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Evaluation of glycodendron and synthetically-modified dextran clearing agents for multi-step targeting of radioisotopes for molecular imaging and radioimmunotherapy

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Abstract

A series of *N*-acetylgalactosamine-dendrons (NAG-dendrons) and dextrans bearing biotin moieties were compared for their ability to complex with and sequester circulating bispecific anti-tumor antibody (scFv₄) streptavidin (SA) fusion protein (scFv₄-SA) *in vivo*, to improve tumor to normal tissue concentration ratios for targeted radioimmunotherapy and diagnosis. Specifically, a total of five NAG-dendrons employing a common synthetic scaffold structure containing 4, 8, 16, or 32 carbohydrate residues and a single biotin moiety were prepared (NAGB), and for comparative purposes, a biotinylated-dextran with average molecular weight (MW) of 500 kD was synthesized from amino-dextran (DEXB). One of the NAGB compounds, CA16, has been investigated in humans; our aim was to determine if other NAGB analogs (e.g. CA8 or CA4) were bioequivalent to CA16 and/or better suited as MST reagents. *In vivo* studies included dynamic positron-emission tomography (PET) imaging of ¹²⁴I-labelled-scFv₄-SA clearance and dual-label biodistribution studies following multi-step targeting (MST) directed at subcutaneous (s.c.) human colon adenocarcinoma xenografts in mice. The MST protocol consists of three injections: first, a bispecific antibody specific for an anti-tumor associated glycoprotein (TAG-72) single chain genetically-fused with SA (scFv₄-SA); second, CA16 or other clearing agent; and third, radiolabeled biotin. We observed using PET imaging of ¹²⁴I-labelled-scFv₄-SA clearance that the

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SUPPORTING INFORMATION

Additional dynamic PET ROI TAC data up to 240 p.i. of ¹²⁴I-scFv₄-SA for vehicle (*n* = 2) and CA16 (for NeoRx compound *n* = 1; for in-house preparation *n* = 2), and thyroid ROI TAC data for all dendron-CA. This material is available free of charge via the Internet at <http://pubs.acs.org>.

spatial arrangement of ligands conjugated to NAG (i.e. biotin) can impact the binding to antibody in circulation and subsequent liver uptake of the NAG-antibody complex. Also, NAGB CA32-LC or CA16-LC can be utilized during MST to achieve comparable tumor- to-blood ratios and absolute tumor uptake seen previously with CA16. Finally, DEXB was equally effective as NAGB CA32-LC at lowering scFv₄-SA in circulation, but at the expense of reducing absolute tumor uptake of radiolabeled biotin.

Keywords

asialoglycoprotein; pretargeting; radioimmunotherapy; bispecific antibodies

INTRODUCTION

Multi-step targeting (MST) of radionuclides is an effective strategy to improve the therapeutic index of oncologic RIT and achieve the benefits of high-dose radiation without the need for stem cell rescue.¹⁻⁵ This can be accomplished by injecting the antibody and radiation source separately, thereby effectively decoupling the pharmacokinetics of the antibody from the radionuclide.

MST typically involves the sequential administration of complementary agents designed to function in concert to optimize tumor targeting of radiation while minimizing normal tissue exposure. First, antibody that contains either a bispecific protein fusion or bioorthogonal chemical functionality is administered systemically and allowed to accrete to maximal concentration at antigen positive tumor sites. After sufficient time has elapsed to enable peak tumor uptake, the excess antibody is removed from circulation by *in vivo* complexation using a “clearing” agent (CA). The resultant complexes are excreted via hepatic catabolism. This clearing step is essential to achieve the highest absolute concentration of antibody receptor at tumor sites with low absolute blood and whole body concentrations. Radiation is delivered to the tumor in a third step by administration of a complimentary radiolabeled ligand. This low MW carrier molecule (<1 kD) readily penetrates the tumor vasculature, where it is captured by the prelocalized antibody construct. By virtue of the ligand pharmacology (namely, renal glomerular excretion and low whole-body retention), the residence time of uncomplexed (free) radioactivity in circulation is significantly shortened, thus allowing treatment planning with higher doses of activity and efficient fractionation schedules. Rapid uptake of the therapeutic isotope at tumor sites (before significant loss of potency attributable to radioactive decay) and efficient renal elimination of excess radioactivity represent the fundamental advantages of MST over conventional RIT, where the radioisotope is directly conjugated to the antibody. This approach has been tested preclinically in mice, producing cures of human small cell lung, colon, and breast cancer xenografts.⁶ Highly promising median tumor-to-normal organ ratios >30:1 have been reported clinically in patients (e.g. with non-Hodgkin's lymphoma (NHL)), but the maximum tolerated dose is generally limited by hematological and renal toxicities.⁷

Two CA strategies have been predominantly investigated: (1) administration of a secondary antibody or protein^{8,9} to which the circulating targeting antibody will bind and form large

aggregates, and/or (2) administration of a bispecific carbohydrate polymer,⁹ which will also bind with the antibody, but has high affinity for endogenous lectins. The resultant antibody-complexes will illicit recognition as immune complexes by Kupffer cells and macrophages in the reticuloendothelium (RES). Specific targeting of galactose receptors with exogenous ligands is a common approach for liver-targeted drug delivery and MST clearing agents, due to the rapid kinetics of hepatocyte uptake and internalization (e.g. by asialoglycoprotein receptors (ASGPr) and Kupffer cells).¹⁰ A popular CA for biotin-SA MST is biotin-(NAG)₁₆, which consists of a single biotin linked to the core of a four-generation dendrimer with 16 terminal NAG residues (CA16). This compound was demonstrated to be non-toxic and highly efficacious in NHL patients undergoing therapeutic MST; within 6 h post injection (p.i.), freely circulating antibody was reduced approximately 20-fold (as the antibody-CA16 complex), with no adverse effects.⁷ One aspect of the current work that remains unknown is whether these CA16 properties can be extrapolated to larger or smaller constructs, e.g. a single biotin linked to the core of a three- generation dendrimer with 8 terminal NAG residues (CA8).

In this study we compare the efficiency of different NAGB to CA16 using familiar MST reagents and animal models, i.e. those used during recent investigations to improve the therapeutic index by reducing the renal dose.¹¹ The MST protocol consists of three steps, including injection of each: a bispecific single-chain antibody-streptavidin (SA) fusion (CC49 scFv-SA, as a homo-tetramer: scFv₄-SA), specific for an anti-tumor associated glycoprotein (TAG-72); a dose of CA16 sufficient to reduce circulating scFv₄-SA, and third, radiolabeled BSD. The NAGB contain a single biotin attached via a single *N*-methyl-amino-hexanoamide linker to a dendrimer core with 4, 6, 16 or 32 NAG residues (relative sizes: CA4< CA8< CA16<CA32-LC), and encompass a molecular weight (MW) range of 2271-17413 (**Figure 1, A & B**). In addition, the linker length separating the biotin-molecule from the NAG- core was extended to potentially allow for greater conformational flexibility and reduced steric- hinderance (CA32-LC and CA16-LC). In the case of the CA32, given its size and carbohydrate density, we were concerned that a single linker might be too short, and the biotin would be buried inside the glycodendron, rendering it inaccessible for scFv₄-SA binding. To circumvent this possibility, we extended the linker by 14 atoms with two more units of the *N*-methyl-amino-hexanoamides.

During the first set of experiments, we conducted dynamic PET imaging to examine the kinetics and mechanism(s) of the clearance of ¹²⁴I- scFv₄-SA following CA injection in biotin-starved athymic nude mice. The second study included dual-isotope MST in biotin-starved athymic nude mice bearing subcutaneous (s.c.) human colon adenocarcinoma xenografts. By using radioiodinated antibody and radiolabeled biotin-DOTA as tracers, the disposition of antibody mediated with and without CA can be determined, as well as determine the influence of CA on ligand uptake at the tumor and other critical organs. This observation is essential to evaluating CA efficacy, as the optimum CA dose is in molar excess to scFv₄-SA during MST. Axworthy et al. reported that the binding of scFv₄-SA to CA16, while still high and kinetically facile, was significantly lower than that of natural biotin or of the subsequently administered biotin-ligand during *in vitro* analysis.¹² While CA16 may also bind to significant amounts of extravascular antibody under conditions of

high doses, and thus in excess of tumor-associated molecules, it does not compromise binding of subsequently administered radiolabeled biotin. Numerous studies have been performed documenting that the ligand can effectively and completely compete off the CA16 under *in vivo* conditions and time frames, but this property is unknown for other NAGB.

