Supplemental Information

"A Pretargeted Approach for the Multimodal PET/NIRF Imaging of Colorectal Cancer"

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Reagents and General Procedures

All chemicals, unless otherwise noted, were acquired from Sigma-Aldrich and Fisher Scientific and used as received. All water used was ultra-pure (>18.2 $M\Omega cm^{-1}$), and dimethylsulfoxide was of molecular biology grade (>99.9%). All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees of Weill Cornell Medical Center, Hunter College, and Memorial Sloan Kettering Cancer Center and followed National Institutes of Health guidelines for animal welfare. Animals were housed in ventilated cages, given food and water *ad libitum*, and allowed to acclimatize for approximately 1 week prior to inoculation.

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 CRC-15R Dose Calibrator (Capintec). For the quantification of activity, experimental samples were counted for 1 min on an Automatic Wizard γ–counter (Perkin-Elmer). Radiolabeling experiments with ^{64}Cu were monitored using radio-HPLC (*vide infra*). NIR-fluorescence imaging was performed on an IVIS Spectrum (Perkin-Elmer). PET images were recording on a MicroPET Focus (Concorde MicroSystem, Inc.). Electrospray ionization mass spectrometry (ESI-MS) spectra were recorded using a Waters Acquity UPLC (Milford, CA) with electrospray ionization SQ detector. High-resolution mass spectrometry (HRMS) spectra were recorded with a Waters LCT Premier system (ESI).

HPLC

HPLC purifications were performed using a Shimadzu HPLC equipped with a C18 reversed-phase column (XTerra® Preparative MS OBDTM; 10 μm, 19×250 mm), a SPD-M20A photodiode array detector, 2 LC-20AP pumps, a CBM-20A communication BUS module, and a FRC-10A fraction collector, and using a flow rate of 7 mL/min. Quality controls of purified compounds were performed using a C18 reversed phase (Phenomenex Jupiter analytical; 5 μm, 4.6 × 250mm) with a flow-rate of 1 mL/min. Radio-HPLC analysis were performed using a Shimadzu HPLC equipped with a C18 reversed-phase column (Phenomenex Luna analytical 4.6 × 250 mm), 2 LC-10AT pumps, a SPD-M10AVP photodiode array detector, a Bioscan Flow Counts radioactivity detector, and a gradient of 0:100 MeCN:H_2O (both with 0.1% TFA) to 100:0 MeCN:H_2O over 15 min.

Synthesis of N^1-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-N^5-(4-(((8-amino-3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosan-1-yl)amino)methyl)benzyl)glutaramide (Tz-SarAr)

Tz-SarAr was synthesized according to methods previously published in the literature.[1] All analytical data were identical to those reported previously.

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)

Tz-PEG_7-NH_2 was synthesized according to methods previously published in the literature.[1] All analytical data were identical to those reported previously.

Synthesis of Tz-PEG_7-AF488

A solution of Tz-PEG_7-NH_2 in DMSO (40 µL, 18 mM, 0.72 µmol) was added to a solution of AlexaFluor488® *N*-hydroxysuccinimidyl ester in DMSO (108 µL, 10 mM, 1.1 µmol, 1.5 equiv.). Diisopropylethylamine (1 µL, 5.8 µmol, 8 equiv.) was then added to the mixture, and the resulting solution was shaken at RT for 4 hours, before purification of the mixture by HPLC [0:100 MeCN/H_2O (both with 0.1% TFA) to 100:0 MeCN/H_2O over 25 min, t_R = 23 min]. Solvent was removed from the collected fraction under reduced pressure, to afford the product as an orange solid (0.55 µmol, 76% yield). ESI+: $[M-2Li+3H]^+$ = 1168.5, calculated = 1168.3.

Radiolabelling of Tz-SarAr with ^{64}Cu

16 µL of a 1 mM solution of Tz-SarAr in DMSO were diluted to 200 µL with 0.1 M ammonium acetate buffer pH 4.5. 10 mCi (370 MBq) of ^{64}Cu were then added to the solution, and the mixture was shaken at room temperature for 20min. The mixture was analyzed by HPLC [0:100 MeCN/H_2O (both with 0.1% TFA) to 100:0 MeCN/H_2O over 15 min, t_R = 9.0 min], producing a labeling yield of 100%.

Preparation of huA33-Dye800

6.7 mg of huA33 in a solution of 350 µL buffer (50 mM Bis-Tris, 100 mM NaCl, pH 6.0) were incubated with 15 µL of β-(*1,4*)-galactosidase (2.0 U/mL) at 37°C overnight. A solution of 25 µL of 1 M Tris, 5 µL of 1 M solution of $MnCl_2$, 50 µL of GalT, 20 µL of a 40 mM solution of UDP-GalNAz, and 150 µL of deionized water was then added to the antibody solution, and the resultant mixture was incubated at 30°C overnight. The excess of UDP-GalNAz was removed by washing the functionalized antibody 4times with TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.6) using a 2 mL Amicon Centrifugal Filter with a 50,000 Da molecular weight cut-off. The solution of antibody was diluted in TBS to 1500 µL, and 100 µL of a 2mM solution of DBCO-IRDye800CW in DMSO were added. The mixture was incubated overnight, before purification via size-exclusion chromatography (Sephadex G-25 M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with 2 × 1 mL fractions of TBS, pH 7.6) and concentration using a 2 mL Amicon Centrifugal Filter with a 50,000 Da molecular weight cut-off, ultimately producing the fluorescent immunoconjugate with a yield of 76%.

