Supplemental Information

SUPPLEMENTAL METHODS:

Generation of DOTA NHS Ester In Situ

In a typical reaction, DOTA (65.6 mg, 10 eq) and Sulfo-NHS (27.79 mg, 10 eq) were dissolved in 600 µl of metal-free water at 4 ˚C. A 10 mg aliquot of EDC (Thermo Scientific) was dissolved in 200 µl of metal-free water, and 49.1 µl (2.45 mg, 1 eq) of this solution was added to the DOTA/Sulfo-NHS solution. The pH of the reaction was adjusted to ~5.7 by repeated additions of sodium hydroxide (NaOH), and the reaction was allowed to proceed for 30 minutes at 4 ˚C. The resulting active ester was added to antibodies directly without further purification.

Quality Control of Antibody-Chelate Constructs

The protein concentration of the product constructs was determined using the Bio-Rad DC assay (Bio-Rad Laboratories, Inc). The average number of chelates per antibody was measured using the lead-arsenazo spectrophotometric method (*1*). For the 4-arm DOTA construct and the DTPA construct we used the free ligands DOTA and DTPA to generate the standard curve. For the 3-arm DOTA construct we used a three-arm DOTA-maleimide construct obtained from Macrocyclics as a standard.

Quality Control of Radioimmunoconjugates

The percent of ^{225}Ac bound to the radioimmunoconjugates was determined by instant thin-layer chromatography (iTLC) using silica gel (iTLC-SG) paper (Agilent Technologies). The paper was cut into strips, and small aliquots of the radioimmunoconjugate were spotted onto these strips at Rf=0. The strips were then developed using two different mobile phases. Mobile phase I was 10 mM EDTA, and mobile phase II was 9% NaCl/10 mM NaOH. After development in mobile phase I, the Rf of the radiolabeled antibody was 0, and the Rf of both the free metal

species and metal in unconjugated chelates was 1.0. After development in mobile phase II, the Rf of both the radiolabeled antibody and free metal species was 0, and the Rf of the metal chelates was 1.0. The strips were cut in the middle, and the halves were counted separately in a Cobra II gamma counter (Packard).

Immunoreactivity of the constructs radiolabeled with ^{225}Ac was determined using a cell-based assay with excess antigen as described previously (*2*). The CD33-positive cell line used was Set2-Luc, and the CD33-negative cell line was Ramos. Briefly, $10x10^6$ cells per tube were first blocked with 1 ml of 2% human AB serum/1% bovine serum albumin in PBS for 20 min on ice. Cells were then pelleted, and to each pellet was added radiolabeled antibody (2 ng) in 20 μl of the blocking buffer. After a 40-minute incubation on ice, 100 μl of blocking buffer was added. The supernatant plus two washes were transferred to a scintillation vial, while the pellet was transferred to a second vial. ScintiSafe Gel (5 ml, Fisher Scientific) was added to each tube, and the tubes were counted on a Tri-Carb 2910 TR liquid scintillation analyzer (PerkinElmer) after equilibrium. The energy window used was 156-1700 keV (32P window), and the samples were counted for 1 or 10 minutes. Scintillation counting was used to increase the signal to noise over our gamma counter, since the former is much more sensitive. This was desirable because of the small quantities of radiolabeled antibody used in the assay. The use of replicates and standards validated the reliability of this approach.

Protein G binding of the antibodies was determined by incubating serum extracted from treated mice (3 μl per sample) with protein G agarose (Thermo Scientific, 10 μl settled resin). It was assumed that the amount of radioimmunoconjugate was small compared to the total IgG in the serum, and the amount of protein G agarose was calculated such that all IgG in the sample should be bound. The remaining volume in the incubation was occupied by 1% HSA (17 μl) for a total of 30 μl per incubation. Samples were incubated at room temperature for 2 h, the supernatant and two washes were separated from the protein G pellet, and the radioactivity was measured on a beta scintillation counter as described for immunoreactivity.

Serum Stability In Vitro

Purified radioimmunoconjugates (0.1 ml) were added to 100% human AB serum (0.9 ml) in 1.8 ml Nunc tubes and incubated at 37 ˚C. At various timepoints, an aliquot (30 µl) was removed from each sample and mixed with 50 mM DTPA (10 µl) to challenge off any metal that was not stably chelated to the antibody. After incubation of this aliquot at 37 ˚C for 15 minutes, 8 µl was spotted in triplicate on iTLC strips and developed with solvent system 1 as described above.

