Supporting Information

"Click-Mediated Pretargeted Radioimmunotherapy of Colorectal Carcinoma"

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Methods and Materials

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification. All water used was ultrapure (>18.2 $M\Omega cm^{-1}$ at 25 °C), all DMSO was of molecular biology grade (>99.9%), and all other solvents were of the highest grade commercially available. Amine-reactive *trans*-cyclooctene [(E)-cyclooct-4-enyl 2,5-dioxo-1-pyrrolidinyl carbonate; TCO-NHS)] and amine-reactive tetrazine [*N*-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-6-(2,5-dioxopyrrolidin-1-yl)-5-oxohexanamide; Tz-NHS] were purchased from Sigma-Aldrich (St. Louis, MO). *p*-SCN-Bn-DOTA chelator was purchased from Macrocyclics, Inc. (Dallas, TX). ^{177}Lu was procured from PerkinElmer (PerkinElmer Life and Analytical Sciences, Wellesley, MA) as $^{177}LuCl_3$ in 0.05 M HCl. ^{64}Cu was obtained from Washington University (St. Louis MO) as $CuCl_2$ in 0.1 M HCl. The huA33 antibody was produced by the Ludwig Institute and the Olivia Newton-John Cancer Research Institute as previously described[1, 2]. Amine-reactive AlexaFluor 680 (AF680-NHS) was purchased from ThermoFisher Scientific (Waltham, MA). All experiments involving laboratory animals were performed in accordance with a protocol approved by the Weill Cornell Medical Center, Hunter College, and Memorial Sloan Kettering Institutional Animal Care and Use Committees.

Instrumentation

All instruments were calibrated and maintained in accordance with standard quality-control procedures. UV-Vis measurements were taken on a Shimadzu BioSpec-Nano Spectrophotometer. Activity measurements were made using a Capintec CRC-15R Dose Calibrator (Capintec, Ramsey, NJ). Biodistribution samples were counted for activity for 1 min on a Perkin Elmer (Waltham, MA) Automatic Wizard^2 Gamma Counter. Instant thin-layer chromatography (iTLC) for radio-iTLC experiments were performed on strips of glass-fiber, silica-impregnated paper (Pall Corp., East Hills, NY), read on a Bioscan AR-2000 Radio-TLC plate reader, and analyzed using Winscan Radio-TLC software (Bioscan Inc., Washington, D. C.). All PET imaging experiments were performed on Focus 120 PET scanner (Siemens Healthcare Global).

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, two LC-20AP pump systems, a CBM-20A communication BUS module, and a FRC-10A fraction collector using a reversed-phase C_{18} XTerra® Preparative MS OBDTM column (10 µm, 19.2 mm × 250 mm; 10 mL/min) or a reversed-phase C_{18} semi-Prep Phenomenex® Jupiter column (5 µm, 10 mm × 250 mm; 2 mL/min). Analytical HPLC runs were performed using a reversed-phase C_{18} Phenomenex® Jupiter column (5 µm, 4.6 mm × 250 mm; 1 mL/min). All radio-HPLC analyses and purifications were performed using a Shimadzu HPLC equipped with a reversed-phase C_{18} column (Phenomenex® Luna analytical 4.6 x 250 mm; 1 mL/min), 2 LC-10AT pumps, a SPD-M10AVP photodiode array detector, and a Bioscan Flow Counts radioactivity detector with a gradient of 5:95 CH_3CN:H_2O (both with 0.1% TFA) to 95:5 CH_3CN:H_2O over 15 min

Synthesis and Characterization

Preparation of huA33-TCO: An aliquot of huA33 (1.6 mg, 10.7 nmol) was dissolved in 500 µL of phosphate buffered saline (PBS, pH 7.4), and the pH of the solution was adjusted to 8.8-9.0 with Na_2CO_3 (0.1 M). A volume of TCO-NHS in DMF (25 mg/mL) was added to yield a TCO-NHS:huA33 reaction stoichiometry of 40:1. The resulting solution was incubated at 25 °C for 1 hour with shaking at 400 rpm. After 1 hour, the modified antibody was purified using size-exclusion chromatography (Sephadex G-25M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with a 2 mL fraction of PBS, pH 7.4) and concentrated using centrifugal filtration units with a 50,000 Da molecular weight cut off (Amicon™ Ultra 2mL, Millipore Corp., Billerica, MA) and PBS (pH 7.4).

