time count losses, positron branching ratio, and physical decay 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.

PET/CT: Mice with orthotopic Capan-2 xenografts were administered 5B1-TCO (200μg in 200μL of 0.9% saline) 72h prior to injection of ^{64}Cu-NOTA-PEG$_7$-Tz (14.9±0.5 MBq [403±14 μCi]) via the lateral tail vein. Images were acquired 20h after injection of the radioligand. Static scans were recorded on an Inveon PET/CT scanner (Siemens Healthcare Global) with a minimum of 30 million coincident events (10-30m total scan time). Data were sorted into 2-dimensional histograms by Fourier re-binning, and the images were reconstructed using a two-dimensional ordered subset expectation maximization (2DOSEM) algorithm (16 subsets, 4 iterations) into a 128 × 128 × 159 (0.78 × 0.78 × 0.80 mm) matrix. The image data was normalized to correct for non-uniformity of response of the PET, dead-time count losses, positron branching ratio, and physical decay 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]) and MIPs were determined by conversion of the counting rates from the reconstructed images. Whole-body CT scans were acquired with a voltage of 80kV and 500 μA. 120 rotational steps for a total of 220° were acquired with a total scan time of 120s and 145ms per frame exposure. Combined PET/CT images were processed using Inveon Research Workplace software and optimized to show localization of the PET signal in the lymph nodes, primary tumor, and metastases.

Acute Biodistribution.

The acute biodistribution of ^{64}Cu-NOTA-PEG$_7$-Tz was determined using a BxPC3 subcutaneous xenograft (right flank, ~200 mm^3) model in athymic, nude mice. Mice (n = 4) were randomized and warmed gently using a heat lamp prior to being injected with radioligand via the lateral tail vein at various times after administration of 5B1-TCO (200μg in 200μL of 0.9% saline). At the appropriate time post injection, mice (n = 4) were euthanized by asphyxiation using CO_2 (g). After euthanization, 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 that was calibrated for ^{89}Zr. A calibration curve that was 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 converted to %ID/g by normalization to the total activity injected into the respective animal.

Ex vivo analysis

Autoradiography: After sacrifice, organs (tumor, muscle, and pancreas) were excised, embedded in Tissue–Plus OCT compound (Scigen, Gardena, CA), and frozen on dry ice. Tissues were cut in a series of 10μm sections. Autoradiography was performed to determine radiotracer distribution in tissue sections by placing the sections in a film cassette beneath a phosphor imaging plate for 48-72 hours at -20°C (BASMS-2325, Fujifilm, Valhalla, NY). Phosphor imaging plates were then read on a Typhoon 7000IP plate reader (GE Healthcare, Pittsburgh, PA) at a pixel resolution of 25μm.

Immunohistochemistry: Tissue sections were washed in PBS and fixed with 4% paraformaldehyde for twelve minutes. Sections were then washed with PBS and incubated in blocking buffer composed of 1% Bovine Serum Albumin (Sigma Aldrich), 5% Goat Serum (Sigma Aldrich), and 0.1% Triton-X100 (Sigma Aldrich) in PBS for 30 minutes. Blocking buffer was removed and slides were incubated in 5B1 antibody (10ug/mL in blocking buffer) for one hour, washed with PBS, and then incubated in Alexa Fluor®488 goat anti-human IgG (Thermo Fisher Scientific) as a secondary antibody (20ug/ml in blocking