For comparison, DEXB was prepared to examine the influence of sugar type, biotin valency, and overall CA size (**Figure 1, C**). Orcutt et al. reported MST of carcinoembryonic antigen (CEA) using a 500 kD dextran-based CA alongside analogous bispecific antibody and radiolabeled haptens, demonstrating highly promising absolute tumor uptake and tumor to organ ratios in mice bearing s.c. LS174T (CEA+) xenografts.¹³ Dextran is naturally-occurring complex glucose polymers, consisting of linear α -1,6-glucan chains and branches with α -1,3 linkages. There are many properties that make dextrans an attractive choice as a drug carrier: (1) their pharmacokinetic fate *in vivo* can vary based on the size; (2) they are very amenable to chemical modification due to the high density of hydroxyl-groups; (3) they have a well-documented safety profile in humans; and (4) preparations of a wide-variety of sizes (e.g. dextran-40; average MW = 40 kD) can be obtained in high purity at low cost. Dextran is metabolized by the enzyme dextran-1,6-glucosidase, which is present predominantly in RES organs including the liver and spleen.¹⁴ Blood clearance of 150 kD dextran-fluorescein conjugates was reported to occur within 12 h in rats, but subsequent metabolism by the RES- route led to slow whole-body clearance, as high concentrations of tracer-dextran were observed to persist >96 h in spleen.¹⁵ This feature can be of concern when utilizing a dextran-ligand conjugate as a CA, because increased radio-toxicity can result if the concentration of intact antibody-CA is sufficient in tissue to bind subsequently administered radiolabeled ligand. In addition, adventitious small MW dextran-CA fragments released into circulation can potentially interfere with antibody-ligand capture.

EXPERIMENTAL SECTION

Materials

The bispecific scFv₄-SA and CA16 was provided by NeoRx Corporation. All NAGB- CA were synthesized by the Organic Synthesis Core of Memorial Sloan-Kettering Cancer Center (MSKCC), and described in Yoo et al.¹⁶ CA16 prepared in-house was observed to be bioequivalent to the original NeoRx CA16 compound (**see Supplemental Information, S1**), and used for all experiments unless specified otherwise. ¹¹¹In, ¹³¹I, and ¹²⁵I were received from Nordion (Ottawa, ON, Canada). The positron-emitting isotope ¹²⁴I ($t_{1/2}$ = 4.18 days) was provided by the Memorial Sloan-Kettering Radiochemistry & Molecular Imaging Probes Core Facility. Pre-coated iodogen tubes, 2 kD MW cutoff dialysis cartridges, 4'-hydroxyazobenzene- 2-carboxylic acid (HABA), and high-performance liquid chromatography (HPLC) grade solvents were obtained from Thermofisher (Waltham, MA). Biotin-sarcosine-DOTA (BSD) was obtained as the HCl salt and used without further purification (purity 94%) from Macrocyclics (Dallas, TX). 500 kD amino-dextran was obtained from Life Technologies/Molecular Probes (Grand Island, NY). Chelex-100 and Bio-Gel P6 (medium 90-180 μ m) was obtained from Bio-Rad (Hercules, CA). Centrifugal Ultracell YM-10 devices were from Millipore (Billerica, MA). Biotinidase-resistant 18-

biotinamino-17-oxo-4,7,10,13-tetraoxa-16-azaicosan-1-oic acid succinimidyl ester (biotin-dPEG(4)-NHS) was obtained from IRIS Biotech GmbH (Marktredwitz, Germany). All other chemicals and solvents were obtained from Sigma-Aldrich Co. LLC. All buffers and solutions were prepared using ultra-pure water (resistivity, 18.2 M Ω cm) and 0.22 μ m-filtered before use. Plastic-ware used for preparation of radiometal-labeled chelates was acid-washed by soaking in 10-20% (v/v) nitric acid for at least 1 h, followed by rinsing with ultra-pure water. In addition, buffers were treated with chelex (batch contact for minimum 1 h, 10 g dry chelex resin/1L buffer) and 0.22 μ m-filtered before use. HPLC was performed on a Shimadzu (models: pumps, LC-20AB; autosampler, SIL-20AC HT) (Shimadzu Instruments) equipped with an Agilent Zorbax Extend-C18 rapid resolution 4.6 $\times$ 250 mm, 3.5 μ m particle size, and a NaI solid scintillation FlowCount Radio-HPLC detector (Bioscan). HPLC solvent A: 0.1% (v/v) glacial acetic acid in water, solvent B: methanol; gradient: 60% B for 2 min, 60-100% B in 8 min; flow-rate 0.4 mL/min; 111 In-BSD retention time: 5.0-5.5 min). Out-bred female athymic nude mice (4-6 weeks of age) were obtained from Harlan Laboratories (Indianapolis, IN). Biotin-deficient diet (catalog- 5836) was obtained from Purina Mills (Gray Summit, MO). For one week immediately prior to experiments, all mice were fed biotin-free diet to minimize competition between BSD and endogenous biotin. The TAG-72-expressing human cell carcinoma line LS174T was obtained from ATCC (CL-188). Matrigel was obtained from BD Biosciences (Franklin Lakes, NJ).

Radiolabeling of antibody and BSD

scFv₄-SA was labeled with radioactive iodine (124 I, 125 I, or 131 I) using the iodogen method^{17, 18} to final specific activities of 70.3-88.8 MBq/mg for $^{125/131}$ I- and 136.2-260.9 MBq/mg for 124 I-scFv₄-SA. The radiochemical purity for all preparations were consistently >95% using instant-thin layer chromatography with normal saline as the running solvent. The immunoreactivity (59.3 $\pm$ 12.9% (mean $\pm$ standard deviation (SD))) was determined using LS174T cells and the method of Lindmo, et al.¹⁹

BSD was radiolabeled with 111 In using a previously described protocol with minor modifications.²⁰ A 3 mg/mL stock solution of BSD was prepared in 500 mM ammonium acetate pH 5.3 and stored at -20°C. To 50 μ g of BSD (17 μ L), an additional 100 μ L of 500 mM ammonium acetate pH 5.3 was added, followed by 5 μ L of [111 In] indium chloride. The pH was confirmed to be 5.3 by paper, and the reaction was allowed to incubate at 85°C for 60 min. The crude reaction was then purified using HPLC equipped with a C18-column as described in the previous section. The overall incorporation yield of 111 In ranged from 69-75%, and the radiochemical purity was consistently >95%. The pooled HPLC fractions of pure 111 In-BSD were dried under vacuum and re-suspended in normal saline for injection.

