Supporting Information

"A Site-Specifically Labeled Antibody-Drug Conjugate for Simultaneous Therapy and ImmunoPET"

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Reagents and General Procedures

All chemicals, unless otherwise noted, were acquired form Sigma-Aldrich and used as received without further purification. All water used was ultra-pure (>18.2 $M\Omega cm^{-1}$), and dimethylsulfoxide was of molecular biology grade (>99.9%). The DIBO-modified DFO and DIBO-modified MMAE were provided by Thermo Fisher Scientific. ^{89}Zr was produced at Memorial Sloan-Kettering Cancer Center on a TR19/9 cyclotron (Ebco Industries Inc.) via the $^{89}Y(p,n)^{89}Zr$ reaction and purified to yield ^{89}Zr with a specific activity of 196-496 MBq/μg. Activity measurements were made using a CRC-15R Dose Calibrator (Capintec). For the quantification of activity, experimental samples were counted on an Automatic Wizard γ–counter (Perkin-Elmer). The labeling of antibodies with ^{89}Zr was monitored using silica-impregnated instant thin-layer chromatography (iTLC) paper (Pall Corp.) and analyzed on an AR-2000 radio-TLC plate reader (Bioscan Inc.). All *in vivo* experiments were performed according to protocols approved by the Hunter College, Weill Cornell Medical College, and Memorial Sloan Kettering Institutional Animal Care and Use Committees.

Cell Culture

Human breast cancer cell line BT474 was obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle Medium: Nutrient mixture F-12 (DMEM:F12), 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% CO2. Cell lines were harvested and passaged when reaching 80% of confluence using a formulation 0.25% trypsin/0.53 mM EDTA in Hank's Buffered Salt Solution without calcium and magnesium.

Xenograft Models

All experiments were performed under an Institutional Animal Care and Use Committee-approved protocol, and the experiments followed institutional guidelines for the proper and humane use of animals in research. Six to eight week-old athymic nude female (Hsd: 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 before subcutaneous implantation of an estrogen pellet (17β-estradiol 0.72 mg/pellet, 60-day release, from Innovative Research of America, Sarasota, Fl). 5 days after the pellet implantation, BT474 tumors were induced on the left flank (therapy cohort) or left shoulder (imaging cohort) by a subcutaneous injection of 10^7 cells in a 150μL cell suspension of a 1:1 mixture of fresh media:BD Matrigel (BD Biosciences, Bedford, MA). The xenografts reached the size of ~100 mm^3 in approximately 30d.

LC/MS: Liquid Chromatography

Water (Fisher Scientific), acetonitrile (Fisher Scientific) and formic acid (Pierce) were purchased from Thermo Fisher Scientific. Intact and fragment samples were separated and analyzed on Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system coupled to the Thermo

Scientific Q Exactive Plus instrument by LC-MS using a Thermo Scientific MabPac-RP (2.1x50mm,4µm, heated at 80°C, PN: 088648) column. For FC-F(ab')$_2$ fragment separation, the antibody was first cleaved using a FragIT^{TM} column (Genovis, Cambridge, MA) and the gradient elution was performed by keeping 20%B over 1 min, from 20–45% over 4 min and from 45-95% over 1 min with ACN supplemented with 0.1% formic acid at a flow rate of 400 uL/min.

Mass Spectrometry

The Q Exactive Plus MS interfaced with H-ESI II ion source was employed for intact and fragment MS analysis. The acquisition method was set with a full scan at 17,500 (FWHM, at m/z 200) for ADC intact mass analysis and 140,000 resolution for monoisotopic mass analysis of ADC fragments in positive mode. The method parameters were: AGC 3e6, IT 200 ms, in-source CID 0ev, scan range: 1000-6000 m/z, spray voltage 3.8kv, sheath gas 50, aux gas 15, capillary temperature 320°C, s-lens 50, probe heater temperature 200°C.