The Dye:mAb ratio was determined via UV-Vis spectrophotometry of the immunoconjugate at 280 nm and 774 nm using the following equation:

$$A_{mAb} = A_{280} - (A_{774} * CF)$$

$$DOL = [A_{max} * MW_{mAb}]/[[mAb] * \varepsilon_{Dye800}]$$

in which the correction factor (CF) for Dye800 was given as 0.03 by the supplier, MW_{huA33} = 150,000, $\varepsilon_{774,Dye800}$= 240,000, and $\varepsilon_{280,\ mAb}$= 210,000.

Preparation of huA33-Dye800-TCO

The amine-reactive ester TCO-NHS (*N*-succinimidyl 5-((4-(1,2,4,5-tetrazin-3-yl)benzyl)amino)-5-oxopentanoate) was obtained from Sigma Aldrich. A solution of huA33-Dye800 (3.56 mg) was prepared in 1 mL of phosphate buffered saline (PBS, pH 7.4). The pH of the resultant solution was adjusted to 8.8-8.9 with 0.1 M $NaHCO_3$. The appropriate amount of TCO-NHS (25 mg/mL in DMF) was then added such that the ratio of TCO-NHS:mAb was 30:1, and the reaction mixture was incubated for 1 h at RT with agitation (550 rpm). The consequent huA33-Dye800-TCO was purified using size-exclusion chromatography (Sephadex G-25 M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with 1.0 mL fractions of PBS, pH 7.4) and concentrated, if necessary, via centrifugal filtration.

Determination of the Number of Active TCO Moieties per mAb

A solution of huA33-Dye800-TCO (75 µg) was prepared in 300 µL of PBS (pH 7.4). The appropriate amount of Tz-PEG_7-AF488 (200 µM in DMSO) was then added such that the ratio of Tz:mAb was 20:1, and the reaction mixture was incubated for 1 hour at RT with agitation (550 rpm). The consequent huA33-Dye800-AF488 was purified using size-exclusion chromatography (Sephadex G-25 M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with 1.0 mL fractions of PBS, pH 7.4) and concentrated, if necessary, via centrifugal filtration. The Dye:mAb ratio was determine via UV-Vis spectrophotometry of the immunoconjugate at 280 nm and 495 nm using the following equation:

$$A_{mAb} = A_{280} - (A_{495} * CF)$$

$$DOL = [A_{max} * MW_{mAb}]/[[mAb] * \varepsilon_{AF488}]$$

in which the correction factor (CF) for AF488 was given as 0.11 by the supplier, MW_{huA33} = 150,000, $\varepsilon_{495,AF488}$= 71,000, and $\varepsilon_{280,\,mAb}$= 210,000. The correction factors for Dye800 at 495 nm and AlexaFluor488 at 774 nm are zero. The degree of labeling of AF488 was taken as the degree of labeling of TCO due to the rapid and quantitative nature of the Tz/TCO reaction.

Ex Vivo Synthesis of ^{64}Cu-SarAr-huA33-Dye800

Freshly prepared and purified ^{64}Cu-Tz-SarAr was combined with huA33-Dye800-TCO in 400 µL PBS (pH 7.4) such that the molar ratio of Tz:mAb was 2:1, and the resultant solution was incubated at 37 °C for 30 min. After 30 min, the reaction progress was assayed using radio-iTLC with an eluent of 50 mM EDTA, pH 5. The ^{64}Cu-SarAr-huA33-Dye800 was then purified using size-exclusion chromatography (Sephadex G-25 M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with 1 mL fractions of PBS, pH 7.4) and concentrated, if necessary, via centrifugal filtration. The radiochemical purity of the crude and final radiolabeled bioconjugate was assayed by radio-iTLC. In the radio-TLC experiments, ^{64}Cu-SarAr-huA33-Dye800 remains at the baseline, while $^{64}Cu^{2+}$ ions and ^{64}Cu-Tz-SarAr elute with or close to the solvent front. Crude radiochemical yields of 80-90% were obtained, and post-purification radiochemical purities were >99% (corresponding to specific activities of ≈3.0 mCi/mg; 111 MBq/mg).

SDS-PAGE Analysis of Antibody Conjugation

5 μg of antibody (2 μL of a 2.5 mg/mL stock) was diluted with 30.5 μL H_2O, 5 μL 500 mM dithiothreitol (NuPAGE® 10× Sample Reducing Agent, ThermoFisher), and 12.5 μL electrophoresis buffer (NuPAGE® 4× LDS Sample Buffer, ThermoFisher, Eugene, OR). This mixture was then denatured by heating to 90 °C for 15 min using a heat block. Subsequently, 20 μL of each sample was then loaded alongside an appropriate molecular weight maker (Novex® Sharp pre-stained standard, ThermoFisher) into a 1 mm, 10 well 4-12% BisTris protein gel (Life Technologies) and run for ~5 h at 10 V/cm in MOPS buffer. The completed gel was washed 3 times with H_2O, stained using SimplyBlue™ SafeStain (Life Technologies) for 1 h, and destained overnight in H_2O. The gel was then analyzed using an Odyssey CLx (Li-Cor Biosciences, Lincoln, NE).

PNGase F Treatment of Antibody

HuA33 antibody constructs (5 μg) were denatured by adding 3 μL 10× Glycoprotein Denaturation Buffer (New England Biolabs, Ipswich, MA), making up the volume to 30 μL with deionized H_2O and incubated for 10 min at 95 °C on a heat block. Subsequently, 10 μL of H_2O, 5 μL of 500 mM sodium phosphate pH 7.5 (G7 reaction buffer from New England Biolabs), and 5 μL of 10% NP-40 were added. This solution was then split into two aliquots: one aliquot was supplemented with 2 μL PNGaseF (New England Biolabs) and incubated for 2 hours at 37 °C, and the other aliquot was not treated. After incubation, SDS-PAGE electrophoresis was employed to analyze the PNGaseF-treated and untreated samples.