Preparation of DEXB

500 kD dextran-biotin was synthesized in a single step by reacting commercially available 500 kD dextran-amine with a *N*-hydroxysuccinimidyl derivative of biotin-PEG (biotin-dPEG(4)-NHS) using previously described methods.¹³ Briefly, a stock solution of 500 kD amino-dextran (146 mol amine/mol dextran according to manufacturer) was prepared by dissolving 50 mg in 20 mL of dimethylsulfoxide (DMSO) with 25 μ L of triethylamine

added, followed by sonication. To 20.9 mg of stock (8.04 mL, 6.1 μ mol amine), 0.45 mL of a 10 mg/mL solution of biotin-dPEG(4)-NHS in DMSO (1.1 mol equivalents to total amines) was added. The reaction was allowed to proceed at room temperature with stirring for 8 h. To purify, aliquots of the crude reaction were diluted with ultra-pure water to reduce the DMSO concentration to 5% (v/v), and concentrated using a centrifugal device. The retentate was lyophilized and re-dissolved for subsequent purification with Superdex 200 gel-filtration. The DEXB was suspended in saline at 1 mg/mL, passed through a 0.22 μ m syringe filter, and stored at -20°C . The mol ratio of biotin:dextran was determined to be 100 using the HABA test.²¹

Dynamic PET imaging of ^{124}I -scFv₄-SA in normal mice

All animal experiments were approved by the Institutional Animal Care and Use Committee of MSKCC, and institutional guidelines for the proper and humane use of animals in research were followed. Each mouse was fitted with a home-made catheter in the tail-vein while under anesthesia using a mixture of 1.5-2% isoflurane (Baxter Healthcare, Deerfield, IL), and placed on the microPET Focus 120 (Concorde Microsystems, Knoxville, TN) scanner. At $[t = 0]$, imaging (list-mode) was initiated during injection of ^{124}I -scFv₄-SA (0.58 nmol, 5.44-9.07 MBq) via the catheter. After 15 min, CA (100 μ g of CA32-LC, CA16 (either prepared in-house or provided by NeoRx), CA8, or CA16-LC, or 50 μ g of CA4) or vehicle ($n = 1$ for all groups) was given (also via catheter), and scanning was continued for an additional 30-45 min (see **Supplemental Information, S1** for demonstration of reproducibility for groups of $n > 1$, as well as a comparison of CA16 prepared in-house to CA16 provided by NeoRx). Imaging data were acquired using an energy window of 350-700 keV and a coincidence timing window of 6 ns. Mice were re-imaged in static mode at 4 h $[t = 240 \text{ min}]$ and 24 h $[t = 1440 \text{ min}]$ p.i. for a minimum of 10 min or when 20 million coincidence events were collected. Early data $[t = 0-45 \text{ or } 60 \text{ min}]$ were binned into 26 total frames ($20 \times 90\text{-s}$, $6 \times 300\text{-s}$). Next, images were sorted into 2-dimensional histograms by Fourier rebinning, and image reconstruction was performed by filtered back-projection with a $128 \times 128 \times 63$ ($0.72 \times 0.72 \times 1.33 \text{ mm}$) matrix. The final data were parameterized (as %ID/g) by first converting the voxel-counting rates to activity concentrations using empirically determined calibration factors for ^{124}I , followed by decay-correction to the time of injection and normalization to the administered activity. No attenuation, scatter or partial-volume averaging correction was applied. Two-dimensional regions of interest (ROI) and time-activity curves (TAC) were manually drawn using ASIPro VMTM software (Concorde Microsystems), and the average $\pm$ SD %ID/g was used for subsequent analysis. Mice were sacrificed immediately following the last imaging time point $[t = 1440 \text{ min}]$ for biodistribution studies of select organs (*vide infra*). Curve-fitting and integration analysis of data were performed using GraphPad Prism 5.00 software.

Dual-isotope MST with radioiodinated scFv₄-SA, CA, and ^{111}In -BSD in mice bearing s.c. LS174T xenografts

To initiate LS174T xenografts in mice, 5×10^6 cells were mixed with Matrigel and injected s.c. in the lower limb. Tumors were observed after 2 weeks ($500\text{-}600 \text{ mm}^3$). All doses of antibody, CA, and ^{111}In -BSD were prepared at 100-120 μ L and administered i.v. via the tail-vein. At $[t = 0]$, scFv₄-SA was injected (500-800 μ g, 2.8-4.5 nmol, with or without 0.74

MBq of radioiodinated antibody added), followed by CA at $[t = 24 \text{ h}]$, and ^{111}In -BSD at $[t = 28 \text{ h}]$ (see Table 1, A-C for reagent details).

Biodistribution Analysis

Animals were sacrificed via CO_2 asphyxiation following PET imaging studies $[t = 240 \text{ min}]$, or 24 h p.i. of ^{111}In -BSD during MST experiments $[t = 52 \text{ h}]$. Samples of blood and RES-associated organs (liver, spleen, and kidney) were harvested for the former experiments, while activities in blood, tumor, heart, lungs, liver (all), spleen, stomach, small intestine, large intestine, kidneys (both), muscle, and bone were determined for the latter studies. Tissues and organs were excised, rinsed with water and allowed to air-dry, weighed, then counted in an automatic well counter (Perkin Elmer Wallac Wizard 3" Automatic Gamma Counter). Crossover between isotope energy windows were corrected using standards of each radionuclide. The radioactive uptake for each tissue was calculated as the percent injected dose per gram (%ID/g) after decay-correcting the net count rate to the time of injection. Data are expressed either as the mean %ID/g $\pm$ one standard error of the mean (SEM) or as the mean pmol/g $\pm$ SEM. Errors for the tumor-to-tissue ratios are calculated as the geometric mean of the SEM.

SPECT/CT imaging of ^{111}In -BSD uptake in tumor-bearing mice

A separate group of tumor-bearing mice underwent single-isotope MST followed with SPECT imaging. Each animal received scFv₄-SA, followed by either: CA16, CA8, CA4, or DEXB, and ^{111}In -BSD (40 MBq, 1.31 nmol) (see Table 1, D for reagent details). At 18-24 h p.i. of ^{111}In -BSD mice were anesthetized using a mixture of 1.5-2% isoflurane (Baxter Healthcare, Deerfield, IL) and oxygen gas, and placed in the scanner (NanoSPECT/CT, Bioscan). The gantry was heated to 37°C and was equipped for continuous administration of anesthesia during imaging. A CT topogram was acquired first, followed by a 360-degree microSPECT using a 4-headed gamma-camera with pinhole-collimators (1.4 mm) in 24 projections. The SPECT scan time was determined by placing the mouse in the camera field of view and adjusting the time per projection image to record ~30,000 counts minimum per frame (20-55 min). Bioscan HiSPECT iterative reconstruction software was used for image reconstruction and fusion of CT and SPECT images.

Statistical Analysis

Differences in tissue uptake between groups were statistically analyzed with Student's *t* test for paired data. Two-sided significance levels were calculated and $P < 0.05$ was considered statistically significant.

RESULTS

PET imaging of NAGB-mediated blood clearance of ^{124}I -scFv₄-SA

Non-invasive PET imaging studies were conducted with non-tumor bearing mice that were starved of biotin. Animals were sequentially administered ^{124}I -scFv₄-SA and CA while in the PET scanner to examine the kinetics and clearance mechanisms of NAGB-antibody complexes in real-time. At $t = 0$, mice were injected i.v. with ^{124}I -scFv₄-SA, followed 15 min later with either CA32-LC, CA16, CA16-LC, CA8, or CA4. Also, a separate group was

injected with vehicle (i.e. saline) instead of CA as a control for normal ^{124}I -scFv₄-SA clearance. Image acquisition was performed in dynamic mode up to 45 min p.i. of the CA [t = 0-60 min], and static images were acquired at later time points [t = 240 and 1440 min].

