Supporting Information

Dual Radionuclide Theranostic Pretargeting

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Materials and Methods

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 MΩcm^{-1} at 25 °C) and all DMSO was of molecular biology grade (>99.9%). 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 and DiAmSar chelators were purchased from Macrocyclics, Inc. (Dallas, TX). Amine-reactive AlexaFluor 680 (AF680-NHS) was purchased from ThermoFisher Scientific (Waltham, MA). ^{64}Cu was purchased from Washington University, St. Louis, as [^{64}Cu]$CuCl_2$ in 0.05 M HCl. ^{177}Lu was procured from PerkinElmer (PerkinElmer Life and Analytical Sciences, Wellesley, MA) as $^{177}LuCl_3$ in 0.05 M HCl. Humanized A33 (huA33) was generously provided by the Olivia Newton-John Cancer Research Institute, and stored at -80 °C prior to use. All experiments involving 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 according to standard quality control practices and procedures. UV-Vis measurements were obtained on a Shimadzu BioSpec-Nano Micro-Volume UV-Vis Spectrophotometer (Shimadzu, Kyoto, Japan). Mass measurements were taken on a Shimadzu AUW120D analytical balance. Radioactivity measurements were obtained using a Capintec CRC-15R Dose Calibrator (Capintec, Ramsey, NJ). Biodistribution samples were counted for activity for 1 min on a calibrated 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.).

HPLC

All HPLC purifications (Eluent A: 0.1% TFA in water, Eluent 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).

High Resolution Mass Spectrometry

HRMS data were obtained on an Agilent 6550 QToF with a dual sprayer ESI source, coupled to an Agilent 1290 Infinity LC system. Samples were analyzed by either FIA (flow injection analysis) using a mobile phase of 50% acetonitrile in water (0.1% formic acid) with a flow rate of 0.4 mL/min; or LCMS with an Agilent Poroshell 120 SB-C18 column (2.7um, 2.1x50mm) at 45 ºC, using a linear gradient of 5-95% acetonitrile in water (0.1% formic acid) with a flow rate of 0.4 mL/min. Mass

spectra were obtained in either positive or negative mode, and acquired using the MassHunter Acquistiion Software (version B.05.01); then analyzed by MassHunter Qualitative Analysis (version B.06.00).

Syntheses

Preparation of huA33-TCO

HuA33-TCO was prepared as previously described.[1,2] An aliquot of huA33 (11.6 mg, 77.3 nmol) was dissolved in 800 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). 25 µl 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 500 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).

Synthesis of SarAr-Tz

The ligand SarAr-Tz was synthesized as previously described.[1] In brief, a solution of Tz-NHS (5.0 mg; 0.013 mmol; 1.0 equiv.; 398.4 g/mol) in anhydrous dimethylformamide (400 µL) was added to a stirred solution of SarAr-Bn-NH_2 (5.4 mg; 0.013 mmol; 1.0 equiv.; 434.7 g/mol) in anhydrous dimethylformamide (200 µL) at room temperature, and the reaction solution was stirred in the dark for 2 h at room temperature. After dilution with water (1.8 mL), purification by HPLC (1.0 mL/min, 95:5 H_2O:CH_3CN to 20:80 H_2O:CH_3CN over 15 min) afforded SarAr-Tz (MW = 716.9; 3.9 mg; 42%) as a pink solid: t_R = 9.5 min. ^{1}H NMR (600 MHz, D_2O), δ, ppm: 10.25 (s, 1H), 8.31 (d, 2H), 7.46 (d, 2H), 7.22-7.27 (m, 4H), 4.39 (m, 2H), 4.24 (m, 3H), 2.46-3.95 (m, 24H), 2.41-2.44 (m, 4H), 1.83 (m, 2H). ^{13}C NMR (125 MHz, D_2O): δ = 21.7, 34.7, 42.5, 42.7, 47.4, 52.5, 55.9, 57.2, 58.8, 60.1, 116.2 (TFA), 127.7, 128.0, 128.5, 130.3, 135.4, 138.5, 143.5, 157.3, 162.9 (TFA), 166.3, 175.7, 175.8. ESI-MS(+): m/z = 717.6 $[M+H]^+$. HRMS (ESI): m/z calcd. for $C_{36}H_{57}N_{14}O_2$: 717.4789; found: 717.4788.

Synthesis of Tz-PEG$_7$-NHBoc

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) was prepared as previously described.[1,2] 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 MeCN:H_2O (both with 0.1% TFA) to 95:5 MeCN: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: ε_{525} = 530 $M^{-1}cm^{-1}$.