Cell Culture

Human colorectal cancer cell line SW1222 was obtained from Sigma-Aldrich and maintained in Iscove's Modified Dulbecco's Medium, supplemented with 10% heat-inactivated fetal bovine serum, 2.0 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 weekly using a formulation of 0.25% trypsin/0.53 mM EDTA in Hank's Buffered Salt Solution without calcium and magnesium.

Subcutaneous Xenograft Models

Six to eight week-old athymic nude female (Athymic Nude-nu) mice were obtained from Charles River Laboratories (Wilmington, MA). Animals were housed in ventilated cages, were given food and water *ad libitum*, and were allowed to acclimatize for approximately 1 week prior to inoculation. SW1222 tumors were induced on the left shoulder by a subcutaneous injection of 5×10^6 cells in suspension in 150 μL of a 1:1 mixture of fresh media:BD Matrigel (BD Biosciences, Bedford, MA). The xenografts reached ideal size for imaging and biodistribution (~100-150 mm^3) in approximately 12-14 days.

Peritoneal Xenograft Model

Six to eight week-old athymic nude female (Athymic Nude-nu) mice were obtained from Charles River Laboratories (Wilmington, MA). After making a 5 mm incision into the skin, SW1222 tumors were

induced by the injection in the peritoneum wall of 5×10^6 SW1222 cells in suspension in 20 µL of BD Matrigel (BD Biosciences, Bedford, MA) supplemented with bupivacaine for analgesia. After implantation, the skin closed with 1-2 wound clips. The clips were removed after one week. The xenografts reached ideal size for imaging (~100-150 mm^3) in approximately 30-45 days.

Immunoreactivity Assays

The immunoreactivity of ^{64}Cu-SarAr-huA33-Dye800 was determined using an antigen saturation assay. Briefly, suspensions of 2.0×10^7 SW1222 colorectal cancer cells in 100 µL of media were prepared in microcentrifuge tubes. In parallel, a solution of 0.4 ng/µL radioimmunoconjugate was prepared in PBS supplemented with 1% bovine serum albumin. 5 µL (2 ng) of the radioimmunoconjugate solution was then added to the cell suspension, and the resulting mixture was agitated via pipetting and subsequently incubated on ice for 1 h. After 1 h, the cells were pelleted via centrifugation (600×g for 5 min). After centrifugation, the media was carefully removed and placed in a separate tube, and 1 mL of ice-cold PBS was added to the original tube containing the cells. The cells were then pelleted again via centrifugation (600×g for 5 min). Following centrifugation, the PBS was removed and placed in a separate tube. These PBS washing steps were repeated two more times. After washing, the amount of radioactivity in all five tubes — cell pellet, media, wash 1, wash 2, and wash 3 — was assayed using a gamma counter. The counting data was then background corrected, and the immunoreactive fraction was calculated by dividing the counts in the cell pellet by the sum of the counts in the cell pellet, media, and three washes. No weighting was applied to the data, and the data were obtained in triplicate.

Multimodality Fluorescence and PET Imaging

Fluorescence imaging was performed on an IVIS spectrum (PerkinElmer). PET imaging was performed on a micro-PET Focus 120 scanner (Concorde Microsystems) and an Inveon PET/CT scanner (Siemens Healthcare Global). Tumor-bearing mice were administrated with 100 µg of huA33-Dye800-TCO (in 200 µL of 0.9% sterile saline). The animals were imaged for near-IR fluorescence ($\lambda_{excitation}$ = 745 nm and $\lambda_{emission}$ = 820 nm) immediately prior to the injection of the immunoconjugate as well as 48 h later. Immediately after this second imaging time point, the animals were injected *via* tail vein with ^{64}Cu-Tz-SarAr (350-400 µCi; 13.0-14.8 MBq; in 200 µL 0.9% sterile saline).

PET data for each mice were recorded via static scans at 4 and 12 h after radiotracer injection. 24 h after the administration of the radiotracer (72 h post-injection of the immunoconjugate), the mice were imaged via PET and subsequently imaged a third time via NIRF. In all cases, the animals were induced at 3% isoflurane:oxygen (Baxter Healthcare, Inc.) approximately 5 minutes before imaging and maintained at 2% isoflurane:oxygen during imaging. Fluorescence imaging was performed using the 745 nm excitation filter and 820 nm emission filter, with an exposure time of 1 second.

The imaging 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 injected dose per gram of tissue; %ID/g) were determined by conversion of the counting rates from the reconstructed images. All of the resulting PET images were analyzed using ASIPro VM™ software. All of the fluorescence images were analyzed using Living Image® software (Perkin-Elmer).

Acute Biodistribution

Athymic nude mice bearing subcutaneous SW1222 xenografts (left shoulder; 100-150 mm^3) were randomized before the study and were warmed gently with a heat lamp for 5 min before the administration of 100 μg of huA33-Dye800-TCO (into 200 μL of 0.9% sterile saline) via tail vein injection. At 48 h post-injection, the animals were injected via tail vein injection with freshly prepared ^{64}Cu-Tz-SarAr (350-400 μCi; 13.0-14.8 MBq in 200 μL 0.9% sterile saline).