Time-activity curves (TAC) were obtained for blood (as cardiac outflow), liver, and kidney following dynamic PET imaging of ^{124}I -scFv₄-SA up to 45 min p.i. of the CA (or 30 min for vehicle). In addition, ROI analysis was performed on static PET images acquired at 240 min and 1440 min. Biodistribution data obtained at 1440 min correlated with ROI analysis of blood, liver, and kidneys (not shown). Also, *ex vivo* analysis of spleen showed that the tracer activity in tissue was significantly greater in vehicle-treated mice compared with CA-treated mice (5 %ID/g and 2 %ID/g, respectively; $P < 0.05$) (not shown). Representative TAC data for vehicle, CA32-LC, CA16, CA8, and CA4 are shown in **Figures 2 & 3**. **Figure 2** shows each TAC with ^{124}I -activity expressed as %ID/g, while **Figure 3** depicts the same data after normalizing each concentration to the maximum (max.) activity recorded for that particular tissue (as 100%). Compared with vehicle, all CA were efficient at decreasing circulating ^{124}I -scFv₄-SA at the doses administered (100 µg per mouse) within 15 min p.i. of the CA. Just prior to injection of CA [t = 14.25 min], ^{124}I -scFv₄-SA in blood ranged from 12.4-20.5 %ID/g (CA4 < CA16 < CA8 < vehicle). At 45 min p.i. of the CA [t = 60 min], the activity had dropped to 3.3 %ID/g or 5.8 %ID/g in the CA32-LC or CA16 groups, respectively, and to a lesser extent for the CA8 or CA4 groups (7.1 %ID/g and 8.4 %ID/g, respectively). For vehicle, the activity in blood dropped from 20.5 %ID/g to 15.8 %ID/g at ~45 min. Alternatively, if the data is described using the normalized TAC, treatment with CA32-LC showed a reduction in blood activity to 20% (of max.) at 60 min while treatment with CA16 or CA8 showed a reduction in blood activity to 40% at 60 min. CA4 was most similar to vehicle, showing changes to 60% and 80%, respectively. At 240 min, the circulating activity in all CA groups had fallen to ~2-3 %ID/g (or 20%), while the circulating activity in vehicle was 5-fold greater (10.5 %ID/g or 60%). The liver TAC data showed for the largest NAGB (i.e. CA32-LC), the ^{124}I -activity quickly increased from 10.2 %ID/g to a peak of 20.7 %ID/g within ~30 min [t = 14.25-47.5 min], then sharply decreased to a plateau of ~15 %ID/g over the next 10 min. In addition, this relatively high liver activity persisted at 240 min. All other groups showed hepatic activities of 6.6-9.9 %ID/g (CA4 < CA8 < vehicle < CA16) just prior to CA injection, and little accumulation within 30 min p.i. (e.g. at [t = 42.5 min]: 7.6-7.9 %ID/g; CA4 < CA8 < vehicle < CA16). Between 60 and 240 min, the liver uptake for CA4 increased more than 2-fold (5.8 to 12.8 %ID/g), but remained steady for CA16 (10.8 to 9.1 %ID/g) and CA8 (8.3 to 7.8 %ID/g); liver uptake was lowest for vehicle (<5.6 %ID/g up to 240 min) in comparison to all other groups. The normalized renal TAC data of the CA32-, CA16-, and CA8 groups revealed that the changes in activity resembled that of the blood, dropping from 80-100% at [t = 14.25 min] to 30-60% at [t = 60 min], while in CA4 and vehicle, activity remained high (> 80%). At 240 min, the activity lowered to 20-30% (2-fold, from 3 to 1.5 %ID/g) in all CA-groups and was the highest in vehicle (> 80%).

Representative TAC data for CA16-LC is shown in **Figures 4&5**, plotted with analogous data from the CA16 group for comparison. Analysis of the heart activity suggests CA16-LC is more efficient at reducing circulating ^{124}I -scFv₄-SA compared to CA16 at an equal dose.

At 60 min, the activity was 5.8 %ID/g (40%) for CA16 and 2.7 %ID/g (20%) for CA16-LC. The hepatic activity in CA16-LC almost doubled (5.7 %ID/g at 14.25 min to 10.3 %ID/g at 60 min), while for CA16, it remained constant (9.9 %ID/g at 14.25 min and 10.8 %ID/g at 60 min). Also, at 60 min, mice given CA16-LC showed lower renal uptake, with activity dropping to 40% compared to a drop to 60% for the CA16 group.

Investigation of thyroid uptake of ^{124}I (**Supplemental Information, S2**) revealed that all groups showed activities of ~3 %ID/g up to 60 min p.i. At 240 min, the thyroid uptake increased to 5-10 %ID/g for CA16, CA8, CA4, and vehicle, and was highest in CA32 and CA16-LC (~30 %ID/g for both).

The TAC data for blood and kidney from [$t = 15.75\text{-}240$ min] were fit to a single-exponential decay function to determine the tracer clearance kinetics from each tissue. These data are summarized in **Table 2**. The best-fit $t_{1/2}$ for blood in mice treated with vehicle was 66.0 min ($R^2 = 0.98$); with CA32-LC was 9.3 min ($R^2 = 0.96$); with CA16-LC was 10.2 min ($R^2 = 0.96$); with CA16 was 5.9 min ($R^2 = 0.87$); with CA8 was 4.5 min ($R^2 = 0.84$); and with CA4 was 58.4 min ($R^2 = 0.99$). The best-fit $t_{1/2}$ for kidney in mice treated with vehicle was 151.0 min ($R^2 = 0.94$); with CA32-LC was 12.2 min ($R^2 = 0.97$); with CA16-LC was 10.0 min ($R^2 = 0.90$); with CA16 was 47.0 min ($R^2 = 0.95$); with CA8 was 22.2 min ($R^2 = 0.87$); and with CA4 was 200.2 min ($R^2 = 0.98$). To estimate the heart, liver and kidney exposure to tracer the area under the curve (AUC) was calculated by integration of TAC data from 0-240 min. For each tissue, the proportion (as %AUC_{tissue}) was determined ($\text{AUC}_{\text{total}} = \text{AUC}_{\text{blood}} + \text{AUC}_{\text{liver}} + \text{AUC}_{\text{kidney}}$). These data are summarized in **Table 3**. For vehicle, the highest %AUC_{tissue} was the heart ($51 \pm 2.1\%$), followed by the liver ($28 \pm 0.5\%$) and kidney ($22 \pm 1.9\%$). For the CA32-LC, the tissue with the highest tracer exposure was liver (67%), followed by heart (21%) and kidney (12%). Similarly, CA16-LC showed 67%, 25%, and 8% for liver, heart and kidney, respectively. For each CA16, CA8, and CA4, the AUC proportions were similar, with the highest being liver (45-48%), followed with heart (34-36%), and kidney (18-20%).

Dual-isotope MST in tumor-bearing mice

All mice were first injected with equal doses of ^{125}I -scFv₄-SA, followed by NAGB doses ranging from 100 μg of CA32 to 13 μg of CA4 (~5.8 nmol; mol ratio CA to antibody ~2:1), and ^{111}In -BSD (see **Table 1, A** for exact amounts). All sizes of NAGB were effective at reducing ^{111}In -BSD in normal tissue compared to vehicle, as evidenced by biodistribution analysis at 24 h injection p.i. (**Figure 6**). ^{111}In -BSD activity in the blood was significantly less in all CA groups (0.23-0.35 pmol/g) compared with vehicle (4.34 pmol/g) (all $P < 0.005$). **Figure 6, A** shows the absolute tumor concentrations of both radiotracers (as pmol/g).

Compared to vehicle (208.8 pmol/g), ^{111}In -BSD in tumor was similar for the CA32-LC group, but reduced ($P < 0.05$) for all other groups given CA (106.2, and 60.7, and 77.7 pmol/g, for CA16, CA8, and CA4, respectively). Interestingly, intra-tumoral ^{125}I -scFv₄-SA in NAGB groups were similar to vehicle (227.6 pmol/g) with the exception of CA16 (168.3 pmol/g), which showed a small decrease ($P = 0.0037$).

Analysis of the ratio of uptake of ^{111}In -BSD to ^{125}I -scFv₄-SA (**Figure 6, B**) in select tissues reveals that all CA significantly reduce the binding of the antibody to ^{111}In -BSD. For blood and liver, the decrease in $^{111}\text{In}/^{125}\text{I}$ ratio compared with vehicle was similar for all CA (approximately 5-fold for blood and 20-fold for liver). In the tumor, both CA32-LC and CA16 showed mean ratios ranging from 0.56-0.65, but CA8 (0.32) and CA4 (0.46) were lower. These data along with tumor concentrations of ^{125}I -scFv₄-SA similar to vehicle ($P>0.05$), suggest CA8 and CA4 can occupy tumor-associated antibody and reduce absolute tumor uptake of subsequently administered ^{111}In -BSD. CA8 and CA4 also showed differences in spleen and kidney ratios compared to the larger sized CA: the $^{111}\text{In}/^{125}\text{I}$ ratio in spleen was lower for both CA16 and CA32-LC groups, while the ratio in kidney was higher for both CA8 and CA4 groups. These data suggest that the antibody is less accessible to biotin while being metabolized in the RES and that the absolute kidney uptake can be reduced by choosing a smaller CA that can occupy SA subunits at the expense of reduced uptake at the tumor.