Flow Cytometry Analysis

For cell surface staining, cells were incubated with appropriate mAbs for 30 min on ice, washed, and incubated with a R-phycoerythrin-labeled goat anti-rat secondary antibody. Flow cytometry data were collected on a FACSCalibur (Becton-Dickinson) or Accuri C6 (BD Biosciences) and analyzed with FlowJo version 10.0.6 software.

Animal Studies

For the biodistribution studies in healthy animals, we obtained female BALB/c mice aged 6-10 weeks from NCI-Frederick or Taconic Labs. For the therapy study we obtained male Nod.Cg-Prkdc^{scid}-Il2rg^{tm1Wjl}/SzJ (Nod *scid* gamma or NSG) mice aged 9 weeks from the Sloan Kettering Mouse Genetics Core Facility, which were originally derived from the Jackson Labs NSG strain.

For biodistribution experiments, female BALB/c mice (*n* = 3/group) were injected retroorbitally with either Ac-3A1S-HuM, Ac-4A1S-HuM, or Ac-4A2S-HuM (11.1 kBq). At various times post-injection (t=2.5 hours; and 1, 3, 6, and 12-13 days), animals were euthanized and blood was collected via cardiac puncture. Organs were harvested, weighed, and separated into 12x75 test tubes. These tubes were counted on a Cobra II gamma counter (Packard) along with injected dose standards. A portion of blood was allowed to coagulate at 4 ˚C overnight and

was spun down at 16,100xg for 10 minutes to obtain serum. This was used in immunoreactivity and protein G binding assays as described above.

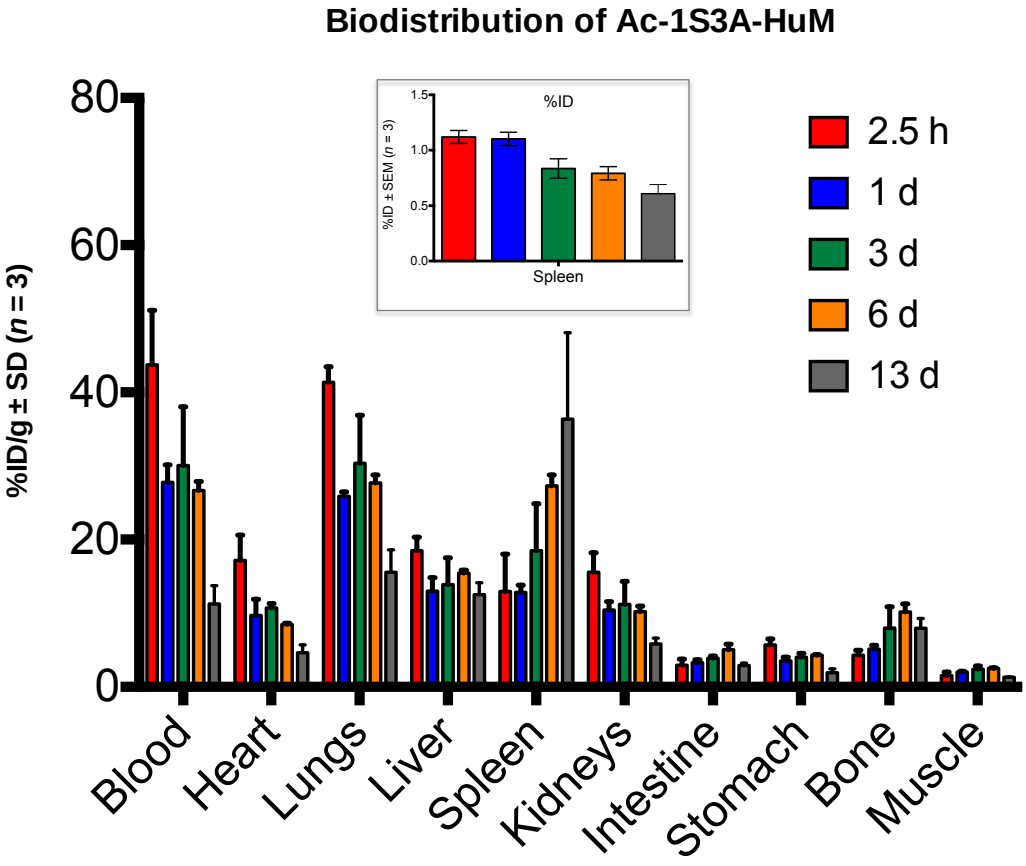
For therapy experiments, male NSG mice (*n* = 5 /group) were injected with 1e6 Set2-Luc cells intravenously via the lateral tail vein. On day 7 post-tumor injection, the animals were injected with D-luciferin potassium salt (Gold Biotechnology, 1 mg/mouse in 0.1ml/mouse IV retroorbitally) and imaged on an IVIS 200 instrument (PerkinElmer). Images were analyzed by drawing regions of interest over the whole animal in Living Image software (PerkinElmer). Mice were divided into groups of 5 such that the average signal intensity was the same for each group. On day 10 post-tumor injection, animals were treated by injecting 0.555 kBq or 1.11 kBq of Ac-1S3A-HuM, Ac-1S3A-Rit + 3A-HuM, Ac-1S4A-HuM, Ac-1S4A-Rit + 4A-HuM, or vehicle control (1 % HSA) retroorbitally. The protein amount of each antibody was at 0.225 μg regardless of the radioactive dose and was adjusted by adding the appropriate unlabeled construct. Bioluminescent imaging was carried out every 5-7 days thereafter, and the experiment was terminated after imaging on day 26, before overt morbidity from tumors was observed.