Subsequently, the mice (n = 4 per group) were euthanized by $CO_{2(g)}$ asphyxiation at 1, 4, 12 and 24 h after radiotracer administration, and 13 tissues (including tumor) were removed, washed, dried, weighed, and counted on a gamma counter. The number of counts in each tissue was background and decayed corrected to the time of injection and converted to activity units (μCi) using a calibration curve generated from known standards. The %ID/g (percentage of injected dose per gram of tissue) for each tissue sample was then calculated by normalization to the total activity injected and the mass of each tissue.

Statistical Analysis

Data were analyzed by the unpaired, two-tailed Student's t-test. Differences at the 95% confidence level ($P < 0.05$) were considered to be statistically significant.

Ex Vivo Autoradiography, Fluorescence Microscopy, and Histology

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

Immunofluorescence staining and imaging was performed essentially as previously described.[2] For detection of IRDye800, samples were mounted in Mowiol 4-88 mounting medium (Sigma-Aldrich, USA), and tiled images acquired at 100× using a Leica SP8 confocal microscope equipped with a tunable white light laser and avalanche photodiode detector (Leica). For detection of the A33 antigen, sequential sections were fixed in 4% paraformaldehyde, and subsequently incubated with huA33 primary antibody (5 μg/ml) overnight at 4°C, followed by secondary detection using goat anti-human Alexa-568 conjugate for 1 h at room temperature (20 μg/ml, Molecular Probes). Following image acquisition, the same sections were stained with hematoxylin and eosin and a further set on bright-field images acquired.

Whole mount fluorescence images were acquired at ×100 magnification using a BX60 fluorescence microscope (Olympus America, Inc.) equipped with a motorized stage (Prior Scientific Instruments Ltd.) and DP80 camera (Olympus). Whole-tumor montage images were obtained by acquiring multiple fields at 100× magnification, followed by alignment using cellSens Dimension software v1.13 (Olympus). Fluorescence, bright-field and autoradiographic images were co-registered using Adobe Photoshop (CS6) as previously described.[3]

Simulated Tumor Resections

Athymic nude mice bearing subcutaneous SW1222 xenografts (left shoulder; 100-150 mm^3) were warmed gently with a heat lamp for 5 min before the administration of 110 μg of huA33-Dye800-TCO

(into 200 μL of 0.9% sterile saline) via tail vein injection. The mice were euthanized by CO_2(g) asphyxiation 96 h after injection of the immunoconjugate. Tumor resection was performed while performing NIRF imaging of the mice carcasses (excitation wavelength = 745 nm and emission wavelength = 820 nm). After the successful removal of the tumor, 8 organs were removed, washed, dried, and imaged alongside the tumor via NIRF using an excitation wavelength of 745 nm and an emission wavelength of 820 nm.

Dosimetry

Mouse biodistribution data were expressed as organ mean standard uptake values (SUVs) versus time post-administration. Assuming that the SUVs are independent of body mass and thus the same among species, the mean SUV in mouse organ i, $SUV_{Organi|Mouse}$, was converted to the fraction of the injected dose (FID) in each human organ I, $FID_{OrganI|Human}$, using the flowing formula:

$$FID_{Organ\ I|Human} = SUV_{Organ\ i|Mouse} \times \frac{\text{Mass of human organ I}}{\text{Mass of human total body}}$$

and the organ and total-body masses of a 70 kg standard man anatomic model.[4]

The data (corrected for radioactive decay to the time of injection) were fit to a mono-exponential or bi-exponential time-activity function, depending on the organ. The residence time τ_i (in μCi·h/μCi), obtained from the cumulated activity in each organ, was then calculated by analytically integrating the time-activity function for the organ, and replacing the biological clearance constant with the corresponding effective clearance constant (λ_e), obtained by correcting the biological clearance constant (λ_b) for the physical decay constant of the radionuclide (λ_p): $\lambda_e = \lambda_b + \lambda_p$. The resulting organ residence times where processed using the OLINDA computer program to yield the mean organ absorbed doses and effective dose in mGy/MBq and mSv/MBq, respectively.[5]