MST comparison of NAGB analogs containing an extended linker between the sugar core and biotin (CA16-LC vs. CA16)

All mice were first injected with ^{131}I -scFv₄-SA, followed 24 h later with 100 μg of either CA16-LC or CA16, and ^{111}In BSD (see **Table 1, B**). Biodistribution of ^{131}I -scFv₄-SA and ^{111}In -BSD in blood, tumor, liver, spleen, and kidney (**Figure 7**) showed that the blood and kidney uptake of both tracers were significantly less (all $P<0.05$) for CA16-LC compared with CA16, but similar for the other tissues. Data for ^{111}In -BSD (as %ID/g) in all excised organs are provided in **Table 6**. The $^{111}\text{In}/^{131}\text{I}$ ratios are similar between groups, suggesting that the tissue radioactivity is reduced by more efficient complexation and removal of circulating antibody in the blood or kidney by CA16-LC. This is in contrast to what was observed with the smaller NAGB (i.e. CA8 and CA4), which showed similar ^{131}I -scFv₄-SA concentrations to vehicle and a decrease in the $^{111}\text{In}/^{131}\text{I}$ ratio.

Comparison of NAGB and DEXB during MST

All mice were first injected with an equal dose of ^{131}I -scFv₄-SA, followed 24 h later with 100 μg of CA32-LC or DEXB, and an equal dose of ^{111}In BSD (see **Table 1, C**).

The biodistribution data for both tracers in select organs 24 h p.i. of ^{111}In BSD is shown in **Figure 8**, and tissue concentrations of ^{111}In -BSD (as %ID/g) in all excised organs are provided in **Table 7**. Concentrations of both ^{125}I -antibody (16.5-30 pmol/g) and ^{111}In -BSD (0.09-0.17 pmol/g) were similar in blood for CA32-LC and DEXB. The tumor uptake of ^{125}I -scFv₄-SA (272-282 pmol/g) was also similar, but ^{111}In -BSD was reduced in mice given DEXB, suggesting SA was occupied with CA, or biotinylated-metabolism products thereof (22 and 53 pmol/g for DEXB and CA32-LC, respectively; $P<0.005$). This trend was also observed in kidney: the ^{125}I activities were similar (54-59 pmol/g), but ^{111}In -BSD (13 pmol/g) was reduced by ~50% in the DEXB group (6 pmol/g). Spleen activity was 2-fold higher ($P<0.005$) for DEXB (184 pmol/g), suggesting greater RES metabolism for DEXB-antibody complexes compared with CA32-LC (82 pmol/g).

***In vivo* SPECT/CT imaging of ^{111}In -BSD**

Representative maximum-intensity SPECT images fused with CT topograms for mice administered antibody, followed by CA16, CA8, CA4, or DEXB, and ^{111}In -BSD are shown in **Figure 9**. In all four animals, the s.c. LS174T xenografts present in the flank can be imaged with sufficient contrast at 18-24 h p.i. of ^{111}In -BSD. In addition, the high amount of activity concentrated in the kidneys compared to blood and other organs can also be clearly visualized.

DISCUSSION

Recently we developed a procedure to allow the facile preparation of highly-pure CA16, as well as potentially other NAG analogs for future MST applications. By making the strategy highly modular, various parameters including the number of antibody ligands (e.g. biotin for antibody-SA); linker length connecting the ligand to the sugar/dendron core; and the sugar density and type can be varied. This is important when designing an ideal CA, since both the antibody-binding and sugar portions can have significant impact on CA-effectiveness. The CA should efficiently and rapidly complex any antibody in circulation, but have limited diffusion from the vascular compartment as to not interfere with radionuclide binding at antibody-tumor sites, and/or re-direct or prolong the antibody in circulation. Furthermore, it is essential that antibody-associated radioactivity is minimized by promoting turnover in normal tissue, especially the blood (marrow), liver, spleen, and kidney.

Specifically targeting hepatocyte ASGPr as a mechanism to remove circulating antibody constructs is theoretically appealing, as parenchymal cell metabolism does not directly result in biliary clearance of a binding-competant antibody, leading to prolonged retention in the RES. The ASGPr receptor is specific for ligands containing terminal clustered galactosyl or NAG with three or more residues in close spatial proximity being requisite for maximal binding efficiency.²²⁻²⁴ The biology of ASGPr is known to be complex, as the kinetics of recognition and uptake has been reported to be dependent on factors such as sugar type, spatial arrangement, and overall CA size^{4, 5, 15, 25} yet, the functionality, constitutive turnover, capacity, and carbohydrate specificity of this receptor make it an apt mechanism to exploit for the aforementioned purpose.

Our goal was to investigate a library of NAGB of various sizes in the context of a familiar biotin-SA system of MST. This system, while promising for clinical application to hematologic malignancies, was found to be functional, but immunogenic in carcinoma cancer patients, precluding repeat treatments. This approach has many unique controllable features that will be challenging to extrapolate to other MST systems. However, the clear functionality of a variety of CA in this study make them a fundamentally useful class of pharmacophores to investigate as MST-reagents for alternative binding pairs, such as antibody-hapten pairs, with the ultimate goal of producing tumor targeting of radiation with high specificity and the potential for multiple courses of therapy.

The effectiveness of RIT depends on the combination of delivery of therapeutic levels of radiation absorbed dose to tumor, and the therapeutic index between tumor and normal tissues. RIT effectiveness benefits from high uptake and retention in tumor, sufficient to

deliver doses to solid tumors in the range of 70-100 Gy. Toxicity of RIT (including MST) has been predominantly of 3 types: (1) hematopoietic, due to the exposure of bone marrow from blood borne reagent as it targets to tumor, typically in the range of 120 Gy for grade IV toxicity; (2) renal, due radiation to sensitive components of the kidney as a result of excretion of small ligands through the kidney, typically in the range of >15 Gy, and (3) antigen specific, due to cross reacting antigen in normal tissues. Animal models are best suited to the study of hematopoietic and renal toxicities, and optimal therapeutic indices for these tissues would be in the range of 70-100 and 10 for hematopoietic and renal tissues, respectively. Numerous studies have shown that these benchmarks can be attained with MST, especially strategies involving a CA step.

During preclinical investigation of a potential CA, the study of antibody clearance from circulation following treatment with CA in normal mice is normally included. Next, it is important to study the CA in a MST-context in tumor bearing mice. This can be explained by the fact that early human serum albumin-galactose (HSA-gal) CA was eventually replaced with CA16. HSA-gal was shown to be quite effective at reducing circulating antibody-SA,⁶ but reduced radiotracer uptake by the tumor was demonstrated to be dependent on sugar and biotin stoichiometry, presumably due to RES degradation and release of free-biotin from the CA. During a more recent study, Wilbur et al. showed in normal mice that a bis-biotin trigalactoside CA was more effective than CA16 at removing a bispecific fusion anti-CD20-SA antibody from the blood, but an improvement of the tumor-targeting index was not seen during MST of ¹¹¹In-BSD in analogous animal models.²⁰

We first used PET imaging to examine the complexation and uptake of radiolabeled scFv₄-SA following administration of various NAGB. During a second set of experiments, all NAGB, as well as a DEXB for comparison, were evaluated in tumor-bearing mice for their effectiveness to reduce normal tissue uptake of the antibody, as well as impact on absolute tumor uptake of the subsequently administered radiolabeled hapten.

For PET studies, normal mice were given ¹²⁴I-scFv₄-SA (0.58 nmol) followed by 50-100 µg of each NAGB. A convenient dose of each CA was chosen to achieve significant (i.e. >10-fold) molar excess of ¹²⁴I-scFv₄-SA for preliminary evaluation of functionality *in vivo*. The study aim was not to directly compare CA in this context, since factors must be taken into account including the clearance kinetics of the CA; CA binding affinity to antibody; and ASGPr recognition of CA-antibody complex. Nonetheless, doses of CA4 and CA8 were equivalent with regards to mol of biotin (50 and 100 µg, respectively), while doses of the larger CA (all 100 µg) were equivalent in mol of sugar. The difference in MW of CA16 and CA16-LC is 255 g/mol, thus 100 µg doses are similar (11.6 and 11.2 mol, respectively).