Synthesis of Tz-PEG$_7$-NH$_2$

N1-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-N5-(23-amino-3,6,9,12,15,18,21-heptaoxatricosyl)glutaramide (Tz-PEG$_7$-NH$_2$) was prepared as previously described.[1,2] Tz-PEG$_7$-NHBoc (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 MeCN:H_2O (both with 0.1% TFA) to 95:5 MeCN: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). ESIMS(+): *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 DOTA-PEG$_7$-Tz

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) was prepared as previously described.[1,2] A portion of Tz-PEG$_7$-NH$_2$ (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 MeCN:H_2O (both with 0.1% TFA) to 95:5 MeCN: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 (600 MHz, DMSO), δ, ppm: 12.2 (bs, 4H), 11.74 (s, 1H) 10.59 (s, 1H), 8.55 (t, 1H, J = 5.9 Hz), 8.51 (t, 1H, J = 5.9 Hz), 8.46 (d, 2H, J = 8.2 Hz), 7.79 (d, 2H, J = 8.2 Hz), 7.55 (d, 2H, J = 8.3 Hz), 7.44 (d, 2H, J = 8.2 Hz), 7.42 (t, 1H, J = 7.7 Hz), 4.42 (d, 2H, J = 5.9 Hz), 3.80-3.40 (m, 38H), 2.51 (s, 1H), 2.69 (t, 1H, J = 6.0 Hz), 2.50-2.30 (m, 16H), 2.09 (t, 4H, J = 7.0 Hz). ^{13}C NMR (600 MHz, DMSO), δ, ppm: 173.88, 173.16, 170.96, 169.99, 169.31, 165.89, 158.61, 146.09, 145.41, 132.69, 130.77, 128.80, 128.47, 128.22, 70.36, 70.13, 70.11, 70.07, 70.06, 67.35, 66.68, 42.25, 42.18, 40.48, 36.65, 36.59, 35.17, 32.64, 31.19, 28.94, 27.28. ESI-MS(-): *m/z* (%) = 1203.0 $[M-H]^-$; 601.8 $[M-2H]^{-2}$ HRMS (ESI): *m/z* calcd. for $C_{50}H_{76}N_{11}O_{15}S$: 1202.5642; found: 1203.5741.

Synthesis of Tz-PEG$_7$-AF680

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) was prepared as previously described.[2] 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 MeCN:H_2O (both with 0.1% TFA) to 95:5 MeCN: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 of SarAr-Tz with ^{64}Cu

A solution of SarAr-Tz (12.9 µg; 18 nmol) in NH_4OAc buffer (0.25 M, pH 5.5, 200 µL) was first prepared. Then, the desired amount of [^{64}Cu]$CuCl_2$ in 0.05 M HCl (296 MBq; 8.0 mCi) was added to the reaction mixture, and the solution was placed on an agitating thermomixer at 500 rpm for 15 min at 37 °C. After this incubation, the progress of the [^{64}Cu]Cu-SarAr-Tz radiolabeling was determined by iTLC which revealed quantitative labeling of >99% radiochemical purity, thus no further purification was deemed necessary. The final molar activity of [^{64}Cu]Cu-SarAr-Tz was 15.3-16.4 GBq/µmol (n = 2).

Radiolabeling of DOTA-PEG$_7$-Tz with ^{177}Lu

A solution of DOTA-PEG_7-Tz (29.9 µg; 24.8 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 0.05 M HCl (215 MBq; 5.8 mCi) was added to the reaction mixture, and the solution was placed on an agitating thermomixer at 700 rpm for 30 min at 37 °C. After this incubation, the progress of the [^{177}Lu]Lu-DOTA-PEG_7-Tz radiolabeling was determined by iTLC which revealed quantitative labeling of >99% radiochemical purity, thus no further purification was deemed necessary. The final molar activity of [^{177}Lu]Lu-DOTA-PEG_7-Tz was 8.6-9.5 GBq/µmol (n = 2).

Functional characterization

Determination of the TCO occupancy of huA33-TCO

The TCO occupancy of huA33-TCO was determined as previously described.[1,2] A solution of huA33-TCO (50 µg; 0.33 nmol) in 300 µL PBS (pH 7.4) was first prepared (1.1 µM). Next, 10 µL of a 0.5 mM solution of Tz-PEG_7-AF680 in DMSO was added (a ~15-fold excess of Tz). This solution was placed on an agitating thermomixer at 500 rpm at 25 °C. After 24 h 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 Da, ε_{AF680} = 82,030 $cm^{-1}M^{-1}$, and $\varepsilon_{280,\,huA33}$ = 225,000 $cm^{-1}M^{-1}$. 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

Human colorectal cell line SW1222 was obtained from Sigma-Aldrich, Inc. and maintained in Iscove's Modified Dulbecco's Medium (IMDM), 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 containing 5% CO_2. Cell lines were harvested and passaged every seven 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 nude mice (5-7 weeks old) were obtained from The Jackson Laboratories and allowed to acclimatize for approximately 1 week prior to inoculation. Animals were group housed in ventilated cages with water and food ad libitum. Mice were anaesthetized with 2% isoflurane/medical air inhalation and 4-5 × 10^6 SW1222 cells were inoculated subcutaneously into the right shoulder in a 150 µl suspension of 1:1 fresh media / Matrigel (Corning Life Sciences). The tumors reached the ideal size for experiments (~100-150 cm^3) after approximately 14 days.

