SUPPORTING INFORMATION

Cucurbituril - ferrocene: host - guest based pretargeted positron emission tomography in a xenograft model

Vilma IJ Jallinoja [1,2], Brandon D Carney [1,2], Meiying Zhu [1], Kavita Bhatt [2], Paul J Yazaki [3], Jacob L Houghton [1,2 *]

1 Department of Radiology and Radiological Sciences, Vanderbilt University Medical Center, Nashville, Tennessee, 37232, USA.

2 Department of Radiology, Stony Brook University, Stony Brook, New York, 11774, USA.

3 Beckman Institute, City of Hope, Duarte, California 91010, USA.

*Corresponding author:

Jacob Houghton, PhD

Stony Brook University Cancer Center

100 Nicolls Rd

Stony Brook, NY 11774

Tel: 6153226215

Fax: 6314447538

Email: jacob.houghton@stonybrookmedicine.edu

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MATERIALS

Instruments

The HPLC purification of the compound **2** was done on a preparative Kinetex 5 μm EVO C18 100Å column, 150 × 21.2 mm (Phenomenex). The HPLC purification of the compounds **3** and **4** were performed on a semipreparative SunFire C18 column, 5 μm, 10 × 150 mm (Waters). The quality control of compounds **2**, **3, 5** and **6** were performed with an analytical Kinetex 5 μm EVO C18 100 Å column, 150 × 4.6 mm (Phenomenex). The quality control of the compound **4** was performed with an analytical Luna 5 μm C18(2) 100 Å, 250 x 4.6 mm column. The HPLC instruments used for the purification of the compounds **3** and **4** and the quality control of the compound **4** included Hitachi Primaide 1110 Pump, Hitachi Primaide 1410 UV detector and Eckert & Ziegler Flow-Count model 106 radiodetector. The HPLC instrument used for the purification of the compound **2** included Gilson 159 UV-VIS detector and Gilson 322 Pump. The quality control of the compounds **2**, **3**, **5** and **6** were performed on an Agilent HPLC 1260 Infinity II LC System including 1260 Quat Pump VL, 1260 DAD WR and LabLogic Flow-RAM radio-HPLC Detector equipped with a LabLogic Systems Limited NaI Detector.

The radio-TLC plates were analyzed with Bioscan AR-2000 radio-TLC imaging scanner. The activity measurements of the injected radioligand doses were performed with Capintec, Inc. CRC-25W dose calibrator. The mass and activity of biodistribution, blood half-life and log D experiment samples were measured with Hidex Automatic Gamma Counter. All antibody concentration measurements were done with Thermo Scientific Nanodrop One.

The mass spectrometers used for the high-resolution mass spectroscopy were Thermo LTQ Orbitrap and Q-Exactive HF. The proton nuclear magnetic spectroscopy was done using a 400 MHz instrument equipped with an 9.4 Tesla Oxford magnet and Bruker AV-400 console and a 500 MHz AvanceIII Bruker instrument equipped with an 11.7 Tesla magnet.

Reagents

Unless otherwise mentioned, all of the reagents were purchased from Sigma Aldrich (St. Louis, MO). The M5A antibody came from City of Hope Medical Center and CB7-azide was prepared by the Chemical Synthesis Core, Vanderbilt University. The NHS-Fluorescein (5/6-carboxyfluorescein succinimidyl ester) was purchased from Thermo Fisher Scientific. The DBCO-C6-NHS ester and t-Boc-N-amido-PEG3-amine were purchased from BroadPharm (San Diego, CA). p-SCN-Bn-NOTA was obtained from Macrocyclics, Inc. (Dallas, TX). $[^{68}Ga]GaCl_3$ in 0.05 M HCl solution was purchased from the Vanderbilt University Institute of Imaging Science Research Radiochemistry Core Laboratory. $[^{67}Ga]GaC_6H_5O_7$ (gallium citrate) was purchased from Jubilant Radiopharma, USA. $[^{89}Zr]Zr(C_2O_4)_2$ was purchased from Washington University School of Medicine MIR Cyclotron Facility. The cell culturing medium was obtained from the Vanderbilt University Medical Center (VUMC) Molecular Cell Biology Resource Core and Fisher Scientific.

