Supporting Information

Harnessing $^{64}Cu/^{67}Cu$ for a Theranostic Approach to Pretargeted Radioimmunotherapy

Outi Keinänen[1,2], Kimberly Fung[1,3], James M. Brennan[1], Nicholas Zia[4], Matt Harris[5], Ellen van Dam[5], Colin Biggin[5], Amos Hedt[5], Jon Stoner[6], Paul S. Donnelly[4], Jason S. Lewis[2,7,8], Brian M. Zeglis[1,2,3,7]

[1] Department of Chemistry, Hunter College, The City University of New York, New York, NY, USA

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

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

[4] School of Chemistry and Bio21 Molecular Science Institute, University of Melbourne, Melbourne, Australia

[5] Clarity Pharmaceuticals, Sydney, New South Wales, Australia

[6] Idaho Accelerator Center, Idaho State University, Pocatello, Idaho, USA

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

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

www.pnas.org/cgi/doi/10.1073/pnas.2009960117

TABLE OF CONTENTS

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 $M\Omega cm^{-1}$ 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) was purchased from Sigma-Aldrich (St. Louis, MO). ^{64}Cu was purchased from Washington University, St. Louis as $[^{64}Cu]CuCl_2$ in 0.05 M HCl. ^{67}Cu was produced in Idaho Accelerator Center (Idaho State University, Pocatello, Idaho, USA) as $[^{67}Cu]CuCl_2$ in 0.01 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. 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 microfiber chromatography paper impregnated with silica gel (SGI0001), read on a Bioscan AR-2000 Radio-TLC plate reader, and analyzed using Winscan Radio-TLC software (Bioscan Inc., Washington, D.C.).

HPLC

Preparative HPLC was performed using an Agilent 1200 HPLC system (12-18% 0.1% TFA in MeCN over 40 minutes, Supelco C18 Discovery 10 x 250 mm semi-preparative column at 5 mL/min). Analytical RP-HPLC traces were acquired using an Agilent 1200 HPLC system (California, USA) equipped with an Alltech Hypersil BDS C18 analytical HPLC column (4.6 × 150 mm, 5 µm) with a flow rate of 1 mL/min and UV absorbance detection at λ = 214 and 254 nm. Retention times (R_t/min) were recorded using a gradient elution of 5-100% B in A (A = water with 0.1% trifluoroacetic acid (TFA), B = acetonitrile with 0.1% TFA) over 30 min.

High resolution mass spectrometry

High resolution mass spectra were recorded on a Thermo Scientific Exactive Plus OrbiTrap LC/MS (Thermo Fisher Scientific, Massachusetts, USA) and calibrated to internal references.

Syntheses

Preparation of huA33-TCO

HuA33-TCO was prepared as previously described.[1,2] An aliquot of huA33 (8.8 mg, 58.7 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 DMSO (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).

Radiolabeling of MeCOSar-Tz with ^{64}Cu

A solution of MeCoAr-Tz (6.8 µg; 11.4 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 (189 MBq; 5.1 mCi) was added to the reaction mixture, and the solution was placed on an agitating thermomixer at 500 rpm for 20 min at 37 °C. After this incubation, the progress of the [^{64}Cu]Cu-MeCOSar-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-MeCOSar-Tz was 16.5 GBq/µmol.

Radiolabeling of MeCOSar-Tz with ^{67}Cu

A solution of MeCOSar-Tz (17.9 µg; 30.0 nmol) in NH_4OAc buffer (0.25 M, pH 5.5, 300 µL) was first prepared. Then, the desired amount of [^{67}Cu]$CuCl_2$ in 0.01 M HCl (2.5 GBq; 66.8 mCi) was added to the reaction mixture, and the solution was placed on an agitating thermomixer at 500 rpm for 20 min at 37 °C. After this incubation, the progress of the [^{67}Cu]Cu-MeCOSar-Tz radiolabeling was determined by iTLC which revealed quantitative labeling of >99% radiochemical purity, thus no further purification was deemed necessary. The molar activity of [^{67}Cu]Cu-MeCOSar-Tz was 82.3 GBq/µmol. The [^{67}Cu]Cu-MeCOSar-Tz was supplemented with MeCOSar-Tz to yield an approximately 1:1 ratio of Tz to huA33-TCO for each radiotherapy cohort.

Radiolabeling of huA33-TCO with [^{67}Cu]Cu-MeCOSar-Tz

A solution of [^{67}Cu]Cu-MeCOSar-Tz (558.7 MBq; 15.1 mCi, 6.8 nmol) was mixed with huA33-TCO (1.3 mg in 1 mL PBS). The solution was placed on an agitating thermomixer at 500 rpm for 30 min at 37 °C. After this incubation, the [^{67}Cu]Cu-MeCOSar-Tz-TCO-huA33 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). The radiochemical purity of [^{67}Cu]Cu-MeCOSar-Tz-TCO-huA33 was >99% (determined by iTLC). For injections, the end product was diluted with huA33-TCO and PBS to yield a dose of 100 µg (0.7 nmol in 100 µL) of the antibody per mouse. For radioimmunotherapy, each mouse received 18.5 MBq (500 µCi, 100 µg, 0.7 nmol), and for *ex vivo* biodistribution, each mouse received 1.9 MBq (50 µCi, 100 µg, 0.7 nmol).