SDS-PAGE Analysis

5 µg antibody (2 µL of a 2.5 mg/mL stock) was diluted with 30.5 µL H_2O, 5 µL 500 mM dithiothreitol (NuPAGE® 10X Sample Reducing Agent, Life Technologies), and 12.5 µL 4X electrophoresis buffer (NuPAGE® 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 Protein Standard, ThermoFisher, Eugene, OR) onto a 1 mm, 10 well 4-12% Bis-Tris 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^{TM} 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).

Surface Plasmon Resonance

Binding kinetics for the association (k_a), dissociation (k_d), and affinity constants (K_A and K_D) of the unmodified trastuzumab versus the immunoconjugate variants — DFO-^{ss}trastuzumab and DFO:MMAE-^{ss}trastuzumab — were determined via surface plasmon resonance (SPR) on a Biacore 2000 (GE Healthcare Life Sciences). Separate SPR experiments (n = 3 per construct) were performed wherein the antibody constructs were used as ligands captured on a Protein A sensor chip (29127558, GE Healthcare Life Sciences). Antibody capture was accomplished by diluting the antibody and the two immunoconjugates to a concentration of 1 µg/mL in HBS-EP buffer (BR100188, GE Healthcare Life Sciences) and flowing the solution over a protein A sensor chip for 1.6 - 2 min at a flow rate of 5 µL/min. Next, purified recombinant human Her2 protein (HE2-H822R Acro Biosystems) was used as the analyte that was flown over the protein A sensor chip having the captured trastuzumab antibody or its immunoconjugates. The binding kinetics of the antibody (ligand) was evaluated across a concentration series of the Her2 (analyte) at 100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM, 3.125 nM, 0 nM in HBS-EP buffer. Each concentration of the analyte was injected for 5 minutes at a flow rate of 5 µL/min to allow it to bind to the antibody

captured on the protein A sensor chip. Next, the binding buffer (HBS-EP) was allowed to flow over the sensor chip for 15 minutes at a flow rate of 5 µL/min to allow dissociation of the antigen from the trastuzumab-Her2 antigen immunocomplex on the chip. Finally, the regeneration buffer (10 mM Glycine-HCl pH 2.0) was passed over the chip surface for 1 min at a flow rate of 5 µL/min to achieve complete dissociation of the captured antibody and any remaining immunocomplexes. HBS-EP buffer was flown (5 µL/min) over the chip for 2 minutes to stabilize the protein A chip surface prior to injection of the next sample in the concentration series of the analyte described above. The BIAcore control software 3.2 was used to analyze the kinetic data, and the 1:1 binding with mass transfer fit was used to derive kinetic constants for the interaction between the various trastuzumab immunoconjugates and the purified Her2 protein.

Flow Cytometry

Flow cytometry experiments were performed with HER2-positive BT474 cells. Native trastuzumab and the modified conjugate ─ DFO:MMAE-^{ss}trastuzumab ─ were incubated at 6 µg/ml in suspension with 10^6 cells/ml, for 30 min on ice. Cells were washed by pelleting and resuspension three times before incubation with a goat anti-human IgG-Alexa-Fluor 568 secondary antibody (Thermo Fisher Scientific) at 4 µg/ml for 30min on ice. Cells were washed by pelleting and resuspension three times, then analyzed on a BD LSR-II (BD Biosciences). Samples were prepared and analyzed in triplicate.