METHODS

Synthesis of NBOC-PEG_3-NH-Fc (2)

Ferrocenecarboxaldehyde (112 mg, 0.523 mmol) was dissolved in 15 mL of methanol in a round bottom glass flask. t-Boc-N-amido-PEG3-amine (42 mg, 0.144 mmol, 0.28 eq.) and triethylamine (7.26 mg, 0.072 mmol) were added to the reaction vial. The reaction solution was stirred for 120 minutes before the addition of sodium triacetoxyborohydride (1.114 g, 5.26 mmol). The reaction was stirred overnight and then the solvent was evaporated. Afterwards, 20 mL of dichloromethane was added to the reaction vial with 10 mL of water. The organic phase was collected and the water phase was washed with dichloromethane (2 x 15 mL). The dichloromethane phases were combined and dried using Na_2SO_4, and dichloromethane was evaporated off. The crude product was redissolved in 5 mL of DMSO : ACN : H_2O (1:1:1) solution. The undissolved material was filtered out and the supernatant was collected for HPLC purification. Synthesized NBOC-PEG_3-NH-Fc (**2**) was purified with HPLC using a preparative C18 column with a gradient of 10:90 (ACN : H_2O) to 95:5 (ACN : H_2O) in 15 minutes (t_r =5.6 min) with a flow rate of 20.0 mL/min. The product fraction was concentrated and yielded a yellow oil. The purity was analyzed with an analytical HPLC C18 column using a gradient 5:95 (0.1% in ACN : 0.1%TFA in H_2O) 95:5 (0.1% TFA in ACN : 0.1% TFA in H_2O) in 16 minutes with a flow rate of 1.0 mL/min and monitored at a wavelength of 254 nm (t_r =10.27 min, chemical purity: 99.5%)

1H NMR (500 MHz, methanol-d_4) δ; 4.314 (s, 2 H), 4.212 (s, 2 H), 4.141 (s, 5 H), 3.922 (s, 2 H), 3.649-3.479 (m, 10 H), 3.413 (t, 2 H), 3.134 (t, 2 H), 3.020 (s, 2 H), 1.364 (s, 9

H) HRMS (ESI)(+) m/z calculated for $C_{24}H_{38}FeN_2O_5$ $[M+H]^+$: 491.2208, measured: 491.2204.

Synthesis of NBOC-PEG$_3$-NMe$_2$-Fc (3)

Iodomethane (68.4 g, 0.482 mol) and triethylamine (7.26 mg, 0.072 mmol) were added to the reaction vial containing the NBOC-PEG$_3$-NH-Fc. The reaction solution was let to stir overnight at room temperature. The solvent was evaporated off. Synthesized NBOC-PEG$_3$-NMe$_2$-Fc (**3**) was purified with HPLC using a semipreparative C18 column using a gradient of 5:95 (0.1% TFA in ACN : 0.1% TFA in H_2O) to 50:50 (0.1% TFA in ACN : 0.1% TFA in H_2O) in 33 minutes to 95:5 (0.1% TFA in ACN : 0.1% TFA in H_2O) in 41 minutes (t_r=18.6 min) with a flow rate of 4.0 mL/min. The product fraction was concentrated and yielded a yellow oil. (30.1 mg, yield: 40.3 %). The purity was analyzed with an analytical HPLC C18 column using a gradient 5:95 (0.1% TFA in ACN : 0.1% TFA in H_2O) 95:5 (0.1% TFA in ACN : 0.1% TFA in H_2O) in 16 minutes with a flow rate of 1.0 mL/min and monitored at a wavelength of 254 nm (t_r =10.35 min, chemical purity: 96.94%)

1H NMR (500 MHz, methanol-d_4) δ; 4.456 (m, 2 H), 4.346 (s, 2 H), 4.193 (s, 5 H), 3.855 (s, 2 H), 3.617 (s, 4 H), 3.597-3.527 (m, 4 H), 3.430 (t, 2 H), 3.368 (t, 2 H), 3.144 (t, 2 H), 2.943 (s, 6 H), 2.851 (s, 1 H), 1.364 (s, 9 H), HRMS (ESI)(+) $C_{26}H_{43}FeN_2O_5^+$ $[M]^+$ calculated: 519.2516, measured: 519.2516.