All animal studies were approved by the Institutional Animal Care and Use Committee of MSKCC under protocol 96-11-044.

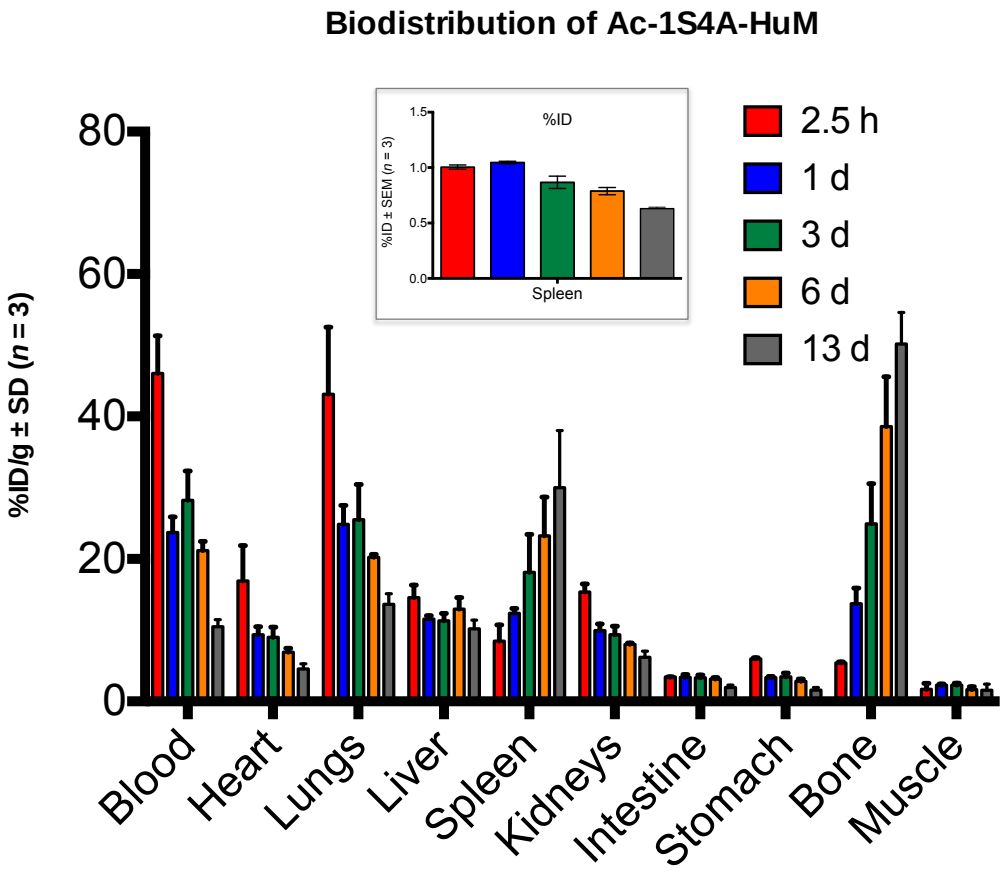
SUPPLEMENTAL REFERENCES

1. Dadachova E, Chappell LL, Brechbiel MW. Spectrophotometric method for determination of bifunctional macrocyclic ligands in macrocyclic ligand-protein conjugates. *Nucl. Med. Biol.* 1999;26:977-982.