Functional characterization

MALDI-ToF mass spectrometry

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (service from the Alberta Proteomics and Mass Spectrometry Facility, University of Alberta, Canada) was used to determine the number of TCO moieties that had been added to each antibody molecule. The immunoconjugates were analyzed using a Bruker Ultraflex MALDI-ToF/ToF (Bruker Daltonic GmbH). 1 µL of each sample (1 mg/mL) was mixed with 1 µL of sinapic acid (10 mg/mL in 50% acetonitrile:water and 0.1% trifluoroacetic acid). 1 µL of the sample/matrix solution was spotted onto a stainless steel target plate and allowed to air dry. All mass spectra were obtained using a Bruker Ultraflex MALDI-ToF/ToF (Bruker Daltonic GmbH). Ions were analyzed in positive mode, and external calibration was performed by use of a standard protein mixture (Bovine Serum Albumin).

Saturation binding assay

HuA33-TCO was conjugated with [^{64}Cu]Cu-MeCOSar-Tz and purified similarly as described in "Radiolabeling of huA33-TCO with [^{67}Cu]Cu-MeCOSar-Tz". After purification [^{64}Cu]Cu-MeCOSar-Tz-TCO-huA33 was diluted to 1 μg/mL with 1% BSA in PBS pH 7.41. 10×10^6 SW1222 cells were pipetted per 1.5 mL microtube (five replicates). The cells were centrifuged (600g, 2 min) at the bottom of the tube, and the supernatant was discarded. 200 μL of ice cold cell media and 1 μL of [^{64}Cu]Cu-MeCOSar-Tz-TCO-huA33 were added, and the mixture was mixed well. The cells were incubated in ice for 60 min, after which they were centrifuged (600g, 2 min) and the supernatant was removed to gamma counter tube. The cells were washed with ice cold PBS three times by putting 200 μL of PBS into each tube, mixing the mixture, centrifuging (600g, 2 min), and collecting the supernatants to gamma counter tubes. All the supernatants and cell pellets were measured with gamma counter. The fraction of radioactivity bound to the cells compared to the total radioactivity was considered the immunoreactive fraction.

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 flank in a 150 μL suspension of 1:1 fresh media / Matrigel (Corning Life Sciences). The tumors reached the ideal size for experiments (~100 mm^3) after approximately 14 days.

Ex vivo biodistribution

[^{67}Cu]Cu-MeCOSar-Tz

Mice were warmed gently using a heat lamp prior to being injected with [^{67}Cu]Cu-MeCOSar-Tz (9.3-10.0 MBq, 0.7 nmol, in 100 μL sterile PBS) via intravenous tail vein injection. At 4, 24, 48, and 72 h post injection, 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 each sample was then counted using a Wizard^2 automatic gamma counter. The mass of each syringe before 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 mass in g.

Pretargeted experiments with huA33-TCO and [^{67}Cu]Cu-MeCOSar-Tz

Mice were warmed gently using a heat lamp prior to being injected with huA33-TCO (100 µg, 0.7 nmol, in 100 µL sterile PBS, 5 TCO/mAb) via intravenous tail vein injection. After an accumulation interval period of 24 h or 72 h, the same mice were then administered with [^{67}Cu]Cu-MeCOSar-Tz (9.5-11.0 MBq, 0.7-0.8 nmol, in 100 µL sterile PBS). At 4, 24, 48, and 72 h post injection of [^{67}Cu]Cu-MeCOSar-Tz, 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 each sample was then counted using a Wizard^2 automatic gamma counter. The mass of each syringe before 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 mass in g.

[^{67}Cu]Cu-MeCOSar-Tz-TCO-huA33

Mice were warmed gently using a heat lamp prior to being injected with [^{67}Cu]Cu-MeCOSar-Tz-TCO-huA33 (1.9 MBq, 100 µg, 0.7 nmol, in 100 µL sterile PBS) via intravenous tail vein injection. At 4, 24, 72, and 120 h post injection, 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 each sample was then counted using a Wizard^2 automatic gamma counter. The mass of each syringe before 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 mass in g.

Longitudinal therapy study

Athymic nude mice bearing SW1222 xenografts were randomly sorted into 9 cohorts — 3 control cohorts and 6 radiotherapy cohorts — of 10 mice each.

Control cohorts

Mice were warmed gently using a heat lamp prior to intravenous tail vein injection. The first control cohort received only saline, the second received only huA33-TCO (100 µg, 0.7 nmol, 5 TCO/mAb), and the third received only [^{67}Cu]Cu-MeCOSar-Tz (55.5 MBq, 0.7 nmol).

Pretargeted radioimmunotherapy with huA33-TCO and [^{67}Cu]Cu-MeCOSar-Tz

Mice were warmed gently using a heat lamp prior to being injected with huA33-TCO (100 µg, 0.7 nmol, in 100 µL sterile PBS, 5 TCO/mAb) via intravenous tail vein injection. After an accumulation interval period of 72 h, the same mice were then administered with the radioligand, [^{67}Cu]Cu-MeCOSar-Tz (0.7 nmol, in 100 µL sterile PBS). In order to see the therapeutic window of the pretargeted methodology, the treatment cohorts received injections of the radioligand loaded with 18.5 MBq, 37 MBq, or 55.5 MBq (0.5 mCi, 1 mCi, or 1.5 mCi) via the lateral tail vein. To explore the efficacy of fractioned dosing, one cohort received two 27.8 MBq doses (0.75 mCi) of [^{67}Cu]Cu-MeCOSar-Tz (0.7 nmol, in 100 µL sterile PBS) separated by a 48 h interval. SPECT imaging was performed at 4, 24, 48, and 96 h post injection of [^{67}Cu]Cu-MeCOSar-Tz.