Synthesis of NOTA-PEG$_3$-NMe$_2$-Fc (4)

NBOC-PEG$_3$-NMe$_2$-Fc (**3**) (28.1 mg, 0.054 mmol) was added to a vial containing dichloromethane (800 μl) and trifluoroacetic acid (149 mg, 1.3 mmol). The reaction was left to stir for 10 minutes at room temperature. The solvent mixture was evaporated. Following the deprotection, the reaction mixture was redissolved in 1 mL of DMSO. p-SCN-Bn-NOTA (2-S-(4-Isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid) (24.5 mg, 0.0392 mmol) and triethylamine (25.4 mg, 0.252 mmol) were added to the reaction solution. The reaction was stirred at room temperature for 90 minutes. The NOTA-PEG$_3$-NMe$_2$-Fc (**4**) was purified with HPLC with a C18 column with a gradient of 5:95 (ACN : 0.1% TFA in H_2O) to 50:50 (ACN : 0.1% TFA in H_2O) in 33 minutes and then to 95:5 (ACN : 0.1% TFA in H_2O) in 41 minutes (t_r=19.6 min) with a flow rate of 4.0 mL/min. The product fraction was concentrated and yielded a yellow oil. (18.8 mg, over all yield: 20.3%) The purity was analyzed with an analytical HPLC C18 column using a gradient 5:95 (ACN : 0.1% TFA in H_2O) to 35:65 (ACN : 0.1% TFA in H_2O) in 28 minutes to 95:5 (ACN : 0.1% TFA in H_2O) in 35 minutes with a flow rate of 1.0 mL/min (t_r =10.3 min).

1H NMR (400 MHz, methanol-d_4) δ 7.30 (d, 4 H), 4.53 (s, 4 H), 4.51 (s, 2 H), 4.42 (s, 5 H), 3.89 (s, 2 H), 3.77 (s, 2H), 3.67 (s, 12 H), 3.60-3.33 (m, 7 H), 3.28-3.06 (m, 8 H), 2.99 (s, 6 H), 2.94-2.49 (m, 4 H); HRMS (ESI)(+) $C_{41}H_{61}FeN_6O_9S^+$ $[M]^+$ calculated for: 869.3570, measured: 869.3559.

^{68}Ga-radiolabeling of NOTA-PEG$_3$-NMe$_2$-Fc (4)

[^{68}Ga]GaCl$_3$ was obtained by eluting the radionuclide from a ^{68}Ge/^{68}Ga generator (ITG) using 0.05 M HCl. 1mL of the [^{68}Ga]GaCl$_3$ eluate (199 - 821 MBq) in 0.05 M HCl was adjusted to pH 5 using 1M NH_4OAc. NOTA-PEG$_3$-NMe$_2$-Fc (15 μg, 0.017 μmol) in 6 μL of DMSO was then added to the ^{68}Ga solution. The reaction solution was incubated at room temperature for 10 minutes. The radiochemical yield was determined by radio-iTLC using 50 mM EDTA as the mobile phase. After the radiolabeling, the pH of the radiolabeling reaction solution was adjusted to pH 7 using phosphate-buffered solution (pH=7.4). The quality control was done on radio-iTLC with the same method as the labeling monitoring.

^{67}Ga-radiolabeling of NOTA-PEG$_3$-NMe$_2$-Fc (4)

The [^{67}Ga]GaC$_6$H$_5$O$_7$ was first converted to [^{67}Ga]GaCl$_3$ using a method previously reported.[1] Briefly, the [^{67}Ga]GaC$_6$H$_5$O$_7$ (59.2 – 155.4 MBq) solution was diluted using chelexed mQ water with 1/3 of its volume. The [^{67}Ga]GaC$_6$H$_5$O$_7$ solution was loaded onto a Sep-Pak Vac Silica cartridge 1cc/100 mg (Waters) which was previously prepared with 2 mL of chelexed mQ water. The silica cartridge was then washed with 5 mL of the chelexed mQ water. The [^{67}Ga]gallium was eluted in two 500-600 μL fractions as [^{67}Ga]GaCl$_3$ using chelexed 0.1 M HCl solution resulting in 78.0 ± 14.9% recovery yield.