Radiolabeling of Antibody Constructs with ^{89}Zr

For each antibody construct, 500 µg of immunoconjugate solution was diluted to 400µL with PBS, pH 7.4. [^{89}Zr]Zr-oxalate (1500 µCi) in 150 µL of 1.0 M oxalic acid was adjusted to pH 7.0-7.5 with 1.0 M Na_2CO_3. After the bubbling of CO_2 stopped, the ^{89}Zr solution was added to the antibody solution, and the resulting mixture was incubated at room temperature for 1h. The reaction progress was then assayed using iTLC and an eluent of 50mM EDTA (pH 5). Subsequently, the reaction was quenched with 13 µL of 50mM of EDTA (pH=5), and the antibody construct was purified using size exclusion chromatography (Sephadex G-25 M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with 500 µL fractions of PBS, pH 7.4) and if necessary concentrated via centrifugal filtration units with a 50,000 molecular weight cut off (Amicon^{TM} Ultra 4 Centrifugal Filtration Units, Millipore Corp. Billerica, MA). The radiochemical purity of the final radiolabeled bioconjugate was assayed by radio-TLC again using 50mM EDTA (pH 5) as an eluent. In the iTLC experiments, free $^{89}Zr^{4+}$ cations and [^{89}Zr]-EDTA elute with solvent front, while radiolabeled antibody construct remains at the baseline.

Immunoreactivity Assays

The immunoreactivity of ^{89}Zr-^{ss}trastuzumab and ^{89}Zr:MMAE-^{ss}trastuzumab was determined using an antigen saturation assay. Briefly, suspensions of 2.0×10^7 BT474 human breast 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. 10 µL (4 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.

Radioimmunoconjugate Stability Assays

The stability of the radioimmunoconjugates with respect to radiochemical purity and loss of radioactivity from the antibody was investigated via incubation of the antibodies in human serum for 7days at 37°C (n = 3). After the prescribed incubation time, the radiochemical purity of the antibodies was determined via radio-TLC with an eluent of 50 mM EDTA pH 5.0.

Ex Vivo Autoradiography and Fluorescence Microscopy

Athymic nude mice bearing subcutaneous BT474 xenografts (left shoulder, 60-120 mm^3, 25-30 days after inoculation) were administrated with the ^{89}Zr-^{ss}trastuzumab (150 µCi, 60-65µg, in solution in 200 µL of saline) via tail vein injection. After 120h, the mice were injected with 50 µL of a solution of Hoechst 33342 and euthanized after 2 min, and 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.

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)

Acute Biodistribution

Athymic nude mice bearing subcutaneous BT474 xenografts (left shoulder; 60-120 mm^3) were randomized before the study and were administrated with the radioimmunoconjugates (150 µCi, 60-65µg, in solution in 200 µL of saline) via tail vein injection. Subsequently, the animals (n

= 4 per group) were euthanized by CO_2(g) asphyxiation at 120 h post-injection, and 13 tissues (including tumor) were removed, washed, dried, weighed, and counted in 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 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.

For the therapy study, pairwise comparisons with the DFO:MMAE-^{ss}trastuzumab 10 mg/Kg group evaluating average relative tumor volumes at Day 20 were performed using the unpaired, two-tailed Student's t-test (not assuming equal variances). The Holm-Sidak correction was applied to adjust for the multiple pairwise comparisons evaluated. Differences at the 95% confidence level ($P < 0.05$) were considered to be statistically significant.