Theranostic pretargeted radioimmunotherapy with huA33-TCO and [^{64}Cu]/[^{67}Cu]Cu-MeCOSar-Tz

Mice were warmed gently using a heat lamp prior to being injected with huA33-TCO (100 µg, 0.7 nmol, in 100 µL sterile PBS, 5 TCO/mAb) via intravenous tail vein injection. After an accumulation interval period of 72 h, the same mice were then administered with the diagnostic radioligand, [^{64}Cu]Cu-MeCOSar-Tz (10.4-11.3 MBq, 0.7 nmol, in 100 µL sterile PBS). The therapeutic radioligand, [^{67}Cu]Cu-MeCOSar-Tz (55.5 MBq, 0.7 nmol in 100 µL sterile PBS), was administered 24 h after the injection of the diagnostic radioligand. PET imaging was performed at 4, 24, and 48 h post-injection of [^{64}Cu]Cu-MeCOSar-Tz. SPECT imaging was performed at 4, 24, 48, 96, and 120 h post-injection of [^{67}Cu]Cu-MeCOSar-Tz.

Radioimmunotherapy with [^{67}Cu]Cu-MeCOSar-Tz-TCO-huA33

Mice were warmed gently using a heat lamp prior to being injected with [^{67}Cu]Cu-MeCOSar-Tz-TCO-huA33 (18.5 MBq; 0.5 mCi, 100 µg, 0.7 nmol, in 100 µL sterile PBS) via intravenous tail vein injection. SPECT imaging was carried out at 4, 24, 48, 96, and 120 h post injection.

Blood analysis

Mice were warmed for 5 min using a heat lamp prior to collecting ~30 µL of blood from the lateral tail vein with a Minivette POCT collection tube coated with K3 EDTA (Braintree Scientific, Inc., Braintree, MA, USA). The samples were analyzed with HemaVet 950 (Drew Scientific, Inc., Miami Lakes, FL, USA).

PET imaging and image reconstruction

PET imaging was carried out 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 anesthesia 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 4, 24, and 48 h after the intravenous injection of [^{64}Cu]Cu-MeCOSar-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).

Quantification of radioactivity at the tumor from PET images

ROI (region of interest) was drawn with ASIPro VM software in transverse, coronal, and sagittal slices at the center of the tumor on the 48 h time point images. Using a system calibration factor derived from the imaging of a mouse-sized water-equivalent phantom containing ^{64}Cu, the total activity in each ROI was determined. An average of total radioactivity in transverse, coronal, and sagittal slices at the center of the tumor was calculated. These average values were compared the tumor growth of each mouse (Manuscript **Fig. 6D**).

SPECT imaging and image reconstruction

SPECT imaging experiments were conducted on a nanoSPECT rodent scanner (Mediso Medical Imaging Systems). Mice were anaesthetized with 2% isoflurane/medical air inhalation approximately 5 min prior to collecting the SPECT images and kept under anesthesia during the SPECT scan. SPECT data was acquired as a 360^{o} image using a 4-head gamma camera with pinhole collimators (1.4 mm). The SPECT scan time was adjusted for each mouse to record 30-60 s per frame (total scan time approximately: 20–60 min). Bioscan HiSPECT software (Bioscan, Inc.) was used for iterative image reconstruction.

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. 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 ^{67}Cu and 0 for the penetrating photon emissions and using an equilibrium absorbed dose constant of 0.33 g * Rad / μCi * h.

Supplemental figures

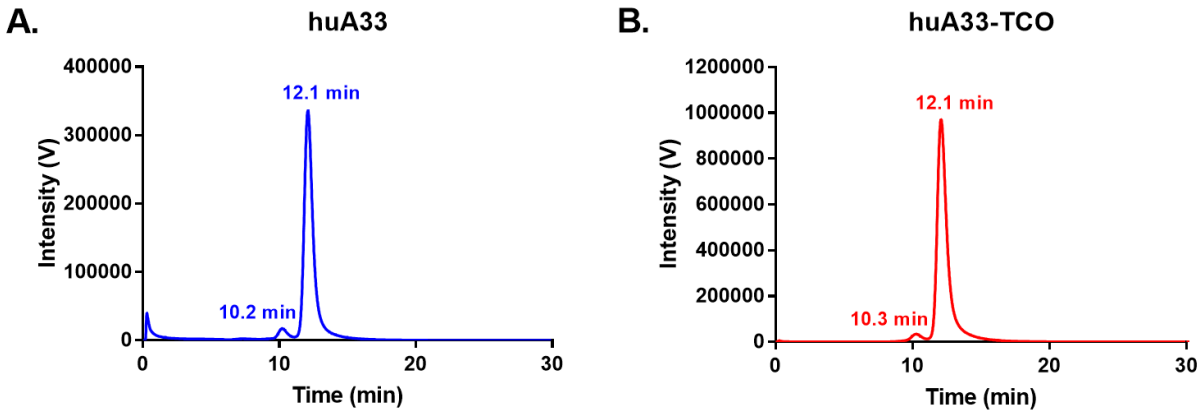

Figure S1. Size exclusion HPLC chromatograms (Shimadzu instrumentation with Superdex^{TM} 200 increase 10/300 GL column, GE Healthcare) for (A) unmodified huA33, and (B) huA33-TCO. Eluent: 1× PBS pH 7.4, flow rate: 1 mL/min, UV detection: 280 nm.