The [^{67}Ga]GaCl$_3$ solution (26.27 – 92.87 ; 500-1000 μL) in 0.1 M HCl was adjusted to pH 5 using 1M NH_4OAc. NOTA-PEG$_3$-NMe$_2$-Fc (75-92 μg, 0.086-0.106 μmol) in 30 μL of DMSO was then added to the ^{67}Ga-solution. The reaction solution was incubated at room

temperature for 10 minutes. Radiochemical yield was determined with radio-HPLC using an analytical HPLC C18 column with a gradient 5:95 (0.1% in ACN : 0.1% TFA in H_2O) to 95:5 (0.1% in ACN : 0.1% TFA in H_2O) in 17 minutes with a flow rate of 1.0 mL/min. (t_R=11.02 min) The [^{67}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc was purified using a Sep-Pak C18 Plus Light Cartridge (Waters) by diluting the reaction solution first with 4 mL of mQ water, loading the solution to the cartridge, washing the cartridge with 7 mL of mQ water and finally eluting the product in five fractions of 200 μL of 100% ethanol. The quality control was performed using the same radio-HPLC method as for the labeling monitoring.

Synthesis of the adamantane-fluorescein (6)

1-Adamantanemethylamine (5.4 mg, 0.033 mmol) was dissolved in 500 μL MeOH. NHS-Fluorescein (15.5 mg, 0.033 mmol) dissolved in 1000 μL DMSO was added to the reaction vial. Finally triethylamine (3.1 mg, 0.031 mmol) was added. The reaction was left at room temperature for 60 minutes with stirring.

The compound adamantane-fluorescein (**6**) was purified with HPLC using a preparative C18 column with a 14 minute gradient of 10:90 (ACN : H_2O) to 95:5 (ACN : H_2O) (t_r =12.8 min) with a flow rate of 20.0 mL/min. The product fractions were combined and the solvent was evaporated overnight under air flow. The purity was analyzed with an analytical HPLC C18 column using a gradient 5:95 (0.1% TFA in ACN : 0.1% TFA in H_2O) 95:5 (0.1% TFA in ACN : 0.1% TFA in H_2O) in 17 minutes with a flow rate of 1.0 mL/min and monitored at the wavelength of 254 nm (t_r=14.05 min, chemical purity: 96.79%). The overall yield of the reaction was 8.7 mg (50.8%).

1H NMR (500 MHz, methanol-d_4) δ 8.34 (s, 1 H), 8.08 (d, 1 H), 7.23 (d, 1 H), 6.61 (d, 2 H), 6.59-6.55 (m, 2 H), 6.47 (d, 1 H), 6.45 (d, 1 H), 3.07 (s, 2 H), 1.92 (s, 3 H), 1.73-1.61 (q, 6 H), 1.55 (m, 6 H) HRMS (ESI)(+) $C_{32}H_{29}NO_6$ $[M+H]^+$ calculated for: 524.2073, measured: 524.2068

Distribution coefficient of [^{67}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc

Freshly synthesized [^{67}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc in ethanol (30 μL, 0.34-0.35 MBq) was measured to an Eppendorf tube. 700 μL of 1-octanol and 700 μL PBS (pH 7.4) buffer solution were added to the tube. The sample was agitated for 10 minutes at room temperature (700 rpm). Next the sample was centrifuged for 5 minutes at 1000 g. 200 μL of each of the phases were measured to pre-weighted Eppendorf tubes. Relative amount of activity in each sample was counted with a gamma counter and the volume of each sample was determined by measuring the mass and adjusting by the density of the solvents. The log D of the radioligand was determined to be -3.2 $\pm$ 0.1 (n=3).

***In vitro* stability of [^{67}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc**

Freshly synthesized [^{67}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc in ethanol (38 μL, 0.29 MBq) was added to an Eppendorf tube along with 120 μL of PBS (pH=7.4). Samples were prepared for four timepoints in triplicate. The samples were incubated at 37 °C and the % of intact radioligand was monitored at four timepoints (30, 70, 120 and 240 min) using radio-HPLC. The % of intact radioligand at each timepoint is presented in the **Table S1** and the graphical presentation of the data is presented in the **Figure S1**.

Table S1. The stability of [^{67}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc in PBS (pH 7.4) at 37 °C

% of intact radioligand at 30 min	96.2 ± 1.2
% of intact radioligand at 70 min	95.9 ± 2.5
% of intact radioligand at 120 min	91.2 ± 1.5
% of intact radioligand at 240 min	86.6 ± 0.7