Preparation of huA33-CHX-A″-DTPA: An aliquot of huA33 (1.2 mg, 8.0 nmol) was dissolved in 500 µL of PBS (pH 7.4), and the pH of the solution was adjusted to ~8.8 using 0.1 M Na_2CO_3. A volume of CHX-A″-DTPA in DMSO (25 mg/mL) was added to yield a CHX-A″-DTPA:huA33 reaction stoichiometry ratio of 20:1. The resulting solution was incubated at 25 °C for 1.5 hours with shaking at 350 rpm. The modified antibody was then purified using size-exclusion chromatography (Sephadex G-25M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with a 2 mL fraction of PBS, pH 7.4) and concentrated with centrifugal filtration units with a 50,000 Da molecular weight cut off (Amicon™ Ultra 2mL, Millipore Corp., Billerica, MA) and PBS (pH 7.4).

*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-PEG_7-NHBoc was prepared as previously described [3]. 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 a thermomixer at 300 rpm at 25 °C. 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$_7$-NH$_2$ was prepared as previously described [3]. Tz-PEG$_7$-NHBoc (**1,** 10 mg; 0.014 mmol; 717.5 g/mol) was first dissolved in 400 µL of 1:1 CH_2Cl_2:TFA and placed on an agitating thermomixer at 300 rpm for 30 minutes at 25 °C. 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: $\varepsilon_{525} = 535\ M^{-1}cm^{-1}$.'

*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**):* DOTA-PEG$_7$-Tz was prepared as previously described [3]. A portion of 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 25 °C. 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 15.4 mg of purified product as 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 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**)*: A portion of 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 (0.0021 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 25 °C. 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).

Radiolabeling

Preparation of ^{177}Lu-DOTA-PEG$_7$-Tz: ^{177}Lu-DOTA-PEG$_7$-Tz was prepared as previously described [3]. 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 0.1 M 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 25 °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 molar activity of ^{177}Lu-DOTA-PEG$_7$-Tz was 17.2 ± 5.7 GBq/μmol (n=4).

Preparation of ^{177}Lu-CHX-A″-DTPA-huA33: An aliquot of prepared huA33- CHX-A″-DTPA (0.3 mg, 2.0 nmol) was buffer exchanged to be suspended in chelex-treated 0.25 M NH_4OAc with 34 mM ascorbic acid (pH 5.5) using centrifugal filtration units with a 30,000 Da molecular weight cut off (Amicon™ Ultra 0.5mL, Millipore Corp., Billerica, MA). To this solution 2.7 mCi (100 MBq) of $^{177}LuCl_3$ was added and incubated for 1 hour at 37 °C with shaking at 400 rpm. The radiolabeled immunoconjugate was then purified using size-exclusion chromatography (Sephadex G-25M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with 2 mL fractions of PBS with 34 mM ascorbic acid, pH 7.4), producing the final product with an overall radiochemical yield of 82.0%, a radionuclidic purity of 99.6% as determined by iTLC, and a molar activity of 31.6 GBq/μmol (n = 1).

Preparation of ^{64}Cu-SarAr-Tz: ^{64}Cu-SarAr-Tz was prepared as previously described [4]. A solution of Tz-SarAr (5-25 μg; 6.9-34.9 nmol) in NH_4OAc buffer (0.2 M, pH 5.5, 200 μL) was first prepared. Then, the desired amount of $^{64}CuCl_2$ in 0.1 M HCl (3000 μCi; 111 MBq) was added to the reaction mixture, and the solution was placed on an agitating thermomixer at 300 rpm for 30 minutes at 25 °C. After this incubation, the ^{64}Cu-SarAr-Tz was purified via reversed phase C_{18} HPLC (tR = 8.7 min). This yielded the completed radioligand with a radionuclidic purity of >99% (as determined by reversed phase HPLC) and a molar activity of 19.4 ± 1.5 GBq/μmol (n = 4).