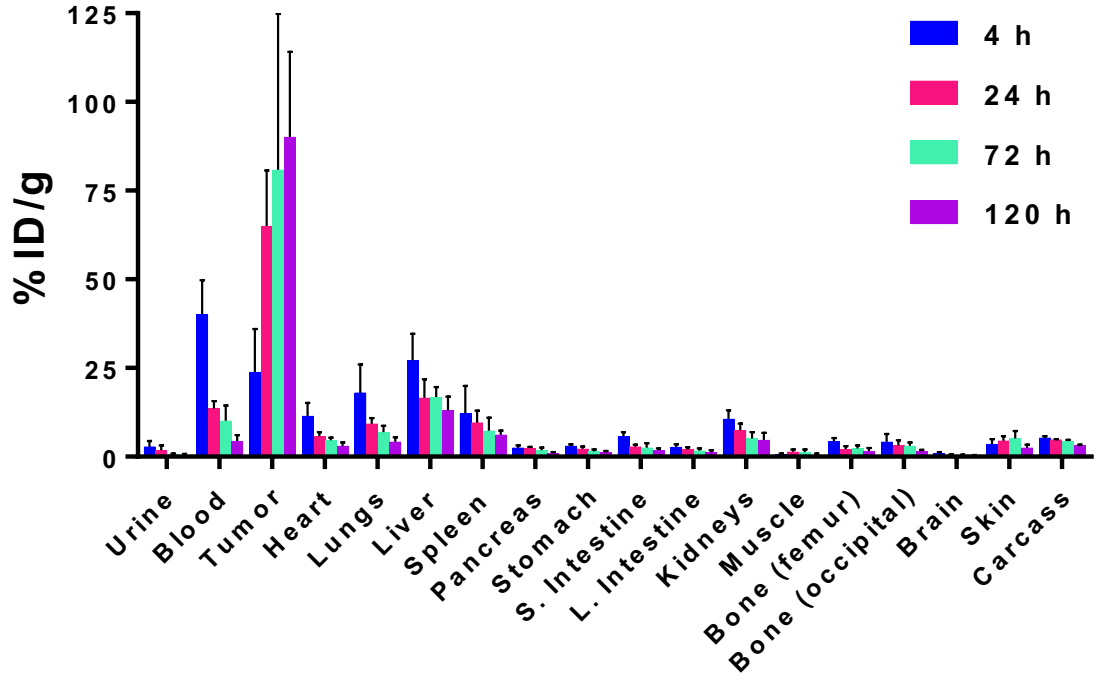

Figure S2. Biodistribution data for [^{67}Cu]Cu-MeCOSar-Tz-TCO-huA33 (1.9 MBq; 50 µCi, 100 µg, 0.7 nmol) in athymic nude mice bearing subcutaneous SW1222 xenografts (n = 5). Data are represented as mean value ± standard deviation. Stomach, small intestine, and large intestine values include contents.