Supplementary Figures

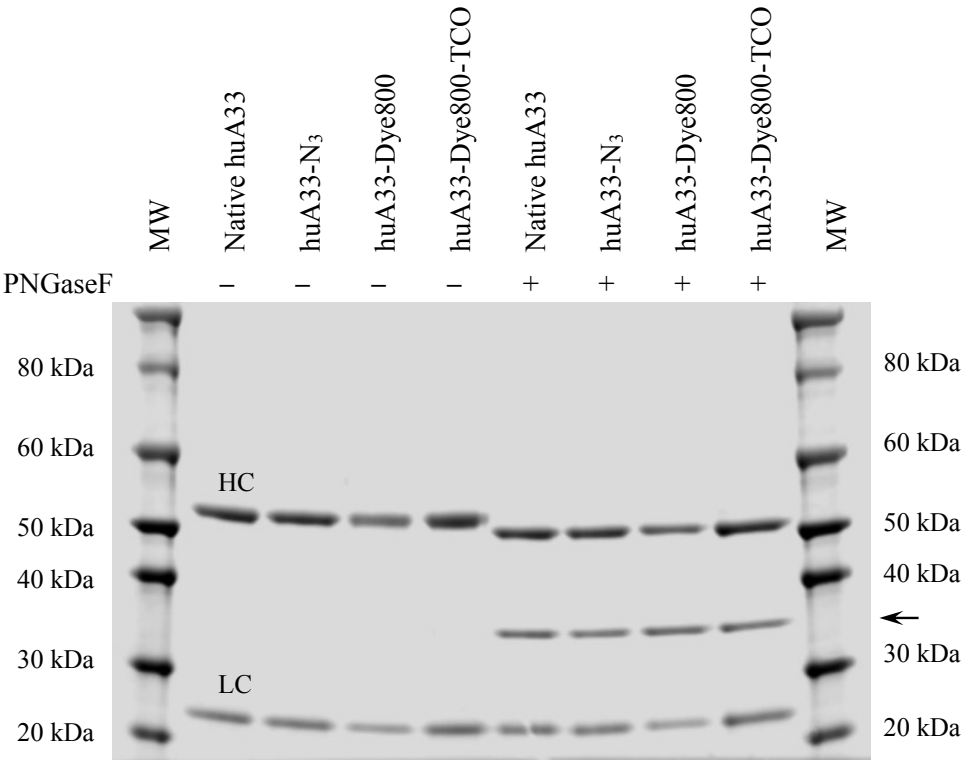

Figure S1. SDS-PAGE gel of (left to right) native huA33, huA33-N_3, huA33-Dye800, and huA33-Dye800-TCO as well as PNGaseF-treated native huA33, huA33-N_3, huA33-Dye800, and huA33-Dye800-TCO. Black arrow = PNGaseF; HC = heavy chains; LC = light chains.

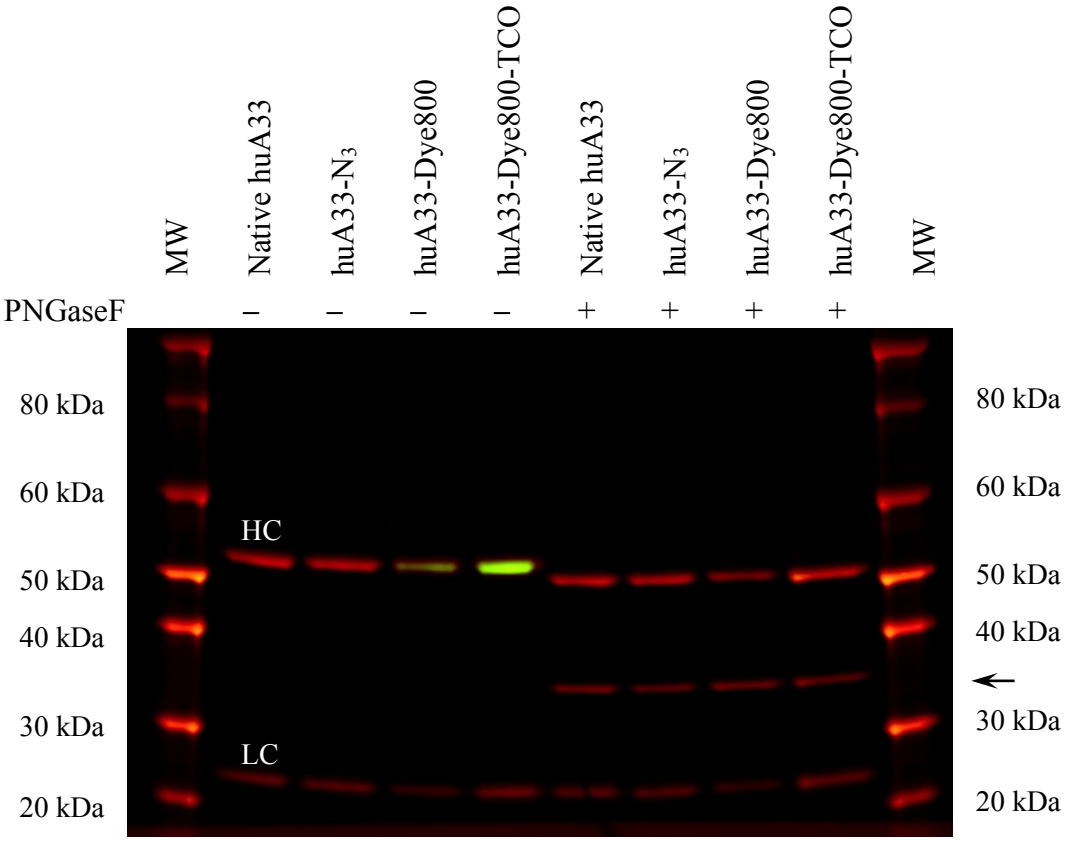

Figure S2. Superposition of images of an SDS-PAGE gel created using excitation wavelengths of 685 nm (red) and 785 nm (green). From left to right: native huA33, huA33-N_3, huA33-Dye800, and huA33-Dye800-TCO as well as and PNGaseF-treated native huA33, huA33-N_3, huA33-Dye800, and huA33-Dye800-TCO. Black arrow = PNGaseF; HC = heavy chains; LC = light chains.