The blood ¹²⁴I activity was reduced in all NAGB groups (20-60% of max.) compared with vehicle (80%) within 45 min p.i of CA. By 240 min, the tracer in blood was reduced to 3 %ID/g; ~20% in all groups given CA, but remained high in vehicle (10 %ID/g or 60%). Liver activity was greater for all CA compared with vehicle, suggesting that all NAGB were effective at increasing uptake of the antibody by the tissue. CA32-LC and CA16-LC were shown be more efficient at removing circulating antibody and directing it to the liver as compared with CA without the extended linker (i.e. CA16-LC vs. CA16), as well as lower-

MW CA. This was also supported by the greater accumulation of tracer-activity in the thyroid for CA32-LC and CA16-LC, suggesting a higher abundance of low-MW iodine catabolites released into circulation.

MST experiments demonstrated that all NAGB were effective at removing antibody from the circulation, as evidenced by the reduction of both ^{111}In -BSD and ^{125}I -scFv₄-SA activity in the blood. CA4 and CA8 were observed to interfere with SA-biotin binding in both tumor and normal tissue compared with larger NAGB. Uptake of ^{111}In -BSD in the liver, kidney, spleen and lungs were reduced with all CA compared with vehicle, suggesting more rapid metabolism and excretion.

To examine the influence of increased spacer length on clearance, tumor-bearing mice were given equal doses of either CA16 or CA16-LC and ^{111}In -BSD following injection of equal doses of tracer-antibody. CA16-LC was more effective than CA16 at reducing both radiotracer concentrations in blood and kidneys, without any reduction in tumor uptake of ^{111}In -BSD. Also, ^{111}In -BSD uptake was reduced in the gut and intestines for CA16-LC, suggesting that the extended linker mediates more rapid ASGPr processing and biliary excretion of antibody-CA complexes.

Additional MST experiments included a direct comparison of CA32-LC and DEXB. The tumor and kidney uptake of ^{111}In -BSD were significantly reduced with DEXB compared to CA32-LC, suggesting that DEXB is catabolized into fragments and biotinylated metabolites are re-introduced into the circulation. This is also supported by significant reduction in tissue $^{111}\text{In}/^{125}\text{I}$ activity ratios for DEXB, although the ^{125}I -antibody activity in all organs was similar to levels seen for CA32-LC, with the exception of spleen.

MST with long-chain (LC) derivatives of CA8 and CA4 could be more effective. DEXB shows promise as a CA for circulating scFv₄-SA, but has limitations for MST with biotin-DOTA, as the tumor uptake of ^{111}In -BSD is significantly reduced compared to MST with CA32-LC. Additional investigation with DEXB may include adjusting the dose and timing of administration; decoration with ASGPr ligands to encourage hepatobiliary excretion; and/or reduced biotin substitution ratio to lead to fewer biotinylated metabolites.

All NAGB investigated were effective at rapidly removing freely-circulating antibody at doses containing equal biotin concentrations, and at CA:antibody ratios of approximately 2:1, similar to those reported in aforementioned preclinical studies. During dual-tracer MST experiments we observed a reduced biotin:antibody ratio at the tumor with NAGB <6000 MW (CA8 and CA4), suggesting that the smaller CA interferes with antibody capture of ^{111}In -BSD. Smaller CA constructs developed by NeoRx employed alternative synthetic strategies (increased steric interference proximal to the biotin) to attenuate this affinity.²¹ Our data was generated to compare directly analogs with a similar molecular presentation of the biotin moiety. The trend in reduced tumor:biotin ratio we observed is consistent with the proposed mechanism (tumor compromise), but the main focus of the current study was primarily to examine *in vivo* binding to scFv₄-SA, clearance kinetics via PET imaging, and biodistribution of both the scFv₄-SA and radionuclide-BSD.

Our studies indicate that incorporation of a longer spacer in the NAG-CA to separate ligand and sugar molecules can allow for more efficient removal of antibody from circulation and normal tissue without any reduction in subsequent ligand-antibody binding stoichiometry in the tumor. We conclude that CA32-LC is an optimum NAG-CA choice for future MST since it shows efficient and rapid complexation antibody in circulation, and has limited diffusion from the vascular compartment as to not interfere with radionuclide binding at antibody-tumor sites, and/or re-direct or prolong the antibody in the circulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A

Clearing Agent	<i>n</i>	<i>m</i>	MW
CA32-LC	32	2	17413
CA16-LC	16	2	8906
CA16	16	1	8651
CA8	8	1	4398
CA4	4	1	2271

B **C** **FIGURE 1.**

A representation of the NAGB, and exact molecular weights of all NAGB investigated (**A**), chemical structure of CA16 (**B**), synthetic scheme of reaction of amino-dextran with biotin-dPEG(4)-NHS to prepare DEXB (**C**).

FIGURE 2.

Representative ^{124}I -scFv $_4$ -SA (5.44-9.07 MBq) TAC of heart (A), and liver (B) and kidney (C) up to 24 h (1440 min) p.i. in mice treated with either: CA32-LC, CA16, CA8, CA4, or vehicle. Activities are expressed as the mean $\pm$ SD of the tissue ROI (as %ID/g) obtained from the PET images. Note that (A) is a semi-log plot, while (B) and (C) have a linear y-axis.

FIGURE 3.

^{124}I -TAC data up to 240 min from figure 2, re-plotted with each ROI-derived concentration normalized to the maximum value recorded during imaging from $t = 0$ -240 min (100%). The mean % of maximum activity versus time is shown.

FIGURE 4.

Pairwise PET imaging comparison of treatment with 100 µg of CA16-LC or CA16 following injection of ^{124}I -scFv $_4$ -SA (5.44-9.07 MBq). TAC data for heart (**A**), and liver (**B**) and kidney (**C**) accumulation up to 24 h (1440 min) p.i. is shown. Activities (as %ID/g) are plotted as the mean $\pm$ SD. Note that (**A**) is a semi-log plot, while (**B**) and (**C**) have a linear y-axis.

FIGURE 5. Normalized TAC curves with data plotted as the mean percentage of the maximum ROI observed for animals treated with CA16 or CA16-LC following injection of ^{124}I -antibody.

A **B** **FIGURE 6.**

The biodistributions of ^{125}I -scFv₄-SA and ^{111}In -BSD following MST in LS174T xenografted athymic nude mice ($n = 3-4$ mice/group). ^{125}I -labeled scFv₄-SA (500 μg , 2.8 nmol, 0.74 MBq) was injected i.v. [$t = 0$], dendron-CA (13-100 μg ; 2 mol ratio of CA to antibody) was injected at [$t = 24$ h], and ^{111}In -BSD (0.74 MBq, 1.04 nmol) was injected at [$t = 28$ h]. Mice were sacrificed at 24 h p.i. of ^{111}In -BSD [$t = 52$ h]. A comparison of ^{125}I -scFv₄-SA in tumor (A, left) and ^{111}In -BSD (A, right) for vehicle, CA32-LC, CA16, CA8, and CA4. The ratio of ^{111}In -BSD/ ^{125}I -scFv₄-SA in blood, tumor, and RES organs (B). Data (as pmol/g) is expressed as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.005$.

FIGURE 7.

The biodistributions of ^{131}I -scFv $_4$ -SA (A) and ^{111}In -BSD (B) at $t = 52$ h in select tissues following MST with ^{131}I -scFv $_4$ -SA and CA16 or CA16-LC in tumor-bearing mice. The ratio of ^{111}In -BSD/ ^{131}I -scFv $_4$ -SA in select tissues (C). Data (as pmol/g) is expressed as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.005$.

FIGURE 8.