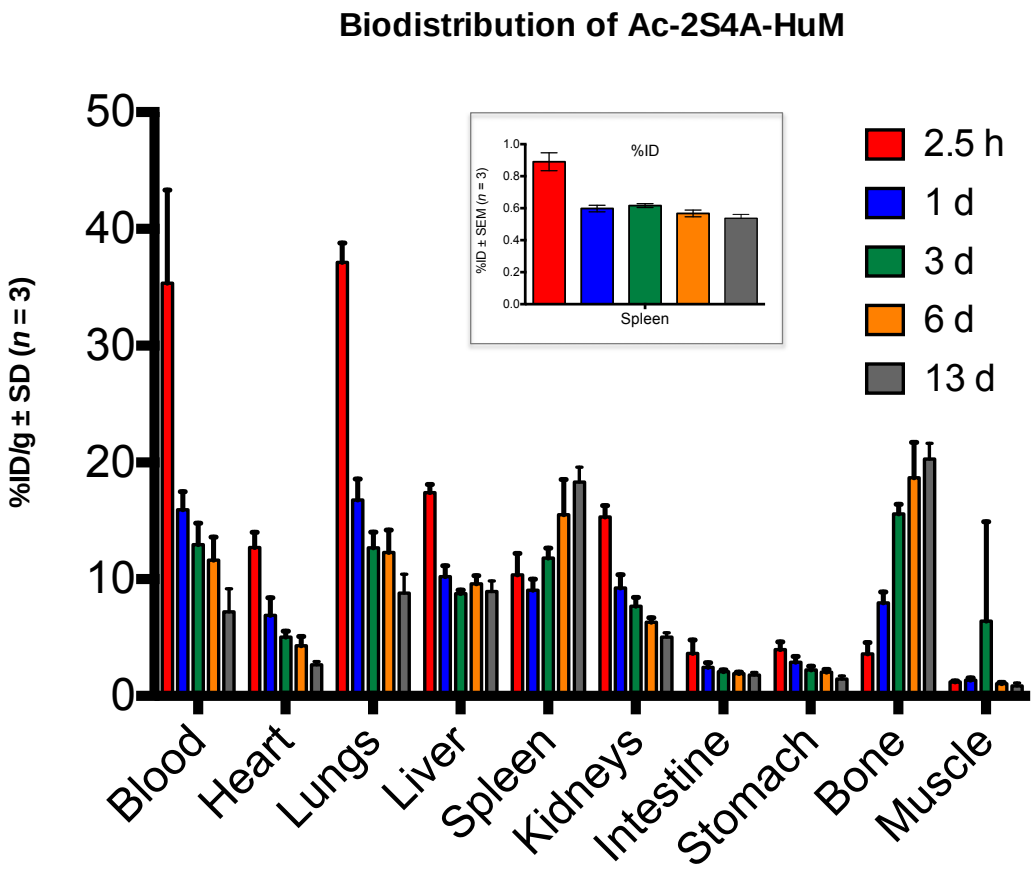
2. Nikula TK, McDevitt MR, Finn RD, et al. Alpha-emitting bismuth cyclohexylbenzyl DTPA constructs of recombinant humanized anti-CD33 antibodies: pharmacokinetics, bioactivity, toxicity and chemistry. *J Nucl Med*. 1999;40:166–176.