Pretargeted biodistribution

Mice were warmed gently using a heat lamp prior to being injected with 100 µg (0.67 nmol, in 100 µL sterile PBS) of huA33-TCO via intravenous tail vein injection. After an accumulation interval period of 24 h, the same mice were then administered with the first radioligand, [^{64}Cu]Cu-SarAr-Tz (*24 h interval*: 10.4-11.3 MBq, 0.64-0.69 nmol in 100 µL sterile PBS, *6 h interval*: 10.4-11.1 MBq, 0.68-0.73 nmol in 100 µL sterile PBS). The second radioligand, [^{177}Lu]Lu-DOTA-PEG$_7$-Tz (*24 h interval*: 5.9-6.4 MBq, 0.69-0.74 nmol in 100 µL sterile PBS, *6 h interval*: 6.2-6.3 MBq, 0.65-0.67 nmol in 100 µL sterile PBS), was administered either 6 h or 24 h after the injection of the first radioligand. At the appropriate time post injection of radioactivity, mice (n = 5) were euthanized via asphyxiation using CO_2(g) and tissues were collected 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. Due to the interference of ^{64}Cu in ^{177}Lu energy window (10-395 keV), the radioactivity measurements for ^{177}Lu were performed after the ^{64}Cu had decayed (5-7 days after administration). ^{177}Lu didn't interfere the ^{64}Cu energy window (425-640 keV). Weight of the syringe prior to injection and after injection was used to determine the mass of injectate. Four aliquots (10 µL) were weighed and counted as internal standards for each radioligand formulation. The total injected dose was found as the mass injected dose × internal standard average counts/g. The percent injected dose (%ID) was determined as the counts for the tissue × 100/total injected dose. The %ID/g was calculated as the %ID/tissue weight.

PET imaging

PET imaging was performed on a microPET Focus 120 dedicated small-animal scanner (Siemens Medical Solutions, Malvern, PA, USA). Mice were anaesthetized with 2% isoflurane/medical air inhalation approximately 5 min prior to recording the PET images and kept under anaesthesia during the PET scan. The energy and coincidence timing windows were 350-750 keV and 6 ns, respectively. Data was acquired as static images at 6, 24, and 48 h after intravenous injection of [^{64}Cu]Cu-SarAr-

Tz. Data were sorted into two-dimensional histograms by Fourier re-binning, and transverse images were reconstructed by filtered back-projection (FBP). The image data were normalized to correct for non-uniformity of response of the detector, 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. The counting rates in the reconstructed images were converted to percent of injected dose per weight (%ID/g) by use of a system calibration factor derived from the imaging of a mouse-sized water-equivalent phantom containing ^{64}Cu. Images were analyzed by using ASIPro VM software (Concorde Microsystems).

Dosimetry

The average of five organ specimens from each time point was fitted to a biexponential function. For most tissues, the best TAC fit comprised a one- or two-phase exponential decay model. For one tissue, the tumor, exhibited marked uptake over the measured time course, and therefore a trapeziodal model was used for this tissue up to the last time point, after which it was assumed that clearance was determined entirely by radioactive decay. Cumulative uptake was calculated from the areas under the uptake curves. Absorbed doses to tumor and all normal organs were estimated assuming absorbed fractions of 1 for the β-emissions of ^{177}Lu and 0 for the penetrating photon emissions and using an equilibrium absorbed dose constant of 0.31 g * Rad / μCi * h.

Calculation of the fraction of available TCO moieties upon the administration of [^{177}Lu]Lu-DOTA-PEG$_7$-Tz

In order to make a rough calculation of the [^{64}Cu]Cu-SarAr-Tz/huA33-TCO pretargeting reaction yield with the available data, two key assumptions must be made:

> (1) That the total amount of TCO available for the *in vivo* IEDDA reaction is the total amount injected per animal minus the amount that has isomerized into *cis*-cyclooctene (CCO).
>
> (2) That at 24 h p.i. all of the radioactivity is due to the [^{64}Cu]Cu- SarAr-Tz that has reacted with the TCO on the surface of huA33.

Each mouse was administered 0.67 nmol of huA33-TCO. On average, each antibody had a degree of labeling of 2.4 TCO/mAb, bringing the two amount of TCO injected to 1.6 nmol. 24 h after the injection of [^{64}Cu]Cu-SarAr-Tz, each mouse (n = 20) was measured with a dose calibrator. The resulting activity values were decay-corrected to the end of the radiosynthesis (also the moment to which molar activity is determined), yielding a decay corrected value for the amount of radioactivity per mouse of 4.0 ± 0.3 MBq (108.9 ± 8.5 μCi). Given that the molar activity of [^{64}Cu]Cu-SarAr-Tz was 16.4 GBq/μmol, we can calculate that each mouse contains ~0.25 nmol of SarAr-Tz 24 hours after injection. The *in vivo* IEDDA yield can be calculated as the ratio of the total amount of [^{64}Cu]Cu-SarAr-Tz remaining *in vivo* at 24 h p.i. (~0.25 nmol) to the total amount of TCO injected (1.6 nmol): ~15%. Meanwhile, another process has also been reducing the amount of TCO on the immunoconjugate: the slow isomerization of TCO to CCO. Rossin, *et al.* have estimated that the *in vivo* half-life of TCO bound to an antibody via a short linker is 6.19 d.[2] Therefore, between t = 0 (the administration of huA33-TCO) and t = 24 h (the administration of [^{64}Cu]Cu-SarAr-Tz), roughly 0.17 nmol (or 11%) of the TCO has become isomerized to CCO. Taking the isomerization of TCO to CCO into account, the *in vivo* IEDDA yield is ~17%. By the time [^{177}Lu]Lu-DOTA-PEG$_7$-Tz is