The biodistribution of $^{125}\text{I-scFv}_4\text{-SA}$ (**A**) and $^{111}\text{In-BSD}$ (**B**) at $[t = 52 \text{ h}]$ in select tissues following MST of $^{111}\text{In-BSD}$ with $^{125}\text{I-scFv}_4\text{-SA}$ and CA32-LC or DEXB in tumor-bearing mice ($n = 4$ mice/group). The ratio of $^{111}\text{In-BSD}/^{125}\text{I-scFv}_4\text{-SA}$ in select tissues (**C**). Data (as pmol/g) is expressed as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.005$.

FIGURE 9.

Maximum-intensity SPECT/CT projection images 18-24 h p.i. of ^{111}In -BSD (40 MBq). Athymic nude mice bearing LS174T xenografts were given scFv₄-SA, CA (CA16, CA8, CA4, or DEXB), and ^{111}In -BSD.

TABLE 1

Overview of MST experiments and reagent details.

		CA	CA dose (μ g)	Sugar (nmol)	Biotin (nmol)
A	<i>MST comparison of NAGB^a</i>	CA32-LC	100	182	5.7
		CA16	50	92	5.8
		CA8	26	47	5.9
		CA4	13	23	5.7
B	<i>MST comparison of CA16 with CA16-LC^b</i>	CA16	100	185	11.6
		CA16-LC	100	180	11.2
C	<i>MST comparison of CA32-LC and DEXB^c</i>	CA32-LC	100	182	5.7
		DEXB	100		20
D	<i>SPECT/CT imaging of ¹¹¹In-BSD following MST^d</i>	CA16	100	185	11.6
		CA8	100	182	22.7
		CA4	6.5	11.4	2.9
		DEXB	100		20

^a All mice received 0.5 mg (2.8 nmol, 0.74 MBq) of ¹²⁵I-scFv4-SA and 0.74 MBq of ¹¹¹In BSD (1.04 nmol). The LS174T tumor size was 0.44 $\pm$ 0.36 g (mean $\pm$ SD).

^b All mice received 0.8 mg (4.5 nmol, 1.11 MBq) of ¹³¹I-scFv4-SA and 1.3 MBq of ¹¹¹In BSD (0.44 nmol). The LS174T tumor size was 1.05 $\pm$ 0.32 g (mean $\pm$ SD).

^c All mice received 0.8 mg (4.5 nmol, 0.74 MBq) of ¹²⁵I-scFv4-SA and 0.37 MBq of ¹¹¹In BSD (0.44 nmol). The LS174T tumor size was 1.07 $\pm$ 0.42 g (mean $\pm$ SD).

^d All mice received 0.5 mg (2.8 nmol) of scFv4-SA and 40 MBq of ¹¹¹In BSD (1.31 nmol). The LS174T tumor size was 1.27 $\pm$ 0.57 g (mean $\pm$ SD).

TABLE 2

Single-phase decay fit using the tissue $\text{ROI}_{\text{mean} \pm \text{sd}}$ from $t = 15.75\text{-}240$ min for heart and kidney.

	Heart		Kidney	
	$t_{1/2}$ (min)	R^2	$t_{1/2}$ (min)	R^2
vehicle *	66.0	0.98	151.0	0.94
CA32-LC	9.3	0.96	12.2	0.97
CA16-LC	10.2	0.96	10.0	0.90
CA16 **	5.9	0.87	47.0	0.95
CA8	4.5	0.84	22.2	0.87
CA4	58.4	0.99	200.2	0.98

* ($n = 3$) Heart $t_{1/2} = 59.4 \pm 14.1$ min; Kidney $t_{1/2} = 161.3 \pm 64.0$ min

** ($n = 3$) Heart $t_{1/2} = 9.9 \pm 5.0$ min; Kidney $t_{1/2} = 48.8 \pm 2.2$ min

TABLE 3

Percent AUC for heart, liver, and kidney from 0-240 min (as the mean $\pm$ SD).

	Heart	Liver	Kidney
vehicle	51 $\pm$ 2.1	28 $\pm$ 0.5	22 $\pm$ 1.9
CA32-LC	21	67	12
CA16-LC	25	67	8
CA16	34 $\pm$ 2.2	48 $\pm$ 3.1	18 $\pm$ 1.0
CA8	36	46	18
CA4	35	45	20

TABLE 4

^{111}In -BSD uptake 24 h p.i. following MST with ^{125}I -scFv4, various NAGB, and ^{111}In -BSD (1.04 nmol) in groups of tumor-bearing mice. The activity concentrations are expressed as %ID/g (mean $\pm$ SEM).

Organ	vehicle (n = 4)	CA32-LC (n = 3)	CA16 (n = 4)	CA8 (n = 4)	CA4 (n = 4)
Blood	0.62 $\pm$ 0.14	0.02 $\pm$ 0.01	0.03 $\pm$ 0.01	0.03 $\pm$ 0.01	0.02 $\pm$ 0.00
Tumor	18.62 $\pm$ 3.95	14.53 $\pm$ 3.47	10.58 $\pm$ 1.17	6.05 $\pm$ 1.29	7.74 $\pm$ 1.67
Heart	0.90 $\pm$ 0.23	0.08 $\pm$ 0.03	0.07 $\pm$ 0.01	0.06 $\pm$ 0.01	0.04 $\pm$ 0.01
Lungs	0.63 $\pm$ 0.13	0.19 $\pm$ 0.03	0.14 $\pm$ 0.02	0.15 $\pm$ 0.03	0.09 $\pm$ 0.01
Liver	3.18 $\pm$ 0.76	0.20 $\pm$ 0.03	0.31 $\pm$ 0.05	0.31 $\pm$ 0.04	0.23 $\pm$ 0.05
Spleen	1.43 $\pm$ 0.27	0.08 $\pm$ 0.01	0.08 $\pm$ 0.01	0.11 $\pm$ 0.02	0.07 $\pm$ 0.02
Stomach	0.73 $\pm$ 0.32	0.10 $\pm$ 0.05	0.15 $\pm$ 0.03	0.08 $\pm$ 0.02	0.73 $\pm$ 0.60
Small int.	0.87 $\pm$ 0.27	0.14 $\pm$ 0.05	0.24 $\pm$ 0.09	0.30 $\pm$ 0.14	0.78 $\pm$ 0.20
Large int.	1.12 $\pm$ 0.75	0.90 $\pm$ 0.37	0.88 $\pm$ 0.61	0.54 $\pm$ 0.20	0.21 $\pm$ 0.07
Kidneys	5.01 $\pm$ 1.44	1.81 $\pm$ 0.22	1.45 $\pm$ 0.20	1.70 $\pm$ 0.28	1.09 $\pm$ 0.21
Muscle	0.34 $\pm$ 0.09	0.04 $\pm$ 0.01	0.03 $\pm$ 0.01	0.04 $\pm$ 0.01	0.04 $\pm$ 0.01
Bone	0.44 $\pm$ 0.05	0.07 $\pm$ 0.02	0.04 $\pm$ 0.01	0.26 $\pm$ 0.19	0.05 $\pm$ 0.02
<i>Tumor-to-tissue ratios</i>					
Blood	29.8 $\pm$ 9.3	639.8 $\pm$ 317.7	381.2 $\pm$ 100.6	174.3 $\pm$ 71.7	337.7 $\pm$ 103.5
Heart	20.7 $\pm$ 6.9	171.9 $\pm$ 66.0	151.5 $\pm$ 21.0	101.2 $\pm$ 32.3	172.3 $\pm$ 42.7
Lungs	29.6 $\pm$ 8.8	76.8 $\pm$ 23.1	77.8 $\pm$ 16.3	41.1 $\pm$ 11.4	86.1 $\pm$ 22.3
Liver	5.9 $\pm$ 1.9	73.4 $\pm$ 21.8	34.5 $\pm$ 6.4	19.5 $\pm$ 4.8	33.2 $\pm$ 10.1
Spleen	13.0 $\pm$ 3.7	173.6 $\pm$ 48.1	137.1 $\pm$ 26.8	56.9 $\pm$ 17.0	103.9 $\pm$ 37.8
Stomach	25.5 $\pm$ 12.5	142.7 $\pm$ 82.1	71.8 $\pm$ 15.0	75.0 $\pm$ 24.2	10.7 $\pm$ 9.2
Small int.	21.4 $\pm$ 8.0	105.6 $\pm$ 43.2	44.2 $\pm$ 17.7	20.4 $\pm$ 10.4	10.0 $\pm$ 3.3
Large int.	16.7 $\pm$ 11.7	16.2 $\pm$ 7.8	12.0 $\pm$ 8.4	11.2 $\pm$ 4.7	37.4 $\pm$ 15.4
Kidneys	3.7 $\pm$ 1.3	8.0 $\pm$ 2.1	7.3 $\pm$ 1.3	3.6 $\pm$ 1.0	7.1 $\pm$ 2.0
Muscle	55.5 $\pm$ 19.5	371.1 $\pm$ 108.1	389.5 $\pm$ 88.5	147.9 $\pm$ 44.6	190.2 $\pm$ 56.6
Bone	42.7 $\pm$ 10.4	219.2 $\pm$ 75.8	238.3 $\pm$ 49.7	27.3 $\pm$ 18.6	148.9 $\pm$ 54.9