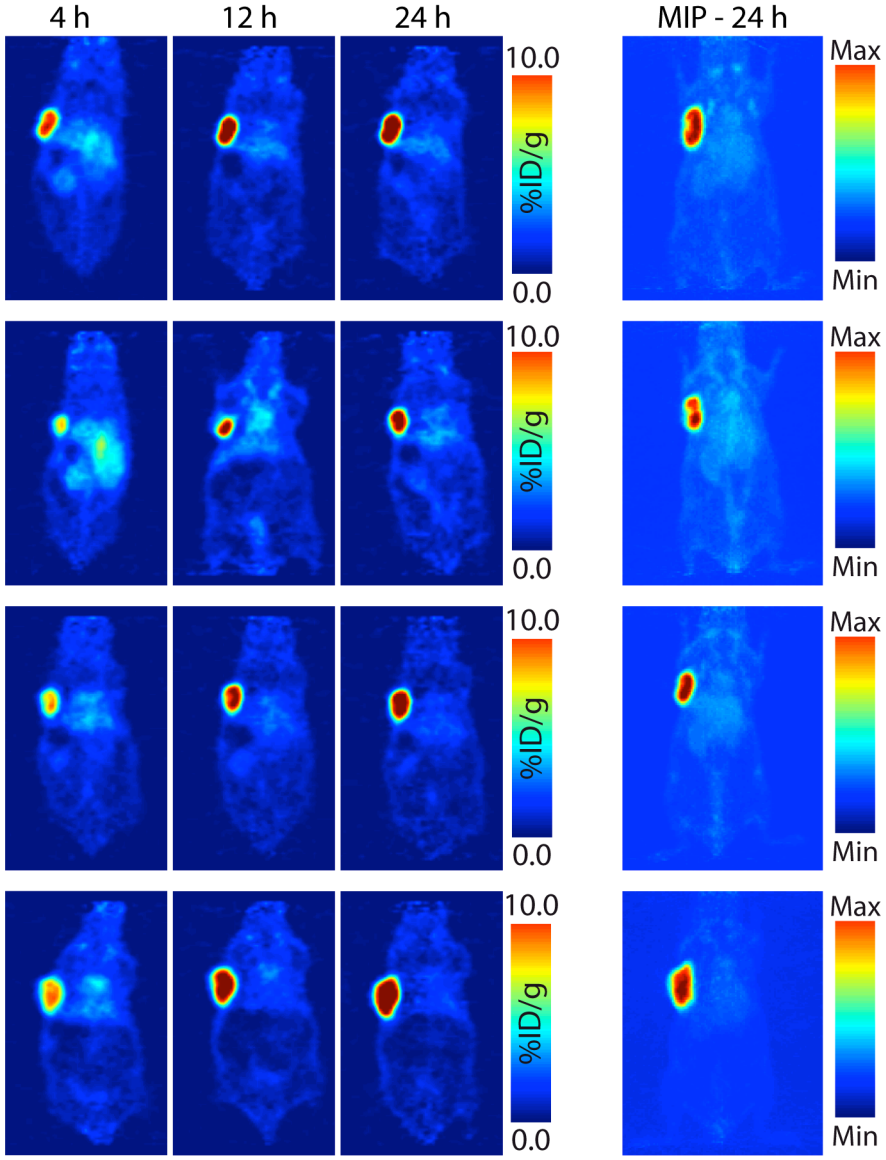

Figure S3. Planar (left) and maximum intensity projection (MIP; right) pretargeted PET images of athymic nude mice bearing subcutaneous SW1222 xenografts on their left shoulder. The mice were injected with huA33-Dye800-TCO (100 µg; 0.66 nmol), followed 48 h later with ^{64}Cu-Tz-SarAr (350-400 µCi; 0.66-0.77 nmol). The coronal slices intersect the center of the tumors.

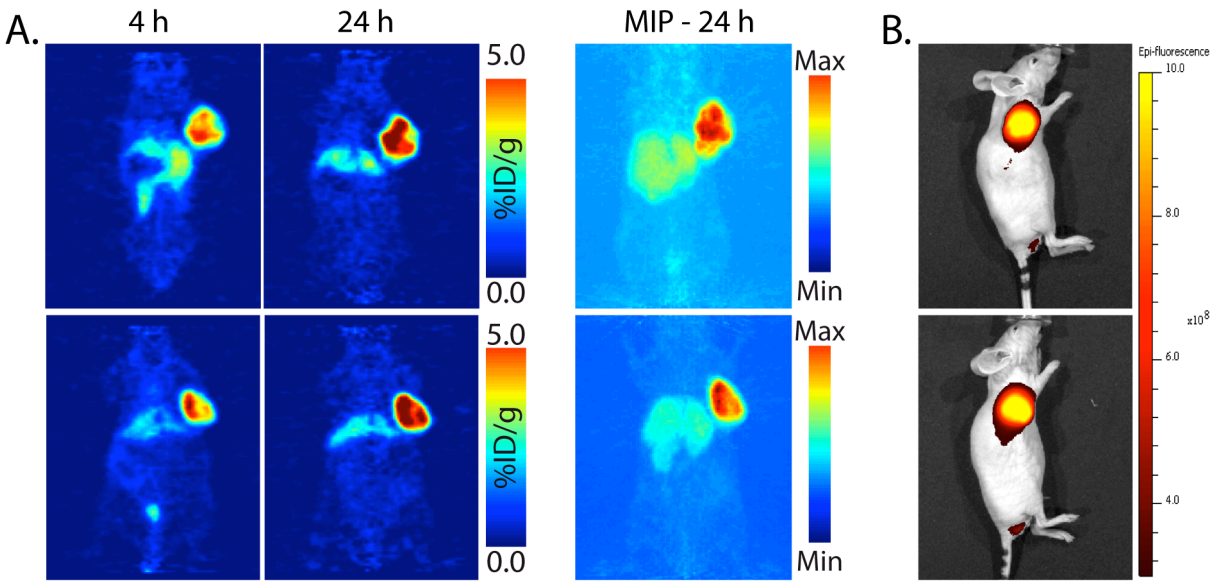

Figure S4. (A) Planar (left) and maximum intensity projection (MIP; right) pretargeted PET images of athymic nude mice bearing subcutaneous SW1222 xenografts on their right shoulder. The mice were injected with huA33-Dye800-TCO (100 µg; 0.66 nmol), followed 96 h later by ^{64}Cu-Tz-SarAr (350-400 µCi; 0.66-0.77 nmol). The coronal slices intersect the center of the tumors. (B) Near-infrared fluorescence images (λ_{ex} = 745 nm; λ_{em} = 820 nm) of the same mice acquired 24 h after the injection of ^{64}Cu-Tz-SarAr (and thus 120 h after the administration of huA33-Dye800-TCO).