Blood half-life of [^{67}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc

^{67}Ga-NOTA-PEG$_3$-NMe$_2$-Fc was synthesized as described earlier. Purified radioligand in 100% EtOH was diluted with PBS (pH 7.4) to result in 20% total volume of EtOH. 200 μL [^{67}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc doses for blood half-life studies were prepared from this solution (0.82-0.84 MBq/nmol; 4.22-4.33 MBq;). A cohort of healthy male nude mice (n=3) received the radioligand via the tail vein and blood was drawn at six timepoints (2, 5, 15, 30, 45, 60 min) post radioligand injection from the saphenous vein. The blood collection tubes were preweighted to determine the mass of the collected blood. Activity of each sample was measured with a gamma counter. The %ID/g values were presented as a function of time and a two-phase decay curved was plotted from the measurement data. (**Figure S2**) The y_0 was set to equal 50 %ID/g, because the total mass of the mouse blood pool was estimated to be 2 g. The blood half-life was calculated with weighted average with equation 1, using the half-life values of the fast and slow phases ($t_{1/2fast}$ and $t_{1/2slow}$) and their relative percentage ($\%_{fast}$ and $\%_{slow}$).

$$t_{1/2} = \frac{(t_{1/2\ slow} \times \%_{fast}) + (t_{1/2\ fast} \times \%_{slow})}{100} \quad (1)$$

The characterization of the CB7-azide

The CB7-azide was characterized with 1H NMR using 400 MHz instrument equipped with an 9.4 Tesla Oxford magnet. The 1H NMR spectrum of the CB7-azide and its chemical structure are presented in the **Figure S3**.

1H NMR (400 MHz, water-d_2) δ; 5.6975-5.5950 (m, 14 H), 5.4790-5.3210 (m, 14 H), 4.2724-4.0252 (m, 14 H), 3.5298 (t, 2 H), 2.2529 (t, 2 H), 1.8406 (s, 3 H), 1.6304 (t, 2 H), 1.2211 (t, 2 H).

Development and characterization of CB7-M5A

CB7 conjugation

3.0 mg (20 nmol) of M5A antibody from 5.0 mg/mL stock was buffer exchanged into 2 mL of PBS (pH 8.7) with a PD10 desalting column (GE Healthcare). DBCO-C6-NHS ester (0.017 mg, 0.039 μmol, 2 eq.) in DMSO (3.1 μL) was added to the antibody solution and the reaction was agitated at 300 rpm for 60 minutes at 37 °C. The DBCO conjugated M5A (DBCO-M5A) antibody was purified with a PD10 column using PBS (pH 7.4) for elution, which resulted in antibody recovery of 2.5 mg (yield: 83.0%). CB7-azide (0.11 mg, 0.083 μmol, 5 eq) in DMSO (18.8 μL) was added to eluted DBCO-M5A (2.5 mg) in 1 mL PBS (pH 7.4). The reaction solution was agitated at 300 rpm overnight at room temperature. The CB7-M5A was purified with a PD10 column using PBS (pH 7.4) for elution, which yielded 2.3 mg in of the modified antibody (overall recovery yield 77.0%).

Characterization of the modified antibody

The immunoreactive fraction of the CB7-M5A was determined via cellular binding assay (Lindmo assay)[2,3] using CEA expressing BxPC3 cell line.[4] Briefly, [^{67}Ga]Ga-NOTA-PEG_3-NMe_2-Fc (10.6 nmol, 4.29 MBq, 0.40 MBq/nmol) in 50 μL of 100% EtOH was mixed with CB7-M5A (3.33 nmol) in 1 mL of PBS (pH 7.4). The solution was incubated at room temperature for 5 minutes. This was followed by purification of the CB7-M5A bound [^{67}Ga]Ga-NOTA-PEG_3-NMe_2-Fc with a PD10 size exclusion column resulting in 28.0% radiochemical yield (1.2 MBq, 0.36 MBq/nmol). The radiochemical purity of the formed [^{67}Ga]Ga-NOTA-PEG_3-NMe_2-Fc-CB7-M5A was determined to be >99% with radio-TLC using iTLC-SG strips (Agilent Technologies) and 50 mM EDTA as the mobile phase. The antibody solution was diluted to 0.019 MBq/mL using 1% BSA in PBS (pH 7.4) solution. Increasing numbers of cells were incubated with a standard amount of radiolabeled antibody. After a 60 minute incubation period, the cells were washed with cold PBS (pH 7.4) and the relative amount of activity bound to the cells was determined with a gamma counter in counts. The total added activity to the bound activity of each sample is presented as function of normalized cell concentration (**Figure S4A**). Immunoreactivity of the cells in determined as the Y-intercept value which corresponds to the total activity to bound activity in a theoretical infinite number of cells. The immunoreactivity of the CB7-M5A was determined to be 95.7 ± 0.7% (n=3).