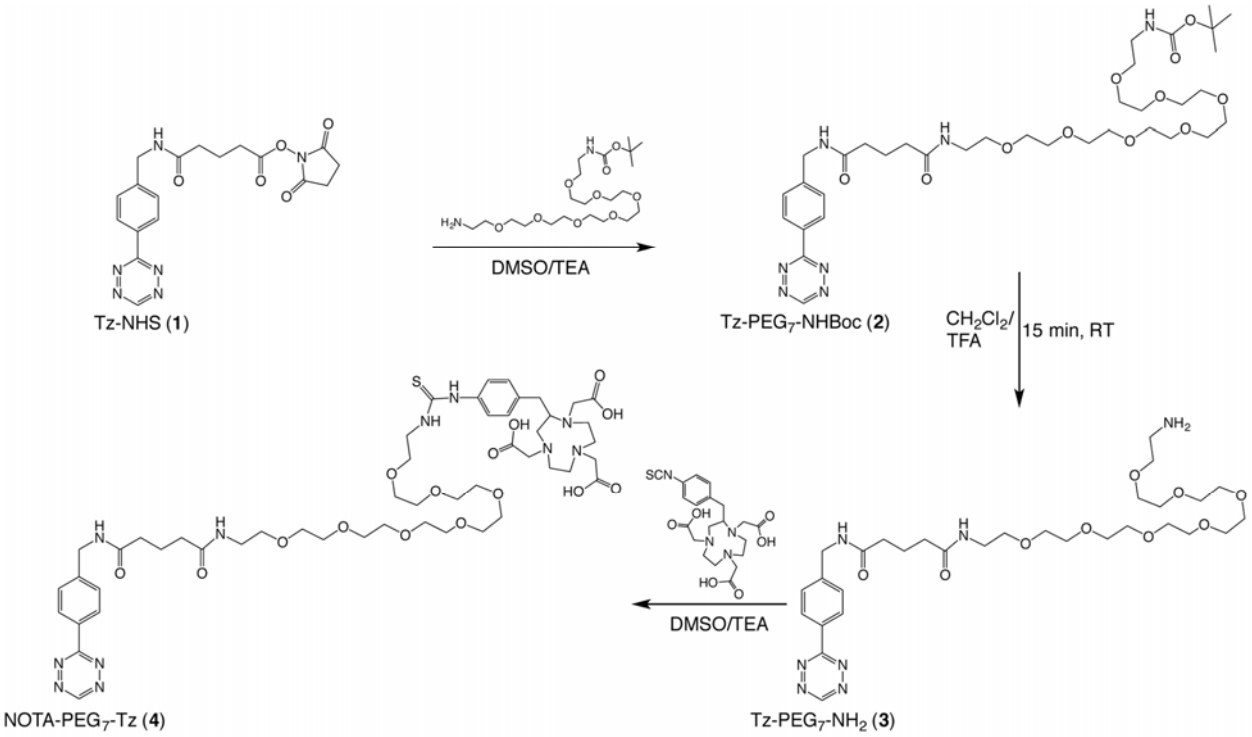
buffer) for forty-five minutes. Slides were washed three times in PBS and one time in H_2O and allowed to air dry.

Microscopy: All microscopic images were acquired using a Zeiss Axioplan2 fluorescence microscope connected to a CCD camera and equipped with a motorized stage (Prior Scientific Instruments, Cambridge, UK). MetaMorph software (Molecular Devices, Sunnyvale, CA) was used to control the microscope and to render the captured image frames into a montage of the entire tumor section. Post-acquisition processing was performed using Photoshop CS6 software (Adobe Systems) and image rebinning and pixel-by-pixel correlations were performed as previously described. (*3*)

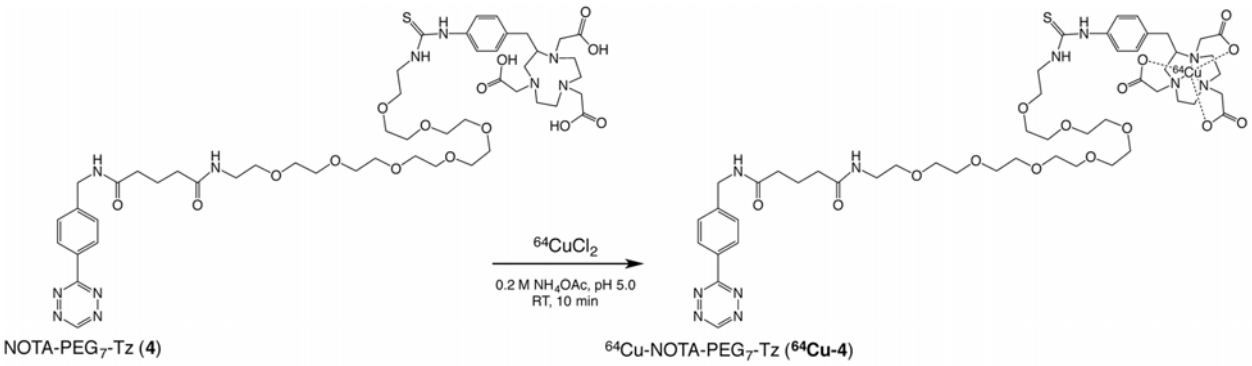
1. Viola-Villegas NT, Rice SL, Carlin S, et al. Applying PET to broaden the diagnostic utility of the clinically validated CA19.9 serum biomarker for oncology. *J Nucl Med.* 2013;54:1876-1882.

2. Stabin MG, Sparks RB, Crowe E. OLINDA/EXM: the second-generation personal computer software for internal dose assessment in nuclear medicine. *J Nucl Med.* 2005;46:1023-1027.