administered (t = 48 h), roughly 0.32 nmol (or 20%) of the TCO has become isomerized to CCO. Ultimately, this means that by the time the [^{177}Lu]Lu-DOTA-PEG$_7$-Tz is injected, 1.6 nmol (amount initially injected) – 0.25 nmol (amount reacted with [^{64}Cu]Cu-SarAr-Tz) – 0.32 nmol (amount isomerized to CCO) = 1.03 nmol is still available for reaction. *This corresponds to 64% of the initial injected TCO.* Critically, however, while we contend that both of the assumptions above are reasonable, this calculation should be used only for guidance rather as an absolute value.

Supplemental Figures

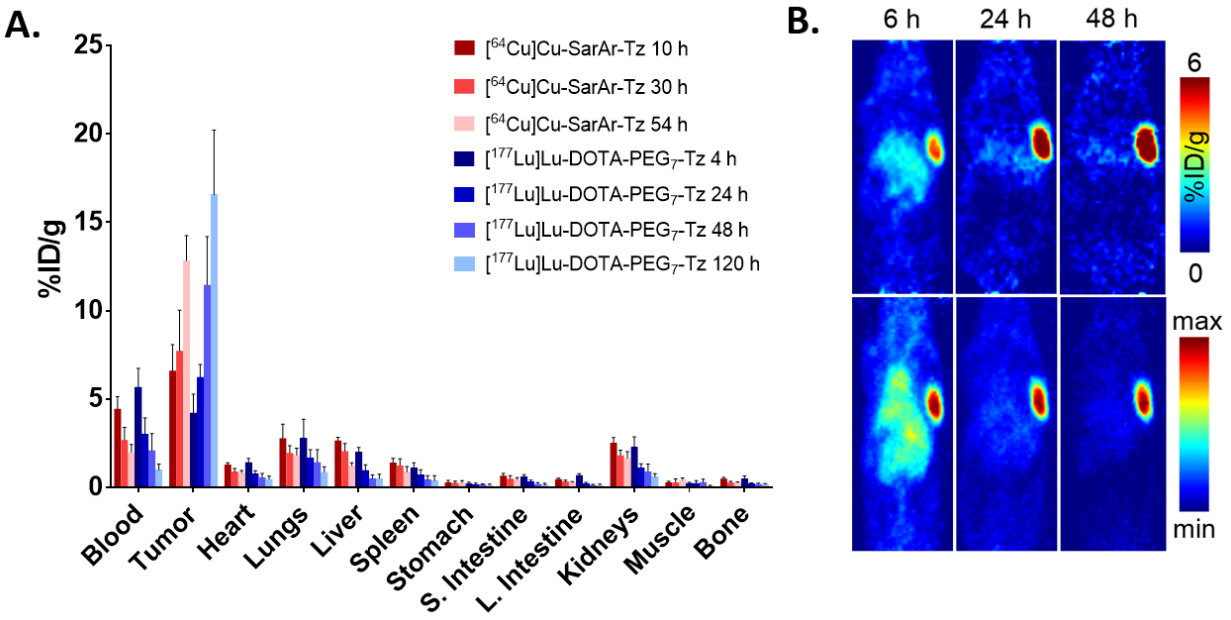

Figure S1. (A) Biodistribution data for *in vivo* pretargeting (n = 5) using two different tetrazine radiotracers — [^{64}Cu]Cu-SarAr-Tz and [^{177}Lu]Lu-DOTA-PEG$_7$-Tz — in athymic nude mice bearing subcutaneous SW1222 xenografts. The mice are first administered huA33-TCO (100 µg, 0.67 nmol, 2.4 TCO/mAb) via tail vein, followed 24 hours later by the *i.v.* administration of [^{64}Cu]Cu-SarAr-Tz (10.4-11.1 MBq, 0.68-0.73 nmol) and 6 h thereafter by the *i.v.* injection of [^{177}Lu]Lu-DOTA-PEG$_7$-Tz (6.2-6.3 MBq, 0.65-0.67 nmol). The time values represent the number of hours after the administration of each Tz radioligand; (B) PET images of the same mice at 6, 24, and 48 h after the injection of [^{64}Cu]Cu-SarAr-Tz (10.4 MBq, 0.68 nmol). Top row: Coronal planar images that intersect the center of the tumors. Bottom row: maximum intensity projections (MIP).