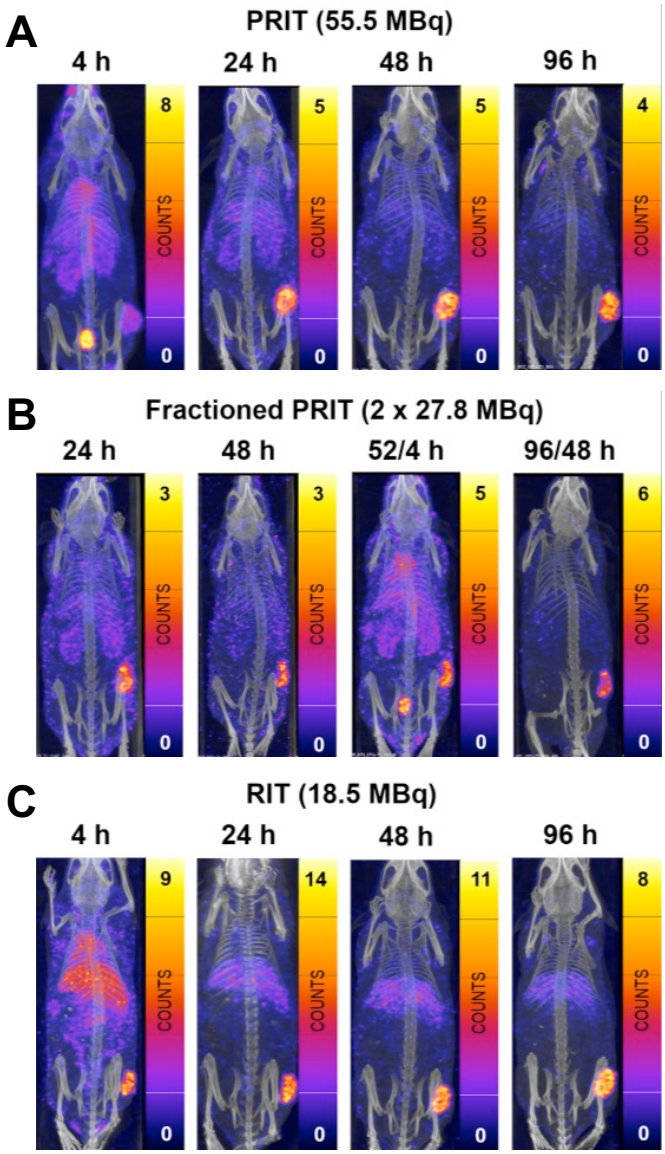

Figure S3. SPECT-CT maximum intensity projection (MIP) images of representative mice from the 55.5 MBq PRIT cohort (A), fractioned dose cohort (B), and RIT cohort (C). The second dose in fractioned dose cohort was administered after the SPECT scan at 48 h. The mice were then imaged 4 h (corresponding to 52 h after first dose) and 48 h (corresponding to 96 h after first dose) after the second dose.

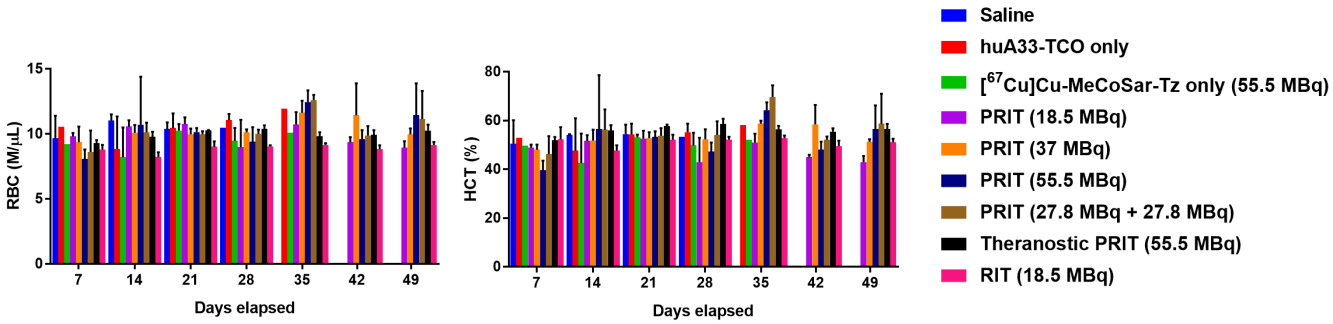

Figure S4. Red blood cell (RBC) counts and hematocrit (HCT) of the mice in the longitudinal radiotherapy study.

Supplemental tables

Table S1. Biodistribution data for [^{67}Cu]Cu-MeCOSar-Tz (9.3-10.0 MBq, 0.7 nmol) in athymic nude mice bearing subcutaneous SW1222 xenografts (n = 5 per timepoint).

	[^{67}Cu]Cu-MeCOSar-Tz			
	4 h	**24 h**	**48 h**	**72 h**
Urine	10.2 ± 2.0	2.4 ± 0.2	1.2 ± 0.1	0.3 ± 0.2
Blood	1.7 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
Tumor	0.5 ± 0.2	0.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1
Heart	0.5 ± 0.0^{4}	0.2 ± 0.0^{3}	0.2 ± 0.0^{3}	0.2 ± 0.0^{2}
Lungs	1.3 ± 0.3	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.0^{4}
Liver	2.9 ± 0.4	1.9 ± 0.7	1.2 ± 0.3	1.2 ± 0.5
Spleen	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.0^{4}
Pancreas	0.2 ± 0.1	0.1 ± 0.0^{3}	0.0^{3} ± 0.0^{2}	0.1 ± 0.0^{3}
Stomach	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0^{3}	0.1 ± 0.0^{1}
S. Intestine	0.3 ± 0.1	0.2 ± 0.0^{2}	0.1 ± 0.0^{4}	0.1 ± 0.0^{1}
L. Intestine	0.8 ± 0.4	0.2 ± 0.1	0.3 ± 0.2	0.2 ± 0.1
Kidneys	2.1 ± 0.1	1.1 ± 0.3	0.7 ± 0.2	0.5 ± 0.1
Muscle	0.1 ± 0.0^{2}	0.0^{4} ± 0.0^{1}	0.0^{4} ± 0.0^{2}	0.0^{2} ± 0.0^{1}
Bone (femur)	0.2 ± 0.0^{2}	0.1 ± 0.0^{2}	0.1 ± 0.0^{3}	0.1 ± 0.0^{4}
Bone (occipital)	0.4 ± 0.2	0.2 ± 0.1	0.1 ± 0.0^{4}	0.1 ± 0.0^{2}
Brain	0.1 ± 0.0^{2}	0.0^{2} ± 0.0^{1}	0.0^{1} ± 0.0^{0}	0.0^{1} ± 0.00
Skin	0.5 ± 0.2	0.3 ± 0.1	0.2 ± 0.0^{4}	0.2 ± 0.1
Carcass	0.3 ± 0.1	0.2 ± 0.0^{2}	0.2 ± 0.0^{3}	0.1 ± 0.0^{2}

Table S2. Biodistribution data for *in vivo* pretargeting with [^{67}Cu]Cu-MeCOSar-Tz in athymic nude mice bearing subcutaneous SW1222 xenografts (n = 5 per timepoint). The mice were first administered huA33-TCO (100 µg, 0.7 nmol, 5 TCO/mAb) via tail vein, followed 24 or 72 hours later by the *i.v.* administration of [^{67}Cu]Cu-MeCOSar-Tz (9.5-11.0 MBq, 0.7-0.8 nmol).