Supplementary Figures and Tables

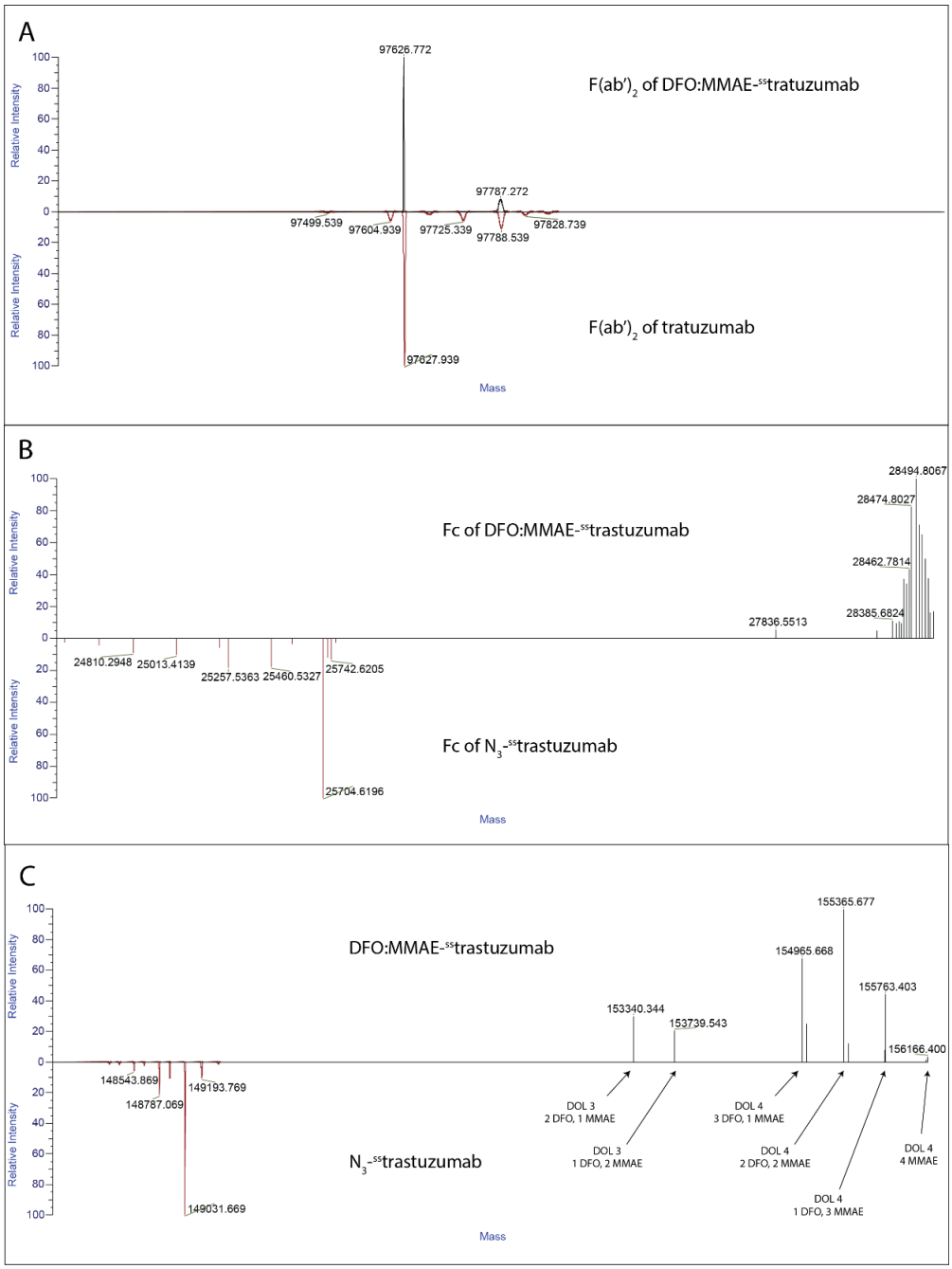

Figure S1. Mass spectra and deconvoluted average masses of the DFO:MMAE-^{ss}trastuzumab conjugate and fragments. (A) Mass spectra of the F(ab')$_2$ of DFO:MMAE-^{ss}trastuzumab (upper spectrum) and the F(ab')$_2$ of trastuzumab (lower spectrum). (B) Deconvoluted average mass spectrum of the Fc fragment of DFO:MMAE-^{ss}trastuzumab (upper spectrum) and mass spectrum of Fc fragment of N_3-^{ss}trastuzumab (lower spectrum). (C) Deconvoluted average mass spectrum of the DFO:MMAE-^{ss}trastuzumab (upper spectrum) and mass spectrum of N_3-^{ss}trastuzumab (lower spectrum).