Supplemental Tables

Table S1. Biodistribution data for [^{64}Cu]Cu-SarAr-Tz (0.9-1.1 MBq, n = 4) in athymic nude mice administered via intravenous tail vein injection.[1]

	[^{64}Cu]Cu-SarAr-Tz		
	4 h	**24 h**	**48 h**
Blood	0.4 ± 0.1	0.2 ± 0.0[1]	0.1 ± 0.0[1]
Heart	0.2 ± 0.1	0.1 ± 0.0[2]	0.1 ± 0.0[2]
Lungs	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
Liver	0.7 ± 0.0[3]	0.4 ± 0.2	0.3 ± 0.1
Spleen	0.4 ± 0.1	0.2 ± 0.1	0.2 ± 0.0[4]
Stomach	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0[1]
S. Intestine	0.1 ± 0.0[1]	0.1 ± 0.0[3]	0.1 ± 0.0[1]
L. Intestine	0.3 ± 0.2	0.2 ± 0.0[3]	0.1 ± 0.0[1]
Kidneys	2.3 ± 0.4	1.6 ± 0.2	1.3 ± 0.4
Muscle	0.1 ± 0.0[3]	0.0[4] ± 0.0[1]	0.0[3] ± 0.0[1]
Bone	0.1 ± 0.0[3]	0.1 ± 0.0[1]	0.1 ± 0.0[2]

Table S2. Biodistribution data for [^{177}Lu]Lu-DOTA-PEG$_7$-Tz (10.4-11.8 MBq, n = 4) in athymic nude mice administered via intravenous tail vein injection.

	[^{177}Lu]Lu-DOTA-PEG$_7$-Tz			
	4 h	**24 h**	**48 h**	**120 h**
Blood	0.6 ± 0.2	0.1 ± 0.0[2]	0.1 ± 0.0[1]	0.0 ± 0.0[1]
Tumor	0.3 ± 0.1	0.1 ± 0.0[1]	0.1 ± 0.0[3]	0.0 ± 0.0[1]
Heart	0.2 ± 0.0[4]	0.1 ± 0.0[1]	0.0 ± 0.0[1]	0.0 ± 0.0[1]
Lungs	0.3 ± 0.1	0.1 ± 0.0[1]	0.1 ± 0.0[1]	0.1 ± 0.0[1]
Liver	0.4 ± 0.0[4]	0.3 ± 0.0[3]	0.2 ± 0.0[2]	0.2 ± 0.0[2]
Spleen	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0[3]	0.1 ± 0.1
Stomach	0.1 ± 0.1	0.0 ± 0.0[1]	0.1 ± 0.1	0.0 ± 0.0[1]
S. Intestine	0.1 ± 0.1	0.0 ± 0.0[1]	0.2 ± 0.4	0.0 ± 0.0[1]
L. Intestine	1.0 ± 0.3	0.1 ± 0.0[1]	0.2 ± 0.3	0.0 ± 0.0[1]
Kidneys	2.0 ± 0.4	1.2 ± 0.1	0.9 ± 0.2	0.4 ± 0.1
Muscle	0.1 ± 0.0[1]	0.0 ± 0.0[1]	0.0 ± 0.0[1]	0.0 ± 0.0[1]
Bone	0.1 ± 0.0[1]	0.1 ± 0.0[1]	0.1 ± 0.0[1]	0.1 ± 0.0[1]

Table S3. Biodistribution data for *in vivo* pretargeting (n = 4) using [^{177}Lu]Lu-DOTA-PEG$_7$-Tz in athymic nude mice bearing subcutaneous SW1222 xenografts. The mice were first administered huA33-TCO (100 µg, 0.7 nmol, 2 TCO/mAb) via tail vein, followed 24 or 48 h later by the *i.v.* administration of [^{177}Lu]Lu-DOTA-PEG$_7$-Tz (11.1 MBq, 0.7 nmol).[3]

	24 h interval: huA33-TCO / [^{177}Lu]Lu-DOTA-PEG$_7$-Tz				**48 h interval: huA33-TCO / [^{177}Lu]Lu-DOTA-PEG$_7$-Tz**			
	4 h	**24 h**	**48 h**	**120 h**	**4 h**	**24 h**	**48 h**	**120 h**
Blood	5.0 ± 1.3	3.7 ± 0.6	3.0 ± 0.6	1.0 ± 0.2	4.4 ± 1.7	2.5 ± 0.8	1.5 ± 0.5	0.4 ± 0.3
Tumor	5.0 ± 1.5	13 ± 1.6	17.0 ± 2.2	21 ± 2.9	5.2 ± 1.2	9.0 ± 2.6	13.0 ± 2.8	11.0 ± 2.4
Heart	1.4 ± 0.4	1.1 ± 0.2	0.8 ± 0.1	0.3 ± 0.1	1.2 ± 0.4	0.7 ± 0.3	0.5 ± 0.2	0.2 ± 0.1
Lungs	2.4 ± 0.7	2.3 ± 0.4	1.9 ± 0.5	0.8 ± 0.1	2.7 ± 1.0	1.5 ± 0.6	1.0 ± 0.3	0.4 ± 0.1
Liver	1.5 ± 0.4	1.4 ± 0.3	1.2 ± 0.1	0.6 ± 0.1	1.1 ± 0.5	0.9 ± 0.2	0.7 ± 0.3	0.3 ± 0.1
Spleen	1.3 ± 0.4	1.4 ± 0.4	1.2 ± 0.3	0.7 ± 0.2	1.0 ± 0.5	0.7 ± 0.3	0.9 ± 0.4	0.2 ± 0.1
Stomach	0.3 ± 0.1	0.7 ± 0.8	0.3 ± 0.1	0.1 ± 0.0^3	0.3 ± 0.1	0.5 ± 0.4	0.2 ± 0.1	0.1 ± 0.0^1
S. Intestine	0.6 ± 0.3	0.7 ± 0.5	0.3 ± 0.1	0.2 ± 0.0^2	1.5 ± 1.9	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.0^2
L. Intestine	0.9 ± 0.4	1.3 ± 1.4	0.4 ± 0.3	0.2 ± 0.1	1.6 ± 0.4	1.5 ± 1.1	0.2 ± 0.1	0.1 ± 0.0^3
Kidneys	2.3 ± 0.5	1.9 ± 0.2	1.6 ± 0.2	0.8 ± 0.0^1	2.5 ± 0.5	1.5 ± 0.3	1.1 ± 0.2	0.6 ± 0.1
Muscle	0.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.1 ± 0.0^1	0.5 ± 0.1	0.3 ± 0.1	0.2 ± 0.0^3	0.1 ± 0.0^2
Bone	0.5 ± 0.2	0.6 ± 0.2	0.7 ± 0.3	0.3 ± 0.0^2	0.5 ± 0.2	0.3 ± 0.1	0.4 ± 0.1	0.1 ± 0.0^3