The number of CB7 moieties per M5A was determined by fluorescence spectroscopy as follows. 100 μg (0.67 nmol) of CB7-M5A was mixed with 50 equivalents of adamantane-fluorescein (compound **6**) ligand (17.5 μg, 33.3 nmol) in an Eppendorf tube. The solution

was left to incubate for 45 min at RT. The CB7-M5A bound compound 6 was purified with a Zeba Spin Desalting Column (7K MWCO, 0.5 mL). The absorbance of the collected eluate was measured at 280 nm and 495 nm with a UV-Vis Spectrophotometer (Thermo Scientific NanoDrop). (**Figure S5**) The number of adamantane-fluorescein molecules bound to the CB7-M5A structure was calculated with the equation 2.

$$\frac{Adma - FL}{CB7 - anti - CEA\ M5A} = \frac{\frac{Abs(495\ nm)}{\varepsilon_{fluorscein}}}{\frac{Abs(280\ nm) - (CF(fluorscein) * Abs(495\ nm)}{\varepsilon_{mAb}}} \qquad (2)$$

The correction factor (CF) for FL is 0.3 and its extinction coefficient ($\varepsilon_{fluorcein}$) is 68 000 $M^{-1}cm^{-1}$. It was assumed that each CB7 moiety was occupied by adamantane-fluorescein due to large excess of the ligand (50 eq.) used and due to the immediate measurement of the absorbance after the purification of the CB7-M5A bound adamantane-fluorescein.

Non-specific binding of the adamantane-fluorescein to the M5A was also measured as described earlier. Briefly, 100 μg (0.67 nmol) of M5A was mixed with 50 equivalents of adamantane-fluorescein (17.5 μg, 33.3 nmol) in an Eppendorf tube. The solution was left to incubate for 45 min at room temperature. The M5A was purified with a Zeba Spin Desalting Column. The absorbance of the collected eluate was measured with a UV-Vis Spectrophotometer. (**Table S2 and Figure S5**)

Table S2. The absorption at 280 nm and 495 nm for the purified M5A samples (DOL; degree of labeling)

	$A_{\lambda 280\ nm}$	$A_{\lambda 495\ nm}$	DOL
water	-0.00033±0.0038	-0.0093±0.0031	N/A
M5A + compound 6	1.0700±0.0174	-0.0303±0.0126	-0.0867±0.0309
CB7-M5A + compound 6	1.0360±0.0094	0.2407+0.0042	0.7733±0.0094

Development and characterization of DFO-M5A

DFO conjugation

M5A (5.0 mg, 33.3 nmol) in PBS (pH 7.4) was buffer exchanged to PBS (pH 8.4) using a PD10 desalting column. p-SCN-Bn-Deferoxamine in DMSO (12.4 μL, 0.124 mg, 164.7 nmol, 5 eq.) was added to the antibody solution (V_{total}=2 mL). The solution was incubated at 37 °C for 60 minutes. The modified antibody (DFO-M5A) was split and purified with two PD10 desalting columns using PBS (pH 7.4) for elution. The antibody solutions were combined and concentrated using an Amicon Ultra-4 centrifugal filter tube (50K cut off). The final amount of the modified antibody was measured to be 4.3 mg (recovery yield: 86.0%).

Characterization of the modified antibody

The immunoreactivity of the DFO-M5A was determined via Lindmo cellular binding assay[2,3] with BxPC3 cell line using a ^{89}Zr-radiolabeled DFO-M5A. ([^{89}Zr]Zr-DFO-M5A radiosynthesis described below) The immunoreactivity was determined to be 89.59 ± 2.07% (n=3). The Lindmo assay described briefly earlier and the assay data presented in the **Figure S4B**.

^{89}Zr-radiolabeling of DFO-M5A

62.53-144.3 MBq of [^{89}Zr]Zr(C_2O_4)$_2$ (20-30 μL) was pH adjusted to pH 7.4 using 1 M Na_2CO_3 and PBS (pH 7.4) (final volume of 200-500 μL). DFO-M5A (200-2000 μg, 1.33-13.3 nmol, 4.3 mg/mL) was added to the pH adjusted ^{89}Zr-solution. The solution was incubated at room temperature for 30-60 minutes. The labeling was monitored with radio-TLC using iTLC-SG strips with 50 mM EDTA as the mobile phase. The [^{89}Zr]Zr-DFO-M5A was purified using a PD10 desalting column and eluted with PBS (pH 7.4). The radiochemical purity was determined with radio-TLC using the same method as for labeling monitoring.