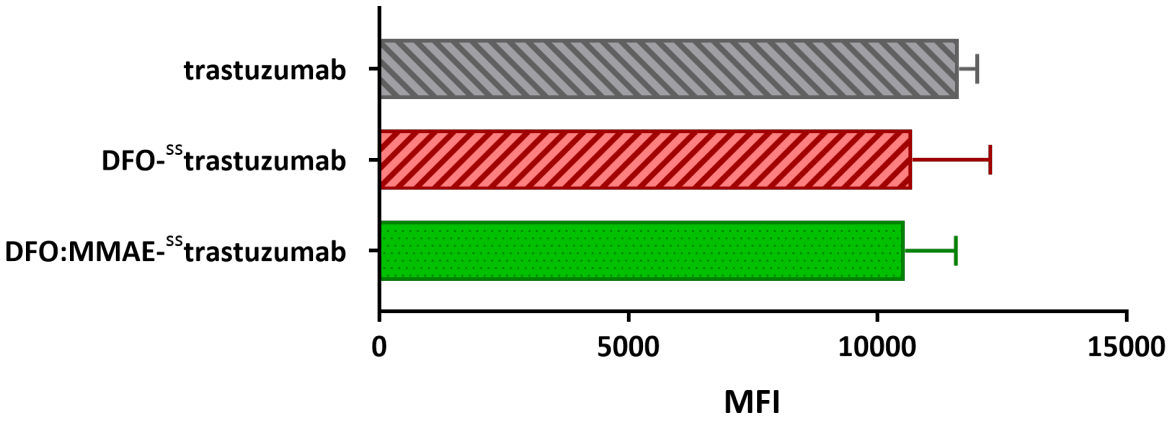

Figure S2. Median fluorescence intensity (MFI) of BT474 cells labeled with native trastuzumab and the conjugates, after incubation with an AF568-labeled anti-human goat antibody. Measurements were done in triplicate.