Functional characterization

Determination of the TCO occupancy of huA33-TCO: The TCO occupancy of huA33-TCO was determined as previously described [3, 4]. A solution of huA33-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 A33-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 25 °C. 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 Da molecular weight cut off (Amicon™ 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_{huA33} = 150,000, ε_{AF680} = 184,000, and $\varepsilon_{280,\ huA33}$ = 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.

Cell culture and xenografts

Human colorectal cell line SW1222 was obtained from Sigma-Aldrich, Inc. and maintained in Minimum Essential Medium, supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin in a 37 °C environment 7 containing 5% CO_2. Cell lines were harvested and passaged every 8-10 days using 0.25% trypsin/0.53 mM EDTA in Hank's Buffered Salt Solution without calcium and magnesium. 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 $5x10^6$ SW1222 cells, suspended in 150 µL of a solution containing a 1:1 mixture of Matrigel (Becton Dickinson, Bedford, MA) and cell culture medium. SW1222 tumors were grown for 14-18 days post-implantation prior to imaging or biodistribution.

PET imaging

One subject from each cohort was administered huA33- TCO (100 µg, 0.6 nmol in 200 µL sterile PBS) via intravenous tail vein injection. After an accumulation interval of 24 h, the same mice were then administered ^{64}Cu-SarAr-Tz (500 µCi; 18.5 MBq in 200 µL sterile PBS), also via intravenous tail vein injection (t = 0). The molar activity of the radiotracer was adjusted using SarAr-Tz such that the molar ratio of Tz:huA33 ≈ 1:1. Static scans were recorded at 24 h after injection of the radioactivity for a total scan time of 15 minutes per scan. 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.

Pretargeted biodistribution

The acute biodistribution of ^{177}Lu-DOTA-PEG$_7$-Tz was determined using a SW1222 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 100 µg (0.6 nmol) of huA33-TCO via intravenous tail vein injection. After an accumulation interval period of 24 h, the same mice were then administered ^{177}Lu-DOTA-PEG$_7$-Tz (300-350 µCi; 11.1-13.0 MBq, 0.6-0.7 nmol, in 200 µL sterile PBS) also via intravenous tail vein injection. At the appropriate time post injection of radioactivity, mice (n = 4) 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.

Biodistribution with ^{177}Lu-CHX-A"-DTPA-huA33

The acute biodistribution of prepared ^{177}Lu-CHX-A″-DTPA-huA33 was determined using a SW1222 subcutaneous xenograft (right flank, ~200 mm^3) model in athymic, nude mice. Mice were randomized into groups and warmed gently using a heat lamp prior to being injected with 5 µg of ^{177}Lu-CHX-A″-DTPA-huA33 (S.A. 5.7 µCi/µg) in PBS with 34 mM ascorbic acid (pH 7.4) via intravenous tail vein injection. At the appropriate time post injection, mice (n = 4 per group) 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/huA33-TCO pretargeting system as well as the directly-labeled ^{177}Lu-CHX-A″-DTPA-huA33 radioimmunoconjugate were 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:

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

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 utilizing the 70-kg Standard Man anatomic model to yield the mean organ absorbed doses and effective dose.[5]

Longitudinal therapy study

Mice bearing established SW1222 subcutaneous tumors (mean tumor volume 250 ± 50 mm^3) were randomly sorted into 5 groups of 10 mice per group. In order to determine the dose dependence of the PRIT methodology, the treatment groups received injections of the radioligand loaded with 500, 1000, or 1500 μCi (18.5, 37.0, 55.5 MBq) via the lateral tail vein 24 h after administration of huA33-TCO (100 μg in 200 μL of 0.9% saline). The remaining two cohorts served as control groups, receiving either huA33-TCO alone (100 μg in 200 μL of 0.9% saline) or 1500 μCi (55.5 MBq) of ^{177}Lu-DOTA-PEG_7-Tz without huA33-TCO. All mice were weighed, and the tumor burden was assessed by caliper measurement every 3-4 days until day 21, and then every 7 days thereafter until the conclusion of the experiment. Tumor volume was calculated using the formula for the volume of an ellipsoid. 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.