Table S4. Biodistribution data for *in vivo* pretargeting (n = 4) using [^{64}Cu]Cu-SarAr-Tz in athymic nude mice bearing subcutaneous SW1222 xenografts. The mice were first administered huA33-TCO (100 µg, 0.7 nmol, 2 TCO/mAb) via tail vein, followed 24 hours later by the *i.v.* administration of [^{64}Cu]Cu-SarAr-Tz (14.8-16.7 MBq, 0.7 nmol).[1]

	huA33-TCO / [^{64}Cu]Cu-SarAr-Tz		
	4 h	**12 h**	**24 h**
Blood	4.0 ± 0.4	2.2 ± 0.4	2.6 ± 0.2
Tumor	5.6 ± 0.9	6.7 ± 1.3	7.4 ± 2.0
Heart	1.6 ± 0.0[3]	0.8 ± 0.1	0.8 ± 0.0[4]
Lungs	1.7 ± 0.5	1.2 ± 0.2	1.0 ± 0.2
Liver	1.5 ± 0.2	1.3 ± 0.5	1.5 ± 0.2
Spleen	0.8 ± 0.2	0.7 ± 0.2	0.6 ± 0.0[4]
Stomach	0.6 ± 0.2	0.2 ± 0.1	0.3 ± 0.1
S. Intestine	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
L. Intestine	0.6 ± 0.1	0.2 ± 0.0[4]	0.3 ± 0.1
Kidneys	2.8 ± 0.6	1.9 ± 0.4	2.0 ± 0.2
Muscle	0.4 ± 0.1	0.2 ± 0.0[1]	0.2 ± 0.1
Bone	0.3 ± 0.2	0.2 ± 0.1	0.3 ± 0.1

Table S5. Tumor-to-tissue activity concentration ratios for *in vivo* pretargeting experiments with two different tetrazine radiotracers — [^{64}Cu]Cu-SarAr-Tz and [^{177}Lu]Lu-DOTA-PEG$_7$-Tz — in athymic nude mice bearing subcutaneous SW1222 xenografts. The mice were first administered huA33-TCO (100 µg, 0.67 nmol, 2.4 TCO/mAb) via tail vein, followed 24 hours later by the *i.v.* administration of [^{64}Cu]Cu-SarAr-Tz (10.4-11.3 MBq, 0.64-0.69 nmol) and 24 h thereafter by the *i.v.* injection of [^{177}Lu]Lu-DOTA-PEG$_7$-Tz (5.9-6.4 MBq, 0.69-0.74 nmol). The time values represent the number of hours following the administration of each Tz radiotracer (*i.e.* the 28 h column under [^{64}Cu]Cu-SarAr-Tz denotes 28 h after the injection of [^{64}Cu]Cu-SarAr-Tz, whereas the 4 h column under [^{177}Lu]Lu-DOTA-PEG$_7$-Tz denotes 4 h after the injection of [^{177}Lu]Lu-DOTA-PEG$_7$-Tz). The values for the stomach and small intestines include contents.