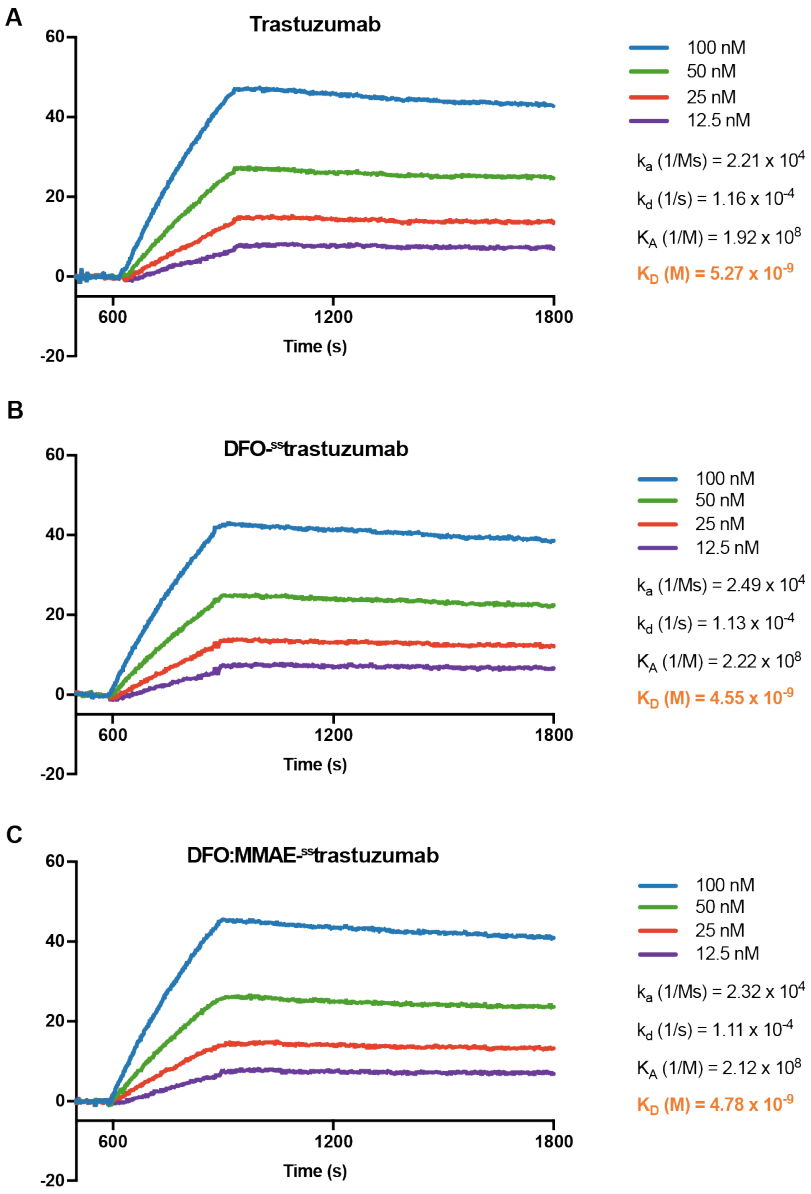

Figure S3. Surface plasmon resonance analyses of trastuzumab immunoconjugates indicates minimal loss of immunoreactivity. Antibody-antigen binding curves fitted from the kinetic analyses of (A) unmodified Trastuzumab; (B) DFO-^{ss}trastuzumab; (C) DFO:MMAE-^{ss}trastuzumab. The closely matched binding curves and kinetic constants for the two immunoconjugates (B and C) indicates a minimal loss of immunoreactivity compared to unmodified trastuzumab (A).

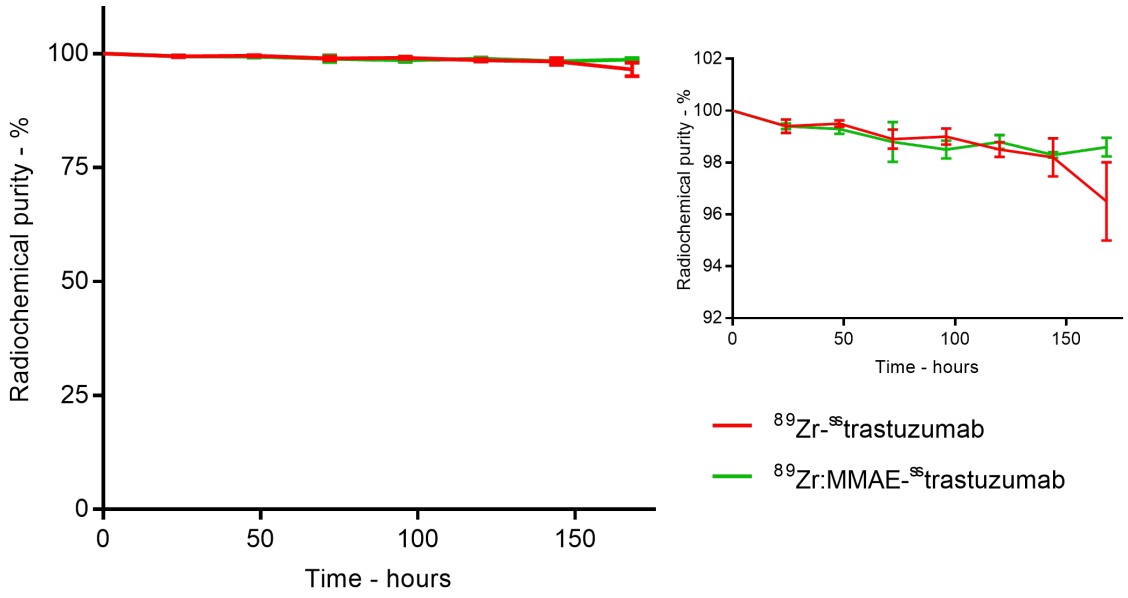

Figure S4. Stability of the 2 site-specifically radiolabeled conjugates in human serum at 37 °C. Measurements were performed in triplicate.

Table S5. Biodistribution data for the ^{89}Zr-^{ss}trastuzumab and ^{89}Zr:MMAE-^{ss}trastuzumab 120 h after their injection in nude mice bearing BT474 tumors.