Statistical Analysis

Biodistribution data were analyzed using an unpaired, two-tailed Student's t-test. Differences at the 95% confidence level ($P < 0.05$) were considered to be statistically significant. For the therapy study, Kaplan-Meier survival analysis was performed with Prism software (version 7.0). Differences at the 95% confidence level ($P < 0.05$) were considered to be statistically significant.

Supplemental Figures and Tables

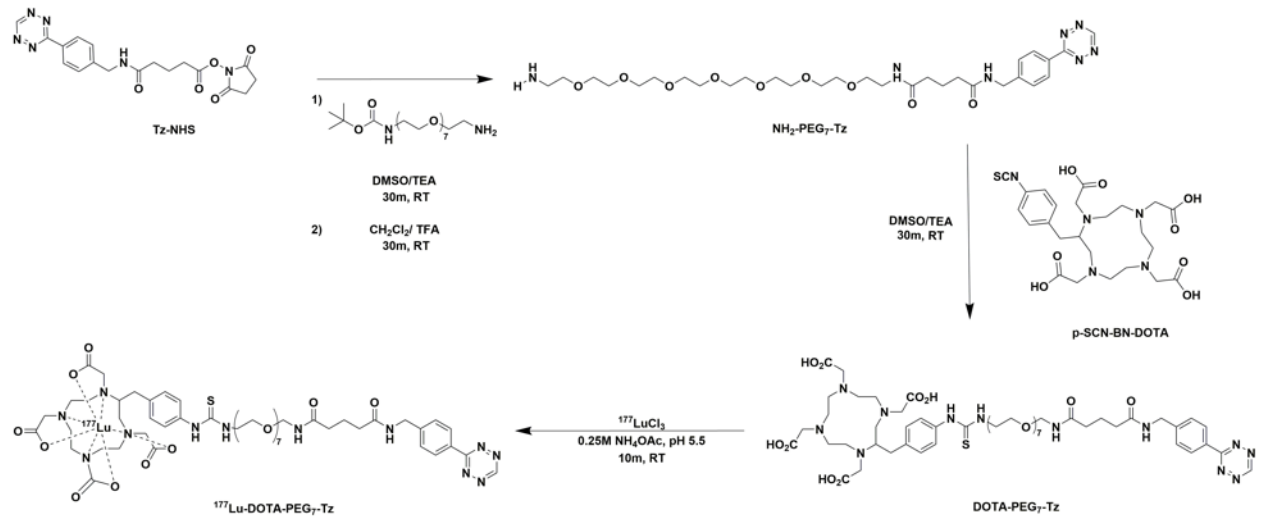

Figure S1: Schematic of the synthesis and radiolabeling of ^{177}Lu-DOTA-PEG$_7$-Tz.

24 h Interval: huA33-TCO/^{177}Lu-DOTA-PEG$_7$-Tz					72 h Interval: huA33-TCO/^{177}Lu-DOTA-PEG$_7$-Tz				
Organ	**4 h**	**24 h**	**48h**	**120 h**	**Organ**	**4 h**	**24 h**	**48h**	**120 h**
Blood	5.0 ± 1.3	3.7 ± 0.6	3.0 ± 0.6	1.0 ± 0.2	Blood	3.3 ± 1.2	2.2 ± 0.4	1.3 ± 0.8	0.7 ± 0.2
Tumor	5.0 ± 1.5	13 ± 1.6	17 ± 2.2	21 ± 2.9	Tumor	3.2 ± 0.7	6.3 ± 3.6	9.8 ± 2.8	13 ± 2.3
Heart	1.4 ± 0.4	1.1 ± 0.2	0.8 ± 0.1	0.3 ± 0.1	Heart	0.8 ± 0.3	0.5 ± 0.1	0.4 ± 0.2	0.3 ± 0.1
Lung	2.4 ± 0.7	2.3 ± 0.4	1.9 ± 0.5	0.8 ± 0.1	Lung	1.4 ± 0.3	1.3 ± 0.4	0.8 ± 0.4	0.6 ± 0.1
Liver	1.5 ± 0.4	1.4 ± 0.3	1.2 ± 0.1	0.6 ± 0.1	Liver	1.1 ± 0.4	0.9 ± 0.2	0.6 ± 0.2	0.4 ± 0.1
Spleen	1.3 ± 0.4	1.4 ± 0.4	1.2 ± 0.3	0.7 ± 0.2	Spleen	0.6 ± 0.2	0.7 ± 0.2	0.5 ± 0.2	0.3 ± 0.1
Stomach	0.3 ± 0.1	0.7 ± 0.8	0.3 ± 0.1	0.1 ± 0.03	Stomach	0.4 ± 0.2	0.4 ± 0.1	0.1 ± 0.04	0.1 ± 0.02
Small Intestine	0.6 ± 0.3	0.7 ± 0.5	0.3 ± 0.1	0.2 ± 0.02	Small Intestine	0.4 ± 0.1	0.3 ± 0.04	0.1 ± 0.05	0.1 ± 0.02
Large Intestine	0.9 ± 0.4	1.3 ± 1.4	0.4 ± 0.3	0.2 ± 0.1	Large Intestine	1.5 ± 0.6	0.6 ± 0.2	0.3 ± 0.1	0.1 ± 0.05
Kidney	2.3 ± 0.5	1.9 ± 0.2	1.6 ± 0.2	0.8 ± 0.01	Kidney	2.0 ± 0.3	1.5 ± 0.2	1.1 ± 0.2	0.7 ± 0.1
Muscle	0.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.1 ± 0.01	Muscle	0.2 ± 0.1	0.2 ± 0.03	0.1 ± 0.05	0.08 ± 0.02
Bone	0.5 ± 0.2	0.6 ± 0.2	0.7 ± 0.3	0.3 ± 0.02	Bone	0.4 ± 0.2	0.4 ± 0.1	0.2 ± 0.1	0.2 ± 0.02