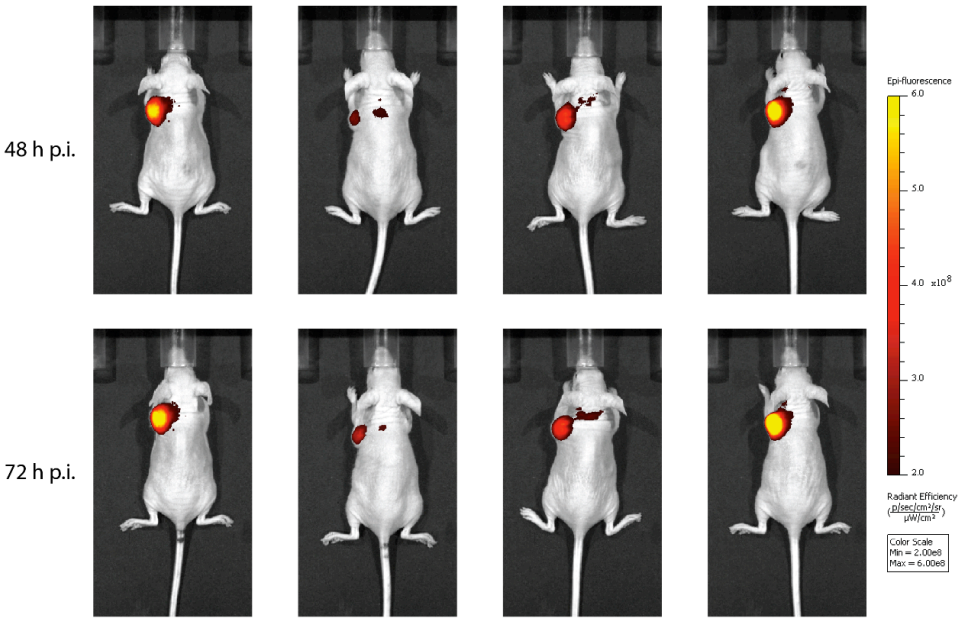

Figure S5. Near-infrared fluorescence images (λ_{ex} = 745 nm; λ_{em} = 820 nm) of mice bearing subcutaneous SW1222 xenografts on their left shoulder acquired 96 h after the injection of huA33-Dye800-TCO (and thus 48 h after the administration of ^{64}Cu-Tz-SarAr; see manuscript *Figure 2B* for temporal scheme).

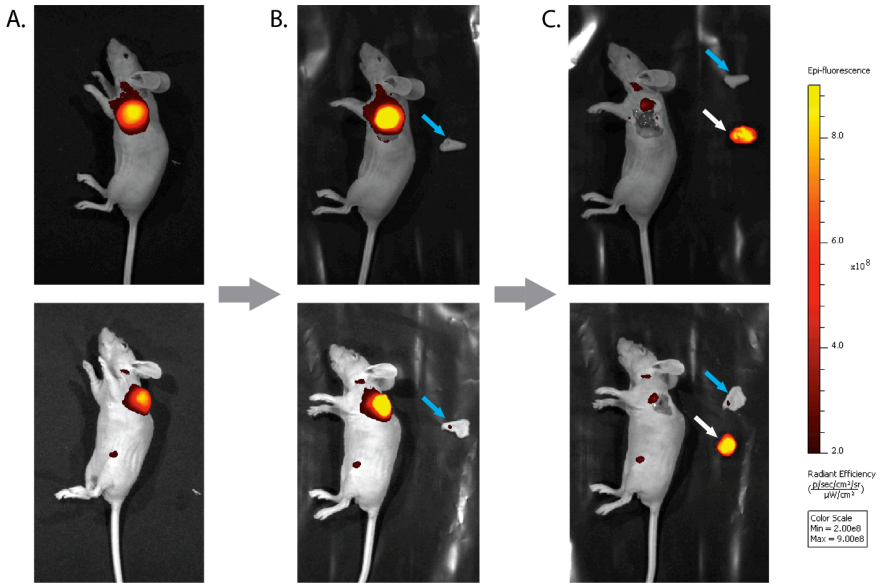

Figure S6. Simulated NIRF image-guided resection of subcutaneous xenografts performed 96 h after the injection of huA33-Dye800-TCO (and thus 48 h after the administration of ^{64}Cu-Tz-SarAr; see manuscript *Figure 2B* for temporal scheme). (A) Intact mouse; (B) mouse after removal of the skin (blue arrow) covering the tumor; (C) mouse after the excision of the tumor (white arrow).