	^{89}Zr-^{ss}trastuzumab	^{89}Zr:MMAE-^{ss}trastuzumab
Blood	6.6 ± 1.2[a]	7.4 ± 1.0
Tumor	76.3 ± 13.1	70.4 ± 7.7
Heart	2.3 ± 0.2	2.8 ± 0.3
Lung	3.1 ± 0.4	3.6 ± 0.3
Liver	2.4 ± 0.1	3.1 ± 0.5
Spleen	2.0 ± 0.3	3.1 ± 1.0
Stomach	1.2 ± 0.8	0.5 ± 0.1
Small Intestine	0.8 ± 0.1	1.0 ± 0.3
Large Intestine	0.9 ± 0.2	0.7 ± 0.1
Kidney	3.2 ± 0.3	4.2 ± 0.7
Muscle	0.5 ± 0.3	1.0 ± 0.2
Bone	3.0 ± 0.9	3.1 ± 1.0

[a]Values are %ID/g ± SD. Nude mice (n = 4) bearing subcutaneous BT474 xenografts were administered the radiolabeled PET-ADC constructs or radiolabeled ^{89}Zr-^{ss}trastuzumab (150 μCi, 65 μg) via tail vein injection. Stomach, small intestine, and large intestine values include contents.

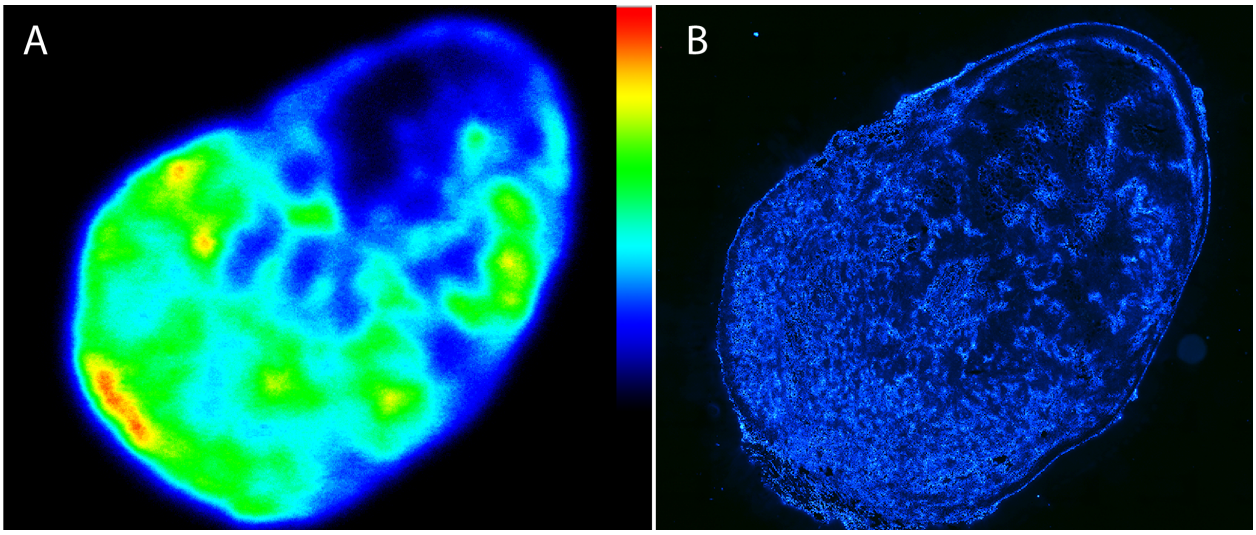

Figure S6. (A) Autoradiography and (B) Hoechst staining of sections of a BT474 tumor excised 120h after the injection of 150 μCi of ^{89}Zr-^{ss}trastuzumab.