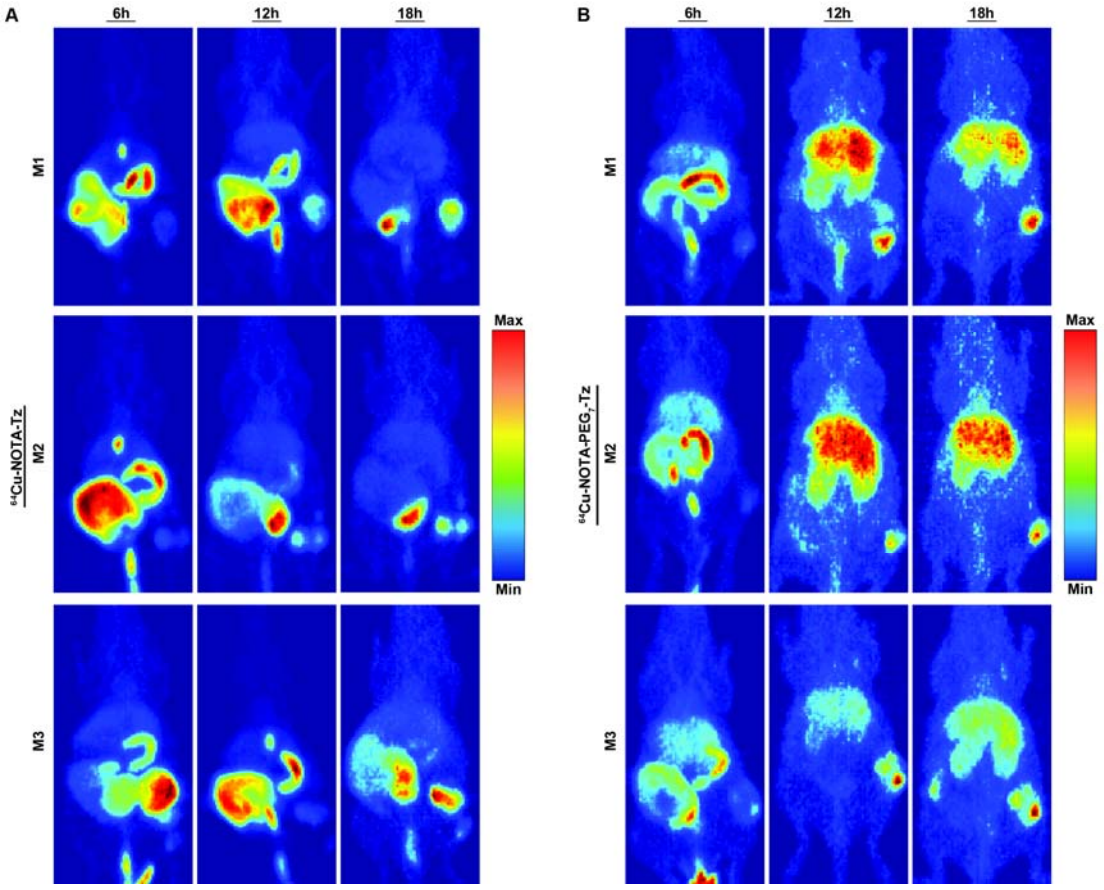
3. Carlin S, Pugachev A, Sun X, et al. In vivo characterization of a reporter gene system for imaging hypoxia-induced gene expression. *Nucl Med Biol.* 2009;36:821-831.