	24 h interval: huA33-TCO + [^{67}Cu]Cu-MeCOSar-Tz				**72 h interval: huA33-TCO + [^{67}Cu]Cu-MeCOSar-Tz**			
	4 h	**24 h**	**48 h**	**72 h**	**4 h**	**24 h**	**48 h**	**72 h**
Urine	11.5 ± 5.2	2.7 ± 0.2	1.0 ± 0.5	0.5 ± 0.2	14.2 ± 1.5	1.0 ± 0.5	0.6 ± 0.1	0.6 ± 0.1
Blood	4.0 ± 0.5	1.6 ± 0.2	1.1 ± 0.4	0.8 ± 0.3	3.0 ± 0.3	1.0 ± 0.4	0.9 ± 0.2	0.5 ± 0.2
Tumor	3.6 ± 0.6	5.3 ± 0.6	7.7 ± 1.6	10.2 ± 2.1	3.3 ± 0.2	5.2 ± 0.5	7.0 ± 1.8	9.2 ± 1.3
Heart	1.0 ± 0.2	0.5 ± 0.1	0.6 ± 0.2	0.4 ± 0.2	1.0 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.1
Lungs	2.5 ± 0.5	1.0 ± 0.4	1.1 ± 0.4	0.9 ± 0.2	2.3 ± 0.2	0.9 ± 0.3	0.9 ± 0.2	0.7 ± 0.1
Liver	3.2 ± 0.5	2.3 ± 0.5	1.9 ± 0.2	1.7 ± 0.3	3.3 ± 0.6	2.2 ± 0.9	1.4 ± 0.2	1.6 ± 0.4
Spleen	0.6 ± 0.1	0.8 ± 0.4	0.4 ± 0.2	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.4	0.3 ± 0.1	0.5 ± 0.3
Pancreas	0.5 ± 0.1	0.3 ± 0.0^{3}	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.2	0.3 ± 0.0^{4}	0.2 ± 0.0^{3}	0.2 ± 0.0^{4}
Stomach	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.1 ± 0.1	0.2 ± 0.1
S. Intestine	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	0.4 ± 0.2	0.2 ± 0.1	0.3 ± 0.2
L. Intestine	0.6 ± 0.2	0.3 ± 0.2	0.4 ± 0.3	0.4 ± 0.1	1.3 ± 0.6	0.4 ± 0.2	0.2 ± 0.0^{2}	0.2 ± 0.1
Kidneys	1.8 ± 0.5	1.3 ± 0.2	0.8 ± 0.2	0.7 ± 0.2	2.4 ± 0.9	1.1 ± 0.2	0.8 ± 0.2	0.8 ± 0.2
Muscle	0.2 ± 0.1	0.2 ± 0.2	0.1 ± 0.0^{3}	0.1 ± 0.0^{4}	0.3 ± 0.1	0.1 ± 0.1	0.1 ± 0.0^{4}	0.1 ± 0.0^{1}
Bone (femur)	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.2	0.2 ± 0.0^{3}	0.2 ± 0.1
Bone (occipital)	1.1 ± 0.3	0.4 ± 0.1	0.4 ± 0.2	0.3 ± 0.1	0.6 ± 0.1	0.4 ± 0.2	0.3 ± 0.1	0.2 ± 0.1
Brain	0.2 ± 0.0^{4}	0.1 ± 0.0^{1}	0.1 ± 0.0^{3}	0.0^{3} ± 0.0^{2}	0.1 ± 0.0^{3}	0.1 ± 0.0^{1}	0.1 ± 0.0^{1}	0.0^{2} ± 0.0^{1}
Skin	1.1 ± 0.3	0.8 ± 0.3	1.0 ± 0.3	0.6 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.2
Carcass	0.7 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.8 ± 0.0^{4}	0.6 ± 0.1	0.4 ± 0.1	0.4 ± 0.1

Table S3. Tumor-to-tissue activity concentration ratios for *in vivo* pretargeting with [^{67}Cu]Cu-MeCOSar-Tz in athymic nude mice bearing subcutaneous SW1222 xenografts (n = 5 per timepoint). The mice were first administered huA33-TCO (100 µg, 0.7 nmol, 5 TCO/mAb) via tail vein, followed 24 or 72 hours later by the *i.v.* administration of [^{67}Cu]Cu-MeCOSar-Tz (9.5-11.0 MBq, 0.7-0.8 nmol).