	[^{64}Cu]Cu-SarAr-Tz			**[^{177}Lu]Lu-DOTA-PEG$_7$-Tz**			
	28 h	**48 h**	**72 h**	**4 h**	**24 h**	**48 h**	**120 h**
Blood	3.2 ± 0.4	5.9 ± 2.0	8.2 ± 1.6	1.1 ± 0.1	3.6 ± 1.1	5.7 ± 1.1	17.9 ± 5.9
Heart	12.2 ± 0.6	17.1 ± 3.2	22.6 ± 4.2	4.1 ± 0.4	13.1 ± 2.8	22.7 ± 8.7	97.6 ± 42.0
Lungs	6.0 ± 0.8	8.9 ± 2.4	13.1 ± 4.3	2.1 ± 0.4	6.4 ± 2.1	13.0 ± 6.9	34.1 ± 19.6
Liver	5.5 ± 1.1	7.3 ± 1.1	11.2 ± 1.4	3.4 ± 0.5	9.3 ± 1.0	17.5 ± 3.3	35.0 ± 5.2
Spleen	8.1 ± 2.1	9.9 ± 3.8	17.1 ± 2.9	5.2 ± 1.2	12.3 ± 5.3	27.7 ± 8.5	49.4 ± 13.7
Stomach	41.6 ± 13.2	51.3 ± 6.7	82.7 ± 17.9	17.7 ± 4.0	43.2 ± 5.2	73.6 ± 35.1	135.5 ± 55.8
S. Intestine	27.8 ± 6.3	36.3 ± 11.8	45.5 ± 7.8	11.9 ± 1.2	35.7 ± 9.8	51.5 ± 10.0	114.7 ± 28.4
L. Intestine	33.0 ± 4.6	35.9 ± 4.8	61.7 ± 6.9	7.5 ± 1.7	33.9 ± 8.3	69.4 ± 21.2	201.1 ± 55.5
Kidneys	5.9 ± 0.7	7.4 ± 1.6	10.6 ± 2.9	3.1 ± 0.6	7.6 ± 1.4	14.3 ± 3.9	29.4 ± 5.5
Muscle	27.6 ± 8.3	34.7 ± 15.4	55.3 ± 12.1	14.4 ± 3.8	32.7 ± 12.2	62.9 ± 14.2	206.7 ± 51.0
Bone	23.9 ± 3.4	36.5 ± 11.8	49.6 ± 15.4	10.7 ± 1.4	30.5 ± 9.5	54.3 ± 11.8	91.6 ± 33.2

Table S6. Biodistribution data for *in vivo* pretargeting (n = 5) using two different tetrazine radiotracers — [^{64}Cu]Cu-SarAr-Tz and [^{177}Lu]Lu-DOTA-PEG$_7$-Tz — in athymic nude mice bearing subcutaneous SW1222 xenografts. The mice were first administered huA33-TCO (100 µg, 0.67 nmol, 2.4 TCO/mAb) via tail vein, followed 24 hours later by the *i.v.* administration of [^{64}Cu]Cu-SarAr-Tz (10.4-11.1 MBq, 0.68-0.73 nmol) and 6 h thereafter by the *i.v.* injection of [^{177}Lu]Lu-DOTA-PEG$_7$-Tz (6.2-6.3 MBq, 0.65-0.67 nmol). The time values represent the number of hours following the administration of each Tz radiotracer (*i.e.* the 10 h column under [^{64}Cu]Cu-SarAr-Tz denotes 10 h after the injection of [^{64}Cu]Cu-SarAr-Tz, whereas the 4 h column under [^{177}Lu]Lu-DOTA-PEG$_7$-Tz denotes 4 h after the injection of [^{177}Lu]Lu-DOTA-PEG$_7$-Tz). The values for the stomach and small intestines include contents.

	[^{64}Cu]Cu-SarAr-Tz			**[^{177}Lu]Lu-DOTA-PEG$_7$-Tz**			
	10 h	**30 h**	**54 h**	**4 h**	**24 h**	**48 h**	**120 h**
Blood	4.5 ± 0.7	2.7 ± 0.7	2.0 ± 0.5	5.7 ± 1.1	3.0 ± 0.9	2.1 ± 1.0	1.00 ± 0.3
Tumor	6.6 ± 1.5	7.7 ± 2.3	12.8 ± 1.4	4.2 ± 1.1	6.3 ± 0.7	11.5 ± 2.7	16.6 ± 3.7
Heart	1.3 ± 0.1	0.9 ± 0.2	0.8 ± 0.1	1.4 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	0.5 ± 0.2
Lungs	2.6 ± 0.8	1.9 ± 0.4	1.8 ± 0.4	2.8 ± 1.1	1.7 ± 0.5	1.4 ± 0.7	0.9 ± 0.3
Liver	2.7 ± 0.2	1.8 ± 0.5	1.3 ± 0.2	2.0 ± 0.3	1.0 ± 0.3	0.5 ± 0.2	0.5 ± 0.3
Spleen	1.4 ± 0.3	1.1 ± 0.2	0.9 ± 0.3	1.1 ± 0.3	0.7 ± 0.3	0.4 ± 0.2	0.4 ± 0.3
Stomach	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
S. Intestine	0.7 ± 0.1	0.5 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
L. Intestine	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.0[2]	0.7 ± 0.1	0.2 ± 0.1	0.1 ± 0.0[4]	0.1 ± 0.1
Kidneys	2.5 ± 0.3	1.8 ± 0.3	1.7 ± 0.4	2.3 ± 0.6	1.1 ± 0.2	0.9 ± 0.4	0.6 ± 0.2
Muscle	0.3 ± 0.1	0.3 ± 0.2	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.1 ± 0.1
Bone	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.0[3]	0.5 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0[4]