48 h Interval: huA33-TCO/^{177}Lu-DOTA-PEG$_7$-Tz					Directly Labeled: huA33-CHX-A″-DTPA-^{177}Lu				
Organ	**4 h**	**24 h**	**48h**	**120 h**	**Organ**	**24 h**	**72 h**	**120 h**	**168 h**
Blood	4.4 ± 1.7	2.5 ± 0.8	1.5 ± 0.5	0.4 ± 0.3	Blood	9.5 ± 1.5	4.0 ± 2.3	2.6 ± 1.9	1.8 ± 1.6
Tumor	5.2 ± 1.2	9.0 ± 2.6	13 ± 2.8	11 ± 2.4	Tumor	57 ± 11	70 ± 15	75 ± 22	69 ± 32
Heart	1.2 ± 0.4	0.7 ± 0.3	0.5 ± 0.2	0.2 ± 0.1	Heart	2.9 ± 0.6	1.0 ± 0.6	1.0 ± 0.3	0.7 ± 0.4
Lung	2.7 ± 1.0	1.5 ± 0.6	1.0 ± 0.3	0.4 ± 0.1	Lung	4.6 ± 0.5	1.9 ± 0.9	1.6 ± 0.8	1.0 ± 0.6
Liver	1.1 ± 0.5	0.9 ± 0.2	0.7 ± 0.3	0.3 ± 0.1	Liver	3.6 ± 0.2	2.9 ± 1.3	2.9 ± 1.4	1.4 ± 0.3
Spleen	1.0 ± 0.5	0.7 ± 0.3	0.9 ± 0.4	0.2 ± 0.1	Spleen	3.1 ± 0.3	1.9 ± 0.5	3.7 ± 3.5	1.3 ± 0.4
Stomach	0.3 ± 0.1	0.5 ± 0.4	0.2 ± 0.1	0.06 ± 0.01	Stomach	0.9 ± 0.3	0.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.2
Small Intestine	1.5 ± 1.9	0.3 ± 0.1	0.2 ± 0.1	0.07 ± 0.02	Small Intestine	1.0 ± 0.2	0.6 ± 0.3	0.8 ± 0.5	0.4 ± 0.2
Large Intestine	1.6 ± 0.4	1.5 ± 1.1	0.2 ± 0.1	0.1 ± 0.03	Large Intestine	0.8 ± 0.1	0.5 ± 0.1	0.6 ± 0.2	0.4 ± 0.1
Kidney	2.5 ± 0.5	1.5 ± 0.3	1.1 ± 0.2	0.6 ± 0.1	Kidney	3.9 ± 0.6	2.3 ± 0.9	1.9 ± 0.6	1.3 ± 0.5
Muscle	0.5 ± 0.1	0.3 ± 0.1	0.2 ± 0.03	0.06 ± 0.02	Muscle	1.0 ± 0.2	0.6 ± 0.2	0.5 ± 0.2	0.3 ± 0.2
Bone	0.5 ± 0.2	0.3 ± 0.1	0.4 ± 0.1	0.1 ± 0.03	Bone	2.3 ± 0.6	0.8 ± 0.3	1.0 ± 0.1	1.1 ± 0.2