	24 h interval: huA33-TCO + [^{67}Cu]Cu-MeCOSar-Tz				**72 h interval: huA33-TCO + [^{67}Cu]Cu-MeCOSar-Tz**			
	4 h	**24 h**	**48 h**	**72 h**	**4 h**	**24 h**	**48 h**	**72 h**
Urine	0.4 ± 0.1	1.9 ± 0.2	8.4 ± 2.5	22.9 ± 11.6	0.2 ± 0.0	8.0 ± 7.9	12.0 ± 4.7	15.3 ± 5.0
Blood	0.9 ± 0.1	3.3 ± 0.1	7.0 ± 1.6	15.2 ± 7.8	1.1 ± 0.2	5.6 ± 1.7	8.8 ± 3.8	20.0 ± 7.7
Heart	3.6 ± 0.9	10.1 ± 0.7	14.4 ± 3.7	42.1 ± 43.5	3.2 ± 0.4	15.9 ± 11.9	17.2 ± 6.2	29.2 ± 6.6
Lungs	1.5 ± 0.2	6.9 ± 5.6	7.4 ± 2.4	12.1 ± 4.2	1.4 ± 0.1	6.3 ± 3.1	8.2 ± 3.0	13.2 ± 3.3
Liver	1.2 ± 0.1	2.4 ± 0.5	4.0 ± 0.7	5.9 ± 1.4	1.0 ± 0.2	2.8 ± 1.1	5.3 ± 1.3	6.0 ± 1.6
Spleen	6.4 ± 2.2	8.3 ± 5.1	23.6 ± 9.8	17.4 ± 7.1	6.3 ± 2.0	16.0 ± 10.8	24.3 ± 13.1	26.1 ± 12.6
Pancreas	8.4 ± 2.0	18.4 ± 2.2	31.8 ± 6.4	74.1 ± 44.9	9.7 ± 5.7	21.0 ± 2.3	39.2 ± 12.4	57.9 ± 17.9
Stomach	18.7 ± 9.0	33.0 ± 14.9	47.9 ± 21.8	41.9 ± 15.5	9.8 ± 1.8	20.2 ± 8.4	76.9 ± 71.6	59.4 ± 50.1
S. Intestine	9.0 ± 1.3	17.6 ± 7.2	29.7 ± 10.5	31.5 ± 9.9	5.6 ± 1.3	15.9 ± 7.3	31.9 ± 11.6	41.0 ± 18.8
L. Intestine	6.6 ± 2.3	24.1 ± 17.8	30.3 ± 18.7	31.9 ± 12.5	3.0 ± 1.1	18.9 ± 11.6	42.5 ± 8.1	66.8 ± 37.7
Kidneys	2.1 ± 0.6	4.3 ± 0.7	10.8 ± 3.0	16.2 ± 5.6	1.6 ± 0.8	4.9 ± 1.3	9.4 ± 4.8	13.1 ± 3.8
Muscle	17.2 ± 5.2	41.7 ± 31.4	68.6 ± 11.9	123.5 ± 43.8	14.5 ± 5.2	63.2 ± 31.3	84.2 ± 63.4	152.9 ± 35.6
Bone (femur)	10.5 ± 2.2	17.6 ± 3.7	37.4 ± 8.1	42.4 ± 9.6	8.6 ± 3.3	20.5 ± 10.7	43.1 ± 14.2	52.4 ± 25.5
Bone (occipital)	3.4 ± 0.5	13.0 ± 3.1	21.1 ± 4.7	32.2 ± 10.8	5.7 ± 0.6	17.5 ± 10.3	23.4 ± 9.6	47.6 ± 18.8
Brain	24.1 ± 5.3	76.3 ± 7.4	268.1 ± 293.9	406.7 ± 239.0	28.4 ± 7.3	110.8 ± 22.8	161.2 ± 73.4	585.3 ± 340.6
Skin	3.4 ± 1.0	7.9 ± 3.5	8.2 ± 2.4	19.7 ± 11.3	5.8 ± 1.0	10.3 ± 2.1	13.7 ± 3.8	24.1 ± 10.2

Table S4. Biodistribution data for [^{67}Cu]Cu-MeCOSar-Tz-TCO-huA33 (1.9 MBq, 100 µg, 0.7 nmol) in athymic nude mice bearing subcutaneous SW1222 xenografts (n = 5 per timepoint).