TABLE 5

Statistical comparison of select data from ^{111}In -BSD biodistribution (table 4).

Organ	CA32 (n = 3)	CA16 (n = 4)	CA8 (n = 4)	CA4 (n = 4)
Blood	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.00
Tumor	14.53 ± 3.47	10.58 ± 1.17	$6.05 \pm 1.29^{a,b}$	7.74 ± 1.67
Liver	0.20 ± 0.03	0.31 ± 0.05	0.31 ± 0.04^c	0.23 ± 0.05
Spleen	0.08 ± 0.01	0.08 ± 0.01	0.11 ± 0.02	0.07 ± 0.02
Kidneys	1.81 ± 0.22	1.45 ± 0.20	1.70 ± 0.28	1.09 ± 0.21^d
<i>Tumor-to-tissue ratios</i>				
Blood	639.8 ± 317.7	381.2 ± 100.6	174.3 ± 71.7	337.7 ± 103.5
Liver	73.4 ± 21.8	34.5 ± 6.4	19.5 ± 4.8	33.2 ± 10.1
Spleen	173.6 ± 48.1	137.1 ± 26.8	56.9 ± 17.0	103.9 ± 37.8
Kidneys	8.0 ± 2.1	7.3 ± 1.3	3.6 ± 1.0	7.1 ± 2.0

No significant differences between CA32 and CA16

No significant differences between CA16 and CA4

^a $P = 0.0205$ compared with CA16

^b $P = 0.0245$ compared with CA32

^c $P = 0.0417$ compared with CA32

^d $P = 0.0325$ compared with CA32

TABLE 6

^{111}In -BSD uptake 24 h p.i. following MST with CA16 or CA16-LC as the CA. The dose of ^{111}In -BSD was 0.44 nmol. The activity concentrations are expressed as %ID/g (mean $\pm$ SEM).

Organ	CA16 (n = 4)	CA16-LC (n = 4)
Blood	1.17 $\pm$ 0.29	0.31 $\pm$ 0.03 ^a
Tumor	12.54 $\pm$ 1.40	15.83 $\pm$ 1.35
Heart	0.94 $\pm$ 0.12	0.76 $\pm$ 0.07
Lungs	1.36 $\pm$ 0.15	0.90 $\pm$ 0.13 ^b
Liver	7.25 $\pm$ 0.18	6.78 $\pm$ 0.55
Spleen	3.21 $\pm$ 0.47	2.47 $\pm$ 0.29
Stomach	8.68 $\pm$ 2.30	2.69 $\pm$ 0.34 ^c
Small int.	1.24 $\pm$ 0.31	0.46 $\pm$ 0.04 ^d
Large int.	2.48 $\pm$ 0.23	1.15 $\pm$ 0.09 ^e
Kidneys	4.95 $\pm$ 0.38	3.74 $\pm$ 0.37 ^f
Muscle	0.65 $\pm$ 0.17	0.35 $\pm$ 0.05
Bone	0.70 $\pm$ 0.24	0.34 $\pm$ 0.08
<i>Tumor-to-tissue ratios</i>		
Blood	10.7 $\pm$ 2.9	51.8 $\pm$ 6.8
Heart	13.3 $\pm$ 2.2	20.8 $\pm$ 2.6
Lungs	9.2 $\pm$ 1.4	15.8 $\pm$ 2.7
Liver	1.7 $\pm$ 0.2	12.5 $\pm$ 1.5
Spleen	3.9 $\pm$ 0.7	1.8 $\pm$ 0.3
Stomach	1.4 $\pm$ 0.4	4.9 $\pm$ 0.7
Small int.	10.1 $\pm$ 2.7	4.5 $\pm$ 0.6
Lg. int.	5.1 $\pm$ 0.7	26.0 $\pm$ 3.0
Kidneys	2.5 $\pm$ 0.3	9.5 $\pm$ 1.2
Muscle	19.3 $\pm$ 5.4	2.7 $\pm$ 0.5
Bone	17.9 $\pm$ 6.4	41.1 $\pm$ 10.4

^a $P = 0.013$

^b $P = 0.031$

^c $P = 0.021$

^d $P = 0.023$

^e $P = 0.00084$

^f $P = 0.031$

TABLE 7

^{111}In -BSD uptake 24 h p.i. following MST with either CA32-LC or DEXB. The dose of ^{111}In -BSD was 0.44 nmol. The activity concentrations are expressed as %ID/g (mean $\pm$ SEM).

Organ	CA32-LC (n = 4)	DEXB (n = 3)
Blood	0.04 $\pm$ 0.01	0.02 $\pm$ 0.01
Tumor	12.07 $\pm$ 0.29	5.03 $\pm$ 0.95 ^a
Heart	0.06 $\pm$ 0.00	0.03 $\pm$ 0.00 ^b
Lungs	0.16 $\pm$ 0.01	0.09 $\pm$ 0.03 ^c
Liver	0.47 $\pm$ 0.02	0.11 $\pm$ 0.02 ^d
Spleen	0.12 $\pm$ 0.00	0.09 $\pm$ 0.01 ^e
Stomach	0.62 $\pm$ 0.43	1.55 $\pm$ 1.44
Small int.	0.26 $\pm$ 0.17	0.37 $\pm$ 0.26
Large int.	2.11 $\pm$ 1.40	0.49 $\pm$ 0.13
Kidneys	3.05 $\pm$ 0.08	1.37 $\pm$ 0.13 ^f
Muscle	0.04 $\pm$ 0.00	0.03 $\pm$ 0.00 ^g
Bone	0.08 $\pm$ 0.00	0.07 $\pm$ 0.01
<i>Tumor-to-tissue ratios</i>		
Blood	319.7 $\pm$ 112.3	243.5 $\pm$ 85.1
Heart	203.3 $\pm$ 9.5	161.8 $\pm$ 37.3
Lungs	75.7 $\pm$ 5.5	56.9 $\pm$ 21.6
Liver	25.8 $\pm$ 1.3	44.1 $\pm$ 11.8
Spleen	102.1 $\pm$ 3.7	58.2 $\pm$ 13.6
Stomach	19.4 $\pm$ 13.3	3.2 $\pm$ 3.1
Small int.	47.2 $\pm$ 32.0	13.7 $\pm$ 10.1
Lg. int.	5.7 $\pm$ 3.8	10.2 $\pm$ 3.4
Kidneys	4.0 $\pm$ 0.1	3.7 $\pm$ 0.8
Muscle	308.7 $\pm$ 26.8	181.0 $\pm$ 37.6
Bone	152.5 $\pm$ 6.1	68.1 $\pm$ 14.0

^a $P = 0.0002$

^b $P = 0.0007$

^c $P = 0.0252$

^d $P = 5\text{E-}05$

^e $P = 0.0158$

^f $P = 4\text{E-}05$

^g $P = 0.0248$