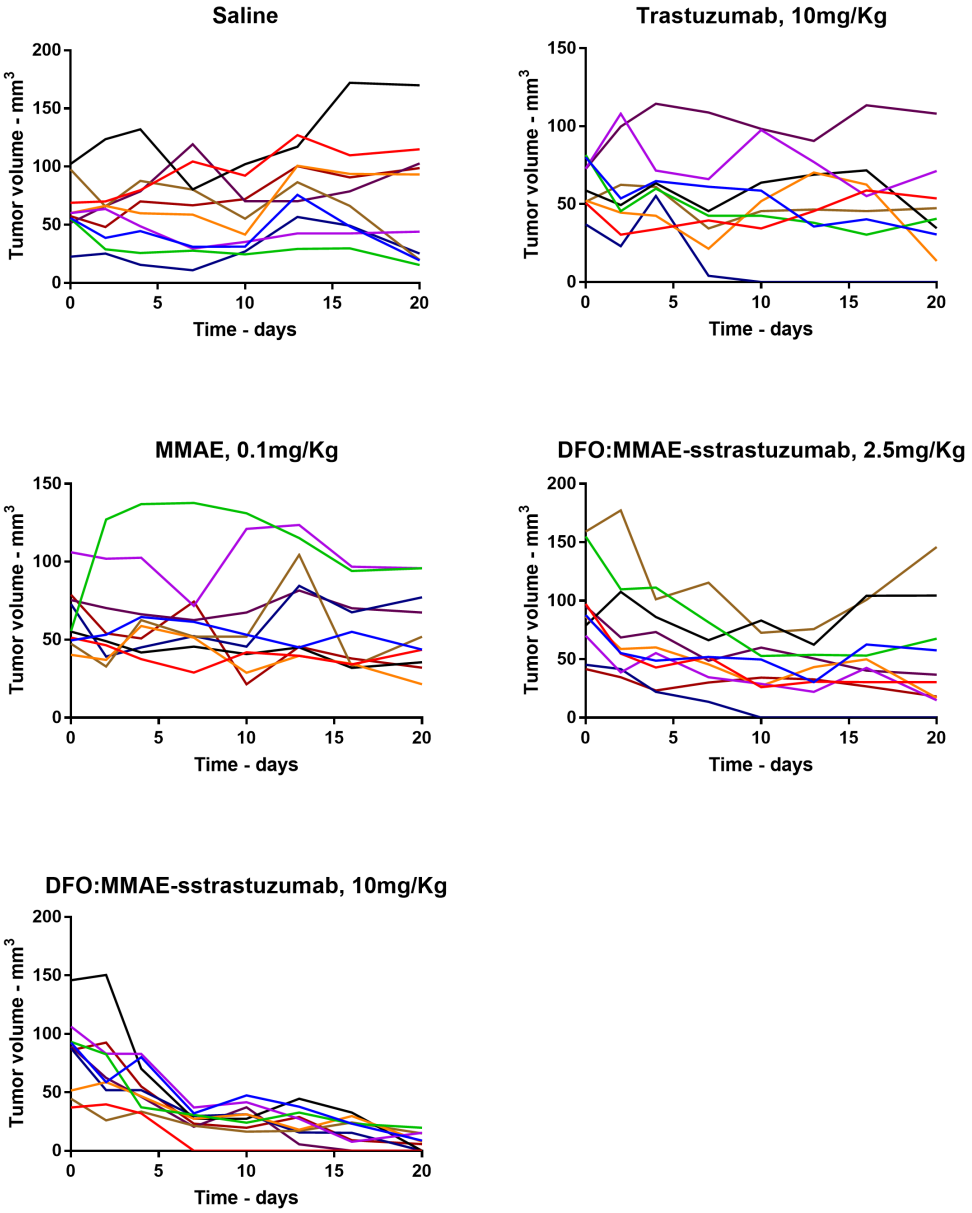

Figure S7. Tumor volume (mm^3) as a function of time for the individual mice in each of the five cohorts of the longitudinal therapy study.