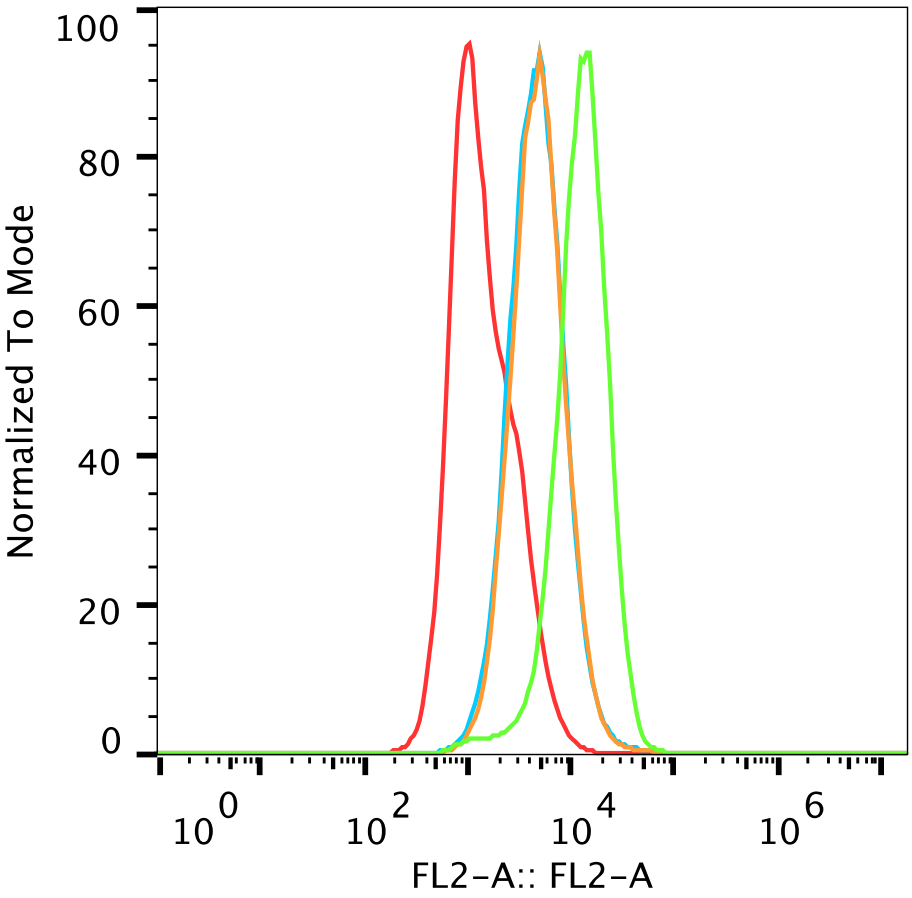
Supplemental Figure 1: Biodistribution of Ac-1S3A-HuM in female BALB/c mice. Inset: %ID values for spleen, not normalized to organ weight. %ID/g = Percent injected dose per gram of tissue.



Supplemental Figure 2: Biodistribution of Ac-1S4A-HuM in female BALB/c mice. Inset: %ID values for spleen, not normalized to organ weight.

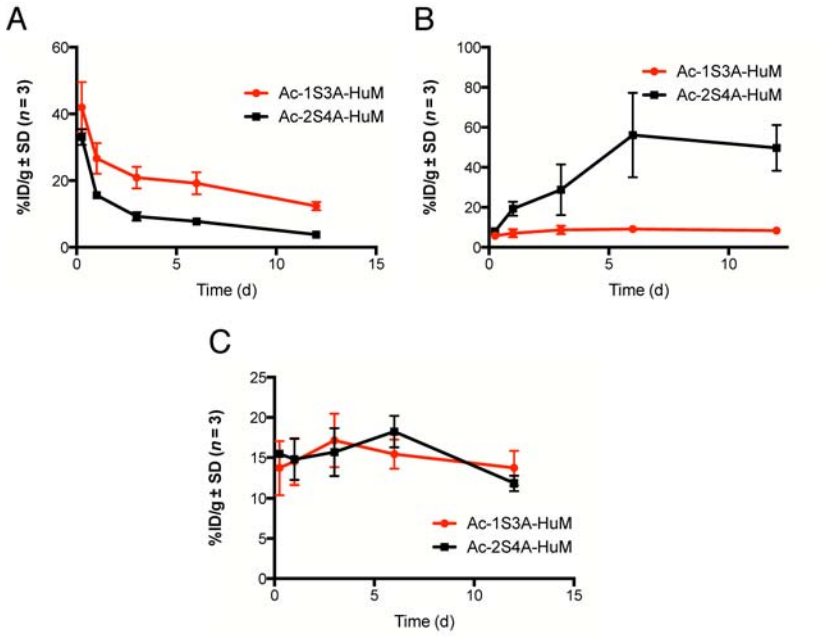


Supplemental Figure 3: Biodistribution of Ac-2S4A-HuM in female BALB/c mice. Inset: %ID values for spleen, not normalized to organ weight.



	Sample Name	Median : FL2-A
	Sample Set2_HUM195 A04.fcs	12931
	Sample Set2_Ritux A03.fcs	4808
	Sample Set2_SecondaryOnly A02.fcs	4662
	Sample Set2_Unstained A01.fcs	1273

Supplemental Figure 4: HuM195 is immunoreactive towards Set2-Luc by flow cytometry, while Rituximab is not.



Supplemental Figure 5: Repeat biodistribution of Ac-1S3A and Ac-2S4A. Organs shown are blood (A), bone plus marrow (B), and liver. %ID/g = percent injected dose per gram of tissue.