	[^{67}Cu]Cu-MeCOSar-Tz-TCO-huA33			
	4 h	**24 h**	**72 h**	**120 h**
Urine	2.8 ± 1.6	1.8 ± 1.3	0.7 ± 0.2	0.6 ± 0.2
Blood	40.2 ± 9.5	13.6 ± 2.1	10.2 ± 4.3	4.4 ± 1.7
Tumor	23.9 ± 12.1	65.0 ± 15.7	80.9 ± 44.0	90.2 ± 23.9
Heart	11.5 ± 3.7	5.8 ± 1.1	4.7 ± 0.7	3.1 ± 0.9
Lungs	18.1 ± 7.9	9.3 ± 1.6	6.9 ± 1.8	4.2 ± 1.3
Liver	27.2 ± 7.4	16.6 ± 5.2	16.8 ± 2.9	13.1 ± 3.9
Spleen	12.2 ± 7.7	9.7 ± 3.3	7.4 ± 3.6	6.2 ± 1.1
Pancreas	2.5 ± 0.6	2.4 ± 0.4	1.8 ± 0.7	1.0 ± 0.3
Stomach	3.1 ± 0.4	2.3 ± 0.6	1.6 ± 0.5	1.2 ± 0.4
S. Intestine	5.8 ± 1.1	2.8 ± 0.6	2.6 ± 1.2	1.8 ± 0.6
L. Intestine	2.8 ± 0.7	2.1 ± 0.6	1.8 ± 0.5	1.3 ± 0.5
Kidneys	10.6 ± 2.4	7.4 ± 1.9	5.2 ± 1.8	4.8 ± 1.9
Muscle	0.7 ± 0.1	1.4 ± 0.7	1.3 ± 0.7	0.6 ± 0.3
Bone (femur)	4.3 ± 1.0	2.2 ± 0.8	2.4 ± 0.8	1.6 ± 0.8
Bone (occipital)	4.2 ± 2.2	3.2 ± 1.4	3.0 ± 1.0	1.6 ± 0.4
Brain	1.0 ± 0.3	0.5 ± 0.1	0.4 ± 0.2	0.3 ± 0.2
Skin	3.6 ± 1.4	4.6 ± 1.2	5.2 ± 2.0	2.5 ± 0.8
Carcass	5.4 ± 0.3	4.8 ± 0.1	4.4 ± 0.3	3.1 ± 0.2

Table S5. Cause of death for animals in the experiment. Each cohort had 10 animals. The endpoint was defined as the point at which the longest dimension of the tumor reached 1 cm, the tumor hampered the movement of the mouse, the tumor was necrotic, mouse had lost more than 10% of body weight compared to previous measurement, or 200 days was reached.

	Tumor	Alive at 200 d	Underweight	Distended abdomen	Hindlimb weakness	Tilted head	Found dead
1. Saline	10	-	-	-	-	-	-
2. TCO-huA33	9	-	1 (day 36)	-	-	-	-
3. [^{67}Cu]Cu-MeCOSar-Tz	10	-	-	-	-	-	-
4. PRIT (18.5 MBq)	9	1	-	-	-	-	-
5. PRIT (37 MBq)	7	-	-	2 (day 127 & 164)	-	-	1 (day 104)
6. PRIT (55.5 MBq)	-	7	-	1 (day 129)	1 (day 185)[a]	-	1 (day 31)
7. PRIT (2 × 27.8 MBq)	4	5	-	1 (day 149)	-	-	-
8. Theranostic (55.5 MBq)	3	7	-	-	-	-	-
9. RIT (18.5 MBq)	1	7	-	-	-	1 (day 86)	1 (day 22)

[a]Tumor had also started regrowing.

Table S6. Dosimetry data derived from the biodistribution data collected in this study as well as dosimetry data obtained by Membreno *et al.*[2] . T.I. = therapeutic index.

	24 h Interval		***72 h Interval***		***^{67}Cu-huA33***		***^{177}Lu-huA33[a]***	
	Absorbed Dose (cGy/MBq)	T.I.	Absorbed Dose (cGy/MBq)	T.I.	Absorbed Dose (cGy/MBq)	T.I.	Absorbed Dose (cGy/MBq)	T.I.
Tumor	63.0	-	57.2	-	574.5	-	1255.6	-
Blood	9.7	6.5	5.9	9.7	71.3	8.1	67.7	18.6
Heart	3.2	19.5	2.6	22.4	31.0	18.5	17.5	71.6
Lungs	6.5	9.7	5.5	10.3	49.0	11.7	29.9	42.0
Liver	14.9	4.2	14.2	4.0	111.5	5.2	39.4	31.8
Spleen	4.5	14.1	3.3	17.6	58.9	9.8	39.5	31.8
Pancreas	1.6	39.6	1.3	43.5	14.3	40.3	-	-
Stomach	1.8	35.4	1.5	38.3	12.9	44.6	6.3	197.8
S. Intestine	2.6	24.1	2.5	22.9	15.7	36.5	9.8	127.7
L. Intestine	2.3	27.1	2.7	21.2	14.0	41.0	8.1	154.7
Kidneys	6.9	9.1	8.0	7.2	42.8	13.4	31.1	40.3
Muscle	1.0	65.3	0.6	94.9	10.0	57.3	8.0	156.9
Bone	1.7	36.4	1.7	33.3	13.7	41.9	15.7	80.2
Carcass	4.9	12.9	3.7	15.4	32.5	17.7	-	-
Bladder	17.7	3.6	15.3	3.8	8.9	64.7	-	-

[a]Membreno *et al.* 2018. *Mol. Pharmaceutics*, 15, 1729−1734

References

(1) Zeglis, B. M.; Brand, C.; Abdel-Atti, D.; Carnazza, K. E.; Cook, B. E.; Carlin, S.; Reiner, T.; Lewis, J. S., Optimization of a Pretargeted Strategy for the PET Imaging of Colorectal Carcinoma via the Modulation of Radioligand Pharmacokinetics. *Mol Pharm* **2015,** *12* (10), 3575-3587.

(2) Membreno, R.; Cook, B. E.; Fung, K.; Lewis, J. S.; Zeglis, B. M., Click-Mediated Pretargeted Radioimmunotherapy of Colorectal Carcinoma. *Mol Pharm* **2018,** *15* (4), 1729-1734.