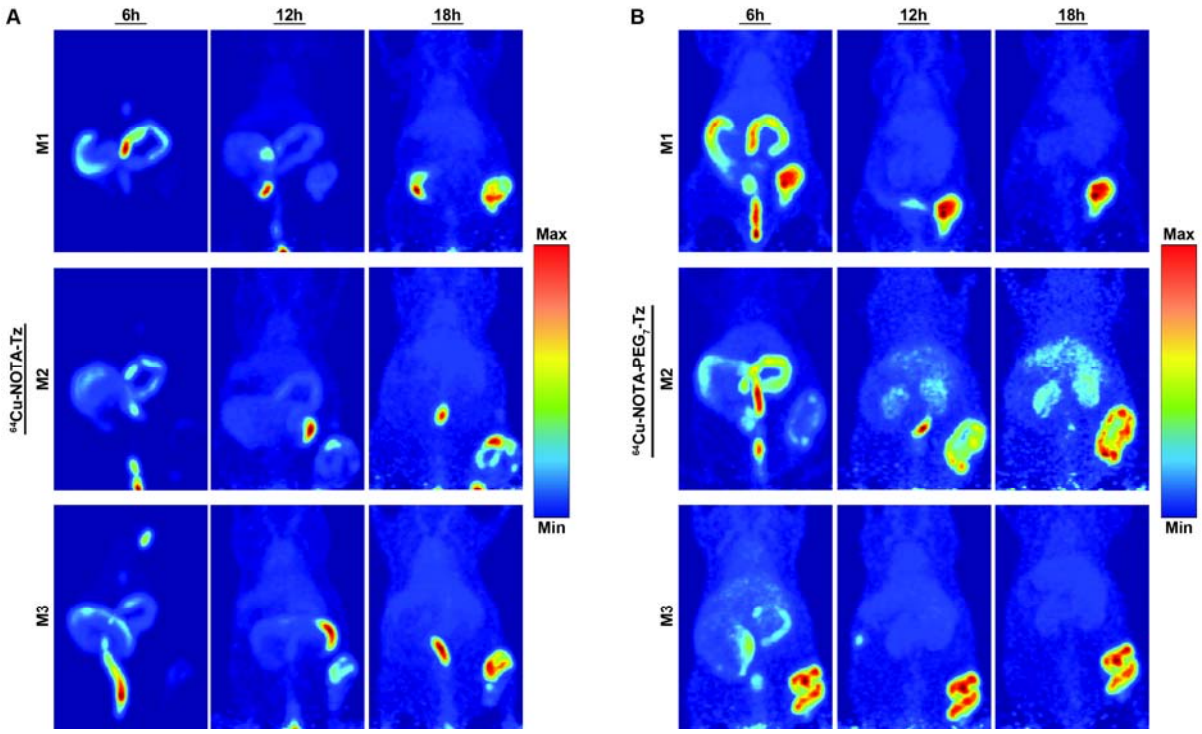
Supplemental Figure 1. The synthetic scheme for preparation of NOTA-PEG$_7$-Tz (**4**).



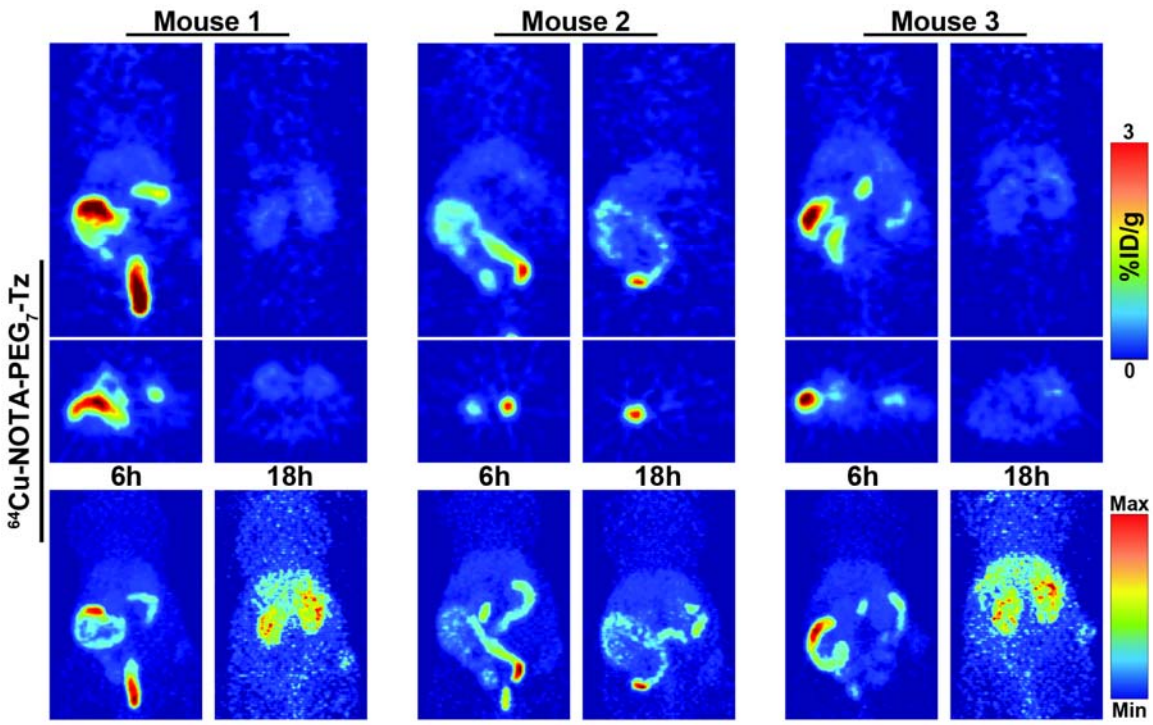
Supplemental Figure 2. The radiolabeling of NOTA-PEG_7-Tz (**4**) using $^{64}CuCl_2$ to give ^{64}Cu-NOTA-PEG_7-Tz (**^{64}Cu-4**).