Table S1. Full biodistribution data in nude mice bearing SW1222 xenografts for the radiopharmaceuticals investigated in this work. The top left, bottom left, and top right panels provide the activity concentration values produced by huA33-TCO/^{177}Lu-DOTA-PEG$_7$-Tz pretargeting with injection intervals of 24, 48, and 72 hours, respectively. The bottom right panel provides the activity concentration values produced by the directly-labeled ^{177}Lu-CHX-A″-DTPA-huA33 radioimmunoconjugate. The values for the stomach, small intestine, and large intestine include the contents.

Tumor-to-Organ	24 h Interval	48 h Interval	72 h Interval	Directly Labeled
Blood	20 ± 5	24 ± 15	17 ± 6	29 ± 23
Heart	67 ± 15	64 ± 28	50 ± 17	77 ± 34
Lung	28 ± 6	27 ± 12	23 ± 6	48 ± 27
Liver	37 ± 7	35 ± 14	30 ± 11	26 ± 14
Spleen	29 ± 10	43 ± 16	38 ± 14	21 ± 20
Stomach	147 ± 34	192 ± 62	150 ± 42	178 ± 68
Small Intestine	141 ± 25	156 ± 70	137 ± 36	94 ± 62
Large Intestine	106 ± 68	86 ± 29	89 ± 34	130 ± 54
Kidney	27 ± 6	19 ± 6	20 ± 5	40 ± 17
Muscle	184 ±30	168 ± 64	153 ± 44	155 ± 67
Bone	82 ± 13	86 ± 32	82 ± 18	71 ± 23

Table S2: Tumor-to-organ activity concentration ratios at 120 h after the administration of the radiopharmaceutical for the huA33-TCO/^{177}Lu-DOTA-PEG$_7$-Tz pretargeting system as well as the directly labeled ^{177}Lu-CHX-A″-DTPA-huA33 radioimmunoconjugate.