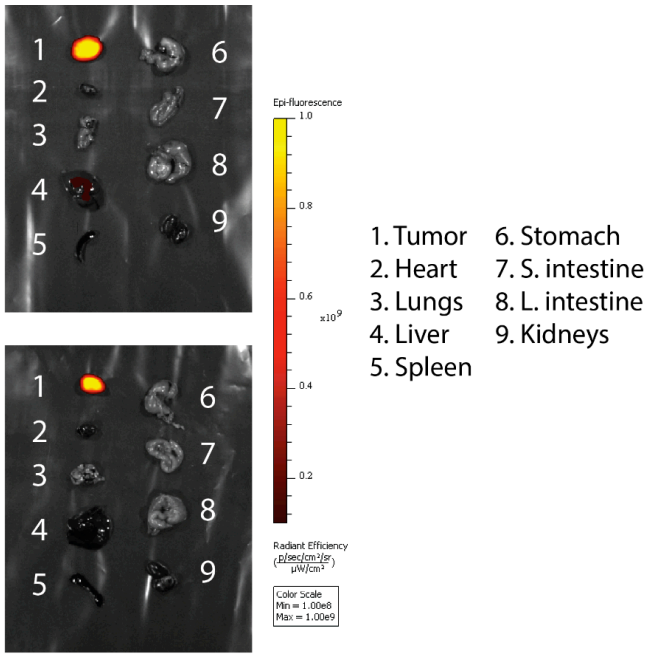

Figure S7. *Ex vivo* near-infrared fluorescence images of a selection of organs from mice bearing subcutaneous SW1222 xenografts on their left shoulder acquired 96 h after the injection of huA33-Dye800-TCO (and thus 48 h after the administration of ^{64}Cu-Tz-SarAr; see manuscript *Figure 2B* for temporal scheme).

Supplementary Tables

Table S1. Tumor-to-background activity concentration ratios produced by *in vivo* pretargeting using huA33-Dye800-TCO, ^{64}Cu-Tz-SarAr, and a 48 h pretargeting interval.

	1 h	4 h	12 h	24 h
Tumor : Blood	0.8 ± 0.3	1.5 ± 0.4	3.0 ± 0.4	5.0 ± 1.5
Tumor : Heart	2.1 ± 1.2	3.6 ± 2.5	7.3 ± 5.7	11.2 ± 21.1
Tumor : Lung	1.6 ± 0.5	2.2 ± 0.5	4.8 ± 0.7	7.9 ± 1.8
Tumor : Liver	2.1 ± 1.3	3.1 ± 1.0	7.1 ± 1.0	6.8 ± 1.6
Tumor : Spleen	3.5 ± 1.9	6.1 ± 2.2	11.9 ± 2.5	10.5 ± 3.8
Tumor : Stomach	10.0 ± 12.2	18.1 ± 18.1	51.3 ± 34.4	41.9 ± 43.0
Tumor : Small Intestine	5.7 ± 1.7	12.4 ± 3.0	25.9 ± 3.3	25.2 ± 6.2
Tumor : Large Intestine	12.3 ± 10.4	9.8 ± 2.0	35.9 ± 6.9	33.1 ± 12.1
Tumor : Kidney	1.4 ± 0.4	2.5 ± 0.5	5.3 ± 0.6	6.1 ± 1.1
Tumor : Muscle	12.1 ± 15.3	15.2 ± 4.4	25.3 ± 11.5	42.2 ± 33.0
Tumor : Bone	7.3 ± 3.0	12.6 ± 3.0	20.9 ± 4.0	22.3 ± 4.4

Table S2. *Ex vivo* fluorescence intensities for a selection of organs collected 96 h after the injection of huA33-Dye800-TCO (and thus 48 h after the administration of ^{64}Cu-Tz-SarAr).

	Radiant efficiency *		Radiant efficiency ratio
Tumor	1393.0 ± 46.0		/
Heart	32.5 ± 3.4	Tumor : Heart	42.8 ± 4.7
Lung	37.0 ± 0.7	Tumor : Lung	37.7 ± 1.4
Liver	107.5 ± 14.0	Tumor : Liver	13.0 ± 1.7
Spleen	34.3 ± 5.2	Tumor : Spleen	40.6 ± 6.3
Stomach	61.6 ± 0.6	Tumor : Stomach	22.6 ± 0.8
Small Intestine	54.5 ± 18.4	Tumor : S. Intestine	25.6 ± 8.7
Large Intestine	35.9 ± 5.3	Tumor : L. Intestine	38.8 ± 5.9
Kidney	52.0 ± 2.6	Tumor : Kidney	26.8 ± 1.6

*Radiant efficiency in 10^6 photons·sec^{-1}·cm^{-2}·sr^{-1}/μW·cm^{-2}.

Table S3. The absorbed doses created by pretargeting using huA33-Dye800-TCO and ^{64}Cu-Tz-SarAr compared to those produced by ^{89}Zr-DFO-huA33.

Target organ *	huA33-Dye800-TCO/^{64}Cu-Tz-SarAr pretargeting **	^{89}Zr-DFO-huA33 ***
Adrenals	0.022	0.443
Brain	0.021	0.207
Breasts	0.019	0.168
Gallbladder wall	0.023	0.389
Lower large intestine wall	0.024	0.362
Small intestine	0.025	0.300
Stomach wall	0.023	0.257
Upper large intestine wall	0.024	0.324
Heart wall	0.025	0.419
Kidneys	0.021	0.684
Liver	0.017	0.768
Lungs	0.019	0.611
Muscle	0.011	0.343
Ovaries	0.024	0.2945
Pancreas	0.023	0.370
Red marrow	0.021	0.843
Osteogenic cells	0.055	1.646
Skin	0.018	0.183
Spleen	0.014	0.681
Testes	0.020	0.185
Thymus	0.021	0.267
Thyroid	0.021	0.256
Urinary bladder wall	0.023	0.223
Uterus	0.024	0.254
Total body	0.022	0.376
Effective dose	*0.021*	*0.416*

*Organ absorbed doses and effective dose are expressed in mGy/MBq and mSv/MBq, respectively.
** 100 µg of huA33-Dye800-TCO injected (in 200 µL 0.9% sterile saline) 48 h before the injection of ^{64}Cu-Tz-SarAr (350-400 µCi; 13.0-14.8 MBq in 200 µL 0.9% sterile saline)
***Values from Zeglis *et al*.[6]

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