Supplemental Figure 3. Maximum intensity projections from serial PET obtained in mice with subcutaneous BxPC3 xenografts on the right flank that were injected with 5B1-TCO followed by ^{64}Cu-NOTA-PEG$_7$-Tz **48h** later.



Supplemental Figure 4. Maximum intensity projections from serial PET obtained in mice with subcutaneous BxPC3 xenografts on the right flank that were injected with 5B1-TCO followed by ^{64}Cu-NOTA-PEG$_7$-Tz **72h** later.



Supplemental Figure 5. Maximum intensity projections from serial PET obtained in mice with subcutaneous BxPC3 xenografts on the right flank that were injected with **IgG-TCO** followed by ^{64}Cu-NOTA-PEG$_7$-Tz **72h** later.

Radioligand	^{64}Cu-NOTA-PEG$_7$-Tz			^{64}Cu-NOTA-Tz	
Lag Time	**72h**			**48h**	
Time post injection	**4h**	**12h**	**20h**	**20h**	**20h**
Tumor : Blood	1.4 ± 0.3	2.2 ± 1.3	4.2 ± 1.2	2.7 ± 0.4	4.2 ± 0.9
Tumor : Heart	4.7 ± 0.8	5.5 ± 3.4	11 ± 3	4.5 ± 0.7	7.1 ± 1.8
Tumor : Lungs	2.5 ± 0.6	3.5 ± 2.0	8.0 ± 5.9	3.4 ± 0.6	5.2 ± 1.3
Tumor : Liver	2.2 ± 0.4	3.2 ± 1.8	5.8 ± 1.3	1.4 ± 0.4	3.9 ± 0.5
Tumor : Spleen	5.4 ± 1.1	6.7 ± 3.9	11 ± 3	3.7 ± 1.2	8.9 ± 1.0
Tumor : Pancreas	11 ± 3	14 ± 9	21 ± 6	16.4 ± 9.9	29.2 ± 12.8
Tumor : Stomach	9.3 ± 2.9	19 ± 17	35 ± 9	5.2 ± 1.1	3.0 ± 1.5
Tumor : Small Intestine	1.3 ± 0.3	4.6 ± 3.2	16 ± 5	7.4 ± 1.9	11.1 ± 1.2
Tumor : Large Intestine	6.9 ± 1.6	10 ± 6	27 ± 8	1.4 ± 0.3	5.7 ± 0.6
Tumor : Kidney	1.7 ± 0.3	2.2 ± 1.3	4.5 ± 1.2	24.6 ± 4.2	35.5 ± 7.7
Tumor : Bone	16 ± 3	26 ± 15	17 ± 15	14.6 ± 2.5	22.1 ± 3.9
Tumor : Muscle	14 ± 3	17 ± 10	26 ± 8	7.0 ± 1.6	11.2 ± 2.2

Supplemental Table 1. Tumor-to-tissue ratios calculated from the biodistribution data for ^{64}Cu-NOTA-PEG$_7$-Tz and ^{64}Cu-NOTA-Tz.

Radioligand	^{64}Cu-NOTA-PEG$_7$-Tz		
Lag Time	**120h**		
Time post injection	**4h**	**12h**	**20h**
Blood	1.20 ± 0.36	0.71 ± 0.11	0.57 ± 0.16
Tumor	1.10 ± 0.46	1.22 ± 0.35	2.02 ± 0.72
Heart	0.53 ± 0.13	0.40 ± 0.11	0.35 ± 0.06
Lungs	1.10 ± 0.20	0.71 ± 0.11	0.55 ± 0.08
Liver	1.07 ± 0.20	0.81 ± 0.07	0.66 ± 0.07
Spleen	0.33 ± 0.02	0.33 ± 0.06	0.34 ± 0.06
Pancreas	0.33 ± 0.04	0.10 ± 0.04	0.12 ± 0.03
Stomach	0.24 ± 0.07	0.18 ± 0.03	0.16 ± 0.02
Large Intestine	3.26 ± 1.43	0.67 ± 0.28	0.33 ± 0.04
Small Intestine	0.44 ± 0.07	0.25 ± 0.04	0.21 ± 0.04
Kidney	1.57 ± 0.45	1.21 ± 0.24	0.83 ± 0.14
Muscle	0.19 ± 0.05	0.10 ± 0.03	0.12 ± 0.02
Bone	0.27 ± 0.02	0.24 ± 0.02	0.22 ± 0.06

Table S2. Values from the acute biodistribution study of mice with subcutaneous BxPC3 xenografts on the right flank that were injected with **5B1-TCO** followed by ^{64}Cu-NOTA-PEG$_7$-Tz **120h** later.