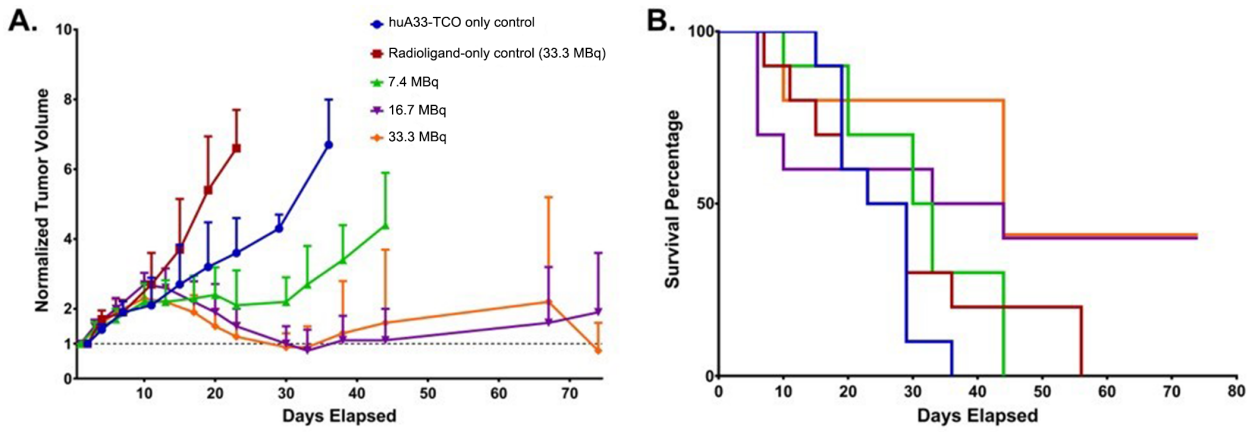

Figure S2: A longitudinal therapy study of 5 groups of mice (n = 10 per cohort) bearing subcutaneous SW1222 tumors depicted in (A) a graph of normalized tumor volume as a function of time and (B) the corresponding Kaplan-Meier survival curve. The control groups received the huA33-TCO immunoconjugate without the ^{177}Lu-DOTA-PEG$_7$-Tz radioligand (blue) or the radioligand without the immunoconjugate (red). The three treatment groups received huA33-TCO (100 μg, 0.6 nmol) followed 72 h later by 7.4 (green), 16.7 (purple), or 33.3 (orange) MBq of ^{177}Lu-DOTA-PEG$_7$-Tz. By log-rank (Mantel-Cox) test, survival was significant ($P < 0.01$) for the PRIT group receiving 33.3 MBq. Median survival was determined to be 21 days for the huA33-TCO-only control group, 15 days for the radioligand-only control group, and 35.5, 41, and 67 days for the cohorts receiving 7.4, 16.7, or 33.3 MBq, respectively.

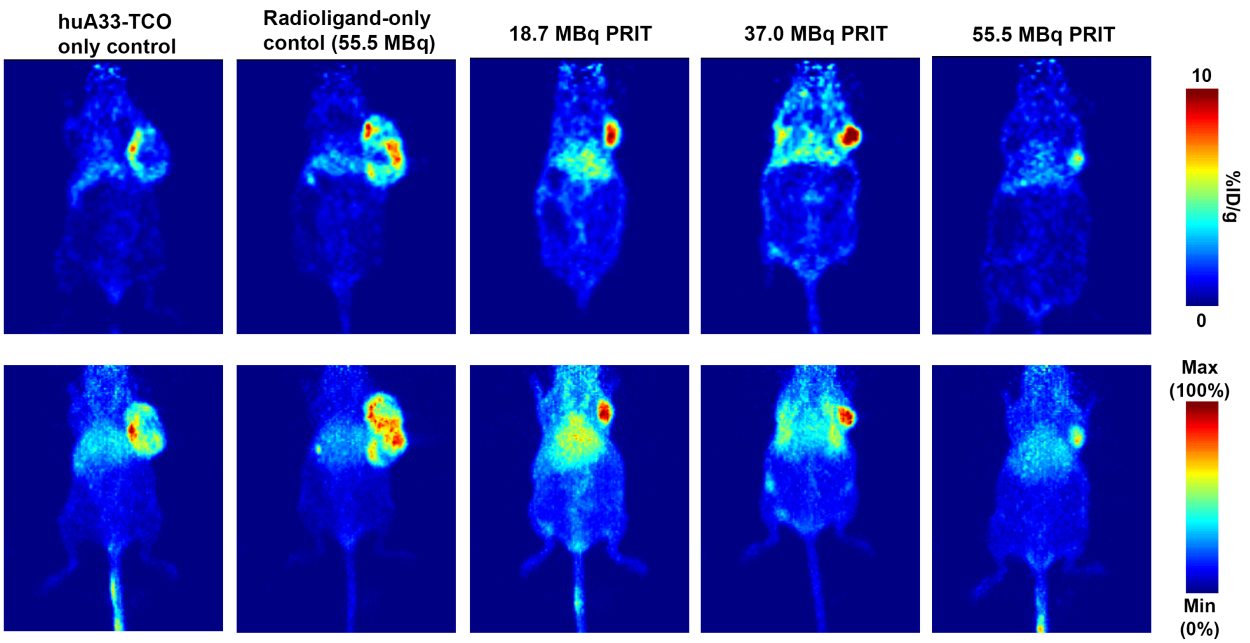

Figure S3: Pretargeted PET maximum intensity projections of mice from the control and treatment arms obtained on day 27 of the longitudinal therapy study (using a 24 h injection interval between huA33-TCO and ^{177}Lu-DOTA-PEG$_7$-Tz). Mice were first injected with huA33-TCO (100 µg, 0.6 nmol), followed 24 h later by the administration of ^{64}Cu-SarAr-Tz (18.5 MBq, 0.7 nmol). PET images were collected 24 h after the administration of the radioligand.

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