Table S7. Tumor-to-tissue activity concentration ratios for *in vivo* pretargeting experiments with two different tetrazine radiotracers — [^{64}Cu]Cu-SarAr-Tz and [^{177}Lu]Lu-DOTA-PEG$_7$-Tz — in athymic nude mice bearing subcutaneous SW1222 xenografts. The mice were first administered huA33-TCO (100 µg, 0.67 nmol, 2.4 TCO/mAb) via tail vein, followed 24 hours later by the *i.v.* administration of [^{64}Cu]Cu-SarAr-Tz (10.4-11.1 MBq, 0.68-0.73 nmol) and 6 h thereafter by the *i.v.* injection of [^{177}Lu]Lu-DOTA-PEG$_7$-Tz (6.2-6.3 MBq, 0.65-0.67 nmol). The time values represent the number of hours following the administration of each Tz radiotracer (*i.e.* the 10 h column under [^{64}Cu]Cu-SarAr-Tz denotes 10 h after the injection of [^{64}Cu]Cu-SarAr-Tz, whereas the 4 h column under [^{177}Lu]Lu-DOTA-PEG$_7$-Tz denotes 4 h after the injection of [^{177}Lu]Lu-DOTA-PEG$_7$-Tz). The values for the stomach and small intestines include contents.

	[^{64}Cu]Cu-SarAr-Tz			**[^{177}Lu]Lu-DOTA-PEG$_7$-Tz**			
	10 h	**30 h**	**54 h**	**4 h**	**24 h**	**48 h**	**120 h**
Blood	1.5 ± 0.5	3.2 ± 1.5	6.7 ± 1.6	0.8 ± 0.3	2.5 ± 1.1	4.8 ± 0.8	17.4 ± 2.6
Heart	5.1 ± 1.4	8.9 ± 2.9	15.5 ± 2.4	3.1 ± 0.9	9.3 ± 3.2	17.9 ± 2.9	36.4 ± 5.5
Lungs	2.7 ± 1.4	4.2 ± 1.6	7.2 ± 1.5	2.0 ± 1.6	4.4 ± 1.8	6.8 ± 0.6	19.9 ± 3.0
Liver	2.5 ± 0.6	4.0 ± 1.7	10.4 ± 1.2	2.2 ± 0.6	7.5 ± 2.9	21.5 ± 4.7	35.2 ± 8.8
Spleen	4.8 ± 1.6	6.4 ± 1.8	15.7 ± 5.0	4.1 ± 1.5	8.4 ± 2.4	25.6 ± 9.6	50.8 ± 19.1
Stomach	25.0 ± 6.7	33.9 ± 15.4	53.3 ± 26.5	21.0 ± 8.3	42.2 ± 12.8	85.9 ± 49.1	140.1 ± 42.8
S. Intestine	10.0 ± 2.4	16.8 ± 7.6	29.2 ± 5.4	6.8 ± 1.7	20.2 ± 6.1	56.0 ± 14.0	133.6 ± 45.5
L. Intestine	14.1 ± 4.2	23.5 ± 8.9	41.3 ± 4.8	6.2 ± 1.8	35.5 ± 16.8	74.1 ± 10.1	144.2 ± 34.4
Kidneys	2.7 ± 0.9	4.4 ± 1.7	8.1 ± 2.3	2.0 ± 0.9	6.3 ± 1.7	11.2 ± 1.6	28.2 ± 6.6
Muscle	23.3 ± 8.8	21.7 ± 6.6	30.3 ± 5.8	18.7 ± 7.0	21.7 ± 1.2	33.7 ± 6.3	214.0 ± 61.2
Bone	13.8 ± 4.2	29.3 ± 10.5	45.3 ± 6.8	8.7 ± 3.4	31.4 ± 11.4	55.1 ± 12.3	87.2 ± 16.9

Table S8. Dosimetry data calculated from the biodistribution data obtained with [^{177}Lu]Lu-DOTA-PEG$_7$-Tz in the theranostic pretargeting experiments in this study (*left* and *center*) and a comparison to the dosimetry data obtained by Membreno *et al* (*right*).[3]

	24 h interval		**6 h interval**		**PRIT with [^{177}Lu]Lu-DOTA-PEG$_7$-Tz[3]**	
	Dose (cGy/MBq)	Therapeutic Index	Dose (cGy/MBq)	Therapeutic Index	Dose (cGy/MBq)	Therapeutic Index
Tumor	310.2	-	274.3	-	363.8	-
Blood	25.0	12.4	22.0	12.5	25.8	14.1
Heart	5.7	54.9	9.6	28.5	7.3	49.9
Lungs	11.7	26.5	12.4	22.1	18.4	19.8
Liver	11.7	26.5	5.2	52.3	12.1	30.2
Spleen	7.2	43.0	8.6	32.1	13.6	26.7
Stomach	2.4	131.2	1.8	152.6	4.0	91.8
S. Intestine	3.5	87.7	6.3	43.8	4.0	91.9
L. Intestine	2.5	124.6	6.8	40.3	6.2	59.1
Kidneys	10.6	29.3	11.3	24.4	15.4	23.6
Muscle	2.3	134.1	2.8	99.7	2.8	128.1
Bone	4.7	65.3	4.3	63.6	7.7	47.4

[3]Membreno *et al.* 2018. *Mol. Pharmaceutics*, 15, 1729−173

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