Cell lines and animal models

The human adenocarcinoma cell line, BxPC3 was purchased from ATCC. The cells were grown in RPMI-1640 medium with 0.3 g/l glutamine, 25 mM HEPES, 1% (vol/vol) Penicillin-Streptomycin and 10% (vol/vol) fetal bovine serum in a 37 °C environment containing 5% CO_2. The cells were extracted using 0.25% Trypsin-EDTA. Pretargeting *in vivo* studies were done using female athymic nude mice (Crl:NU(NCr)-Foxn1nu, The Jackson Laboratory) bearing BxPC3 xenografts. *In vivo* biodistribution and imaging studies of the directly radiolabeled M5A were done using male athymic nude mice (NU/J, Jackson Laboratory) bearing BxPC3 xenografts. The mice were xenografted subcutaneously on the right flank with 4x10^6 cells in 0.1 mL mixture of Matrigel Matrix : RPMI-1640 medium (1:1) per mouse. The tumors were allowed to grow to 100 mm^3 in size (8-9 weeks) before the mice were used in imaging or biodistribution studies. The *in*

vivo blood half-life of the [^{67}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc radioligand was studied in healthy male athymic nude mice (NU/J, Jackson Laboratory). All experiments involving laboratory animals were performed in accordance with either the Vanderbilt University Medical Center Institutional Animal Care and Use Committee or Institutional Animal Care and Use Committee at Stony Brook Medicine.

Biodistribution of [^{89}Zr]Zr-DFO-M5A

The study included two cohorts of subcutaneous BxPC3 tumor bearing (right flank) male nude mice (n=4). The experimental cohort was injected with 1 nmol; 1.85 MBq/nmol of [^{89}Zr]Zr-DFO-M5A and the blocking control group was injected with 2.33 nmol; 0.79 MBq/nmol of [^{89}Zr]Zr-DFO-M5A in PBS (pH 7.4) via the tail vein. The molar activity of the doses for the control cohort was adjusted to the desired antibody concentration using unmodified M5A. Both of the cohorts were sacrificed 72 h post radiotracer injection. The relevant organs were harvested and their mass and activity measured using an automated gamma counter to determine the %ID/g of each organ.

Table S3. Activity biodistribution data of male nude mice with subcutaneous BxPC3 xenografts. The mice were sacrificed 72 hours post [^{89}Zr]Zr-DFO-M5A injection.

	72 h; [^{89}Zr]Zr-DFO-M5A 1.85 MBq/nmol; 1 nmol (%ID/g)	**72 h; [^{89}Zr]Zr-DFO-M5A 0.79 MBq/nmol; 2.3 nmol (%ID/g) (blocking)**
blood	14.65 ± 1.43	15.43 ± 0.46
tumor	31.76 ± 5.81	18.63 ± 5.10
heart	4.65 ± 0.38	4.79 ± 0.40
lungs	5.06 ± 1.55	5.32 ±0.92
stomach	1.64 ± 0.14	1.38 ± 0.24
pancreas	1.85 ± 0.120	2.13 ± 0.14
spleen	5.18 ±1.50	5.00 ± 0.44
liver	6.65 ± 0.40	7.18 ± 3.78
s. intestine	1.07 ± 0.20	1.21 ± 0.15
l. intestine	0.93 ± 0.29	0.99 ±0.26
kidneys	3.52 ± 0.73	4.01 ± 0.79
muscle	1.53 ± 0.58	1.47 ± 0.48
bone	5.58 ± 1.05	4.55 ± 0.69

Table S4. Tumor to organ data of male nude mice with subcutaneous BxPC3 xenografts injected with [^{89}Zr]Zr-DFO-M5A. The mice were sacrificed 72 hours post radiotracer injection.

	72 h; [^{89}Zr]Zr-DFO-M5A 1.85 MBq/nmol; 1 nmol (%ID/g)	**72 h; [^{89}Zr]Zr-DFO-M5A 0.79 MBq/nmol; 2.3 nmol (%ID/g) (blocking)**
tumor / blood	2.2 ± 0.4	1.2 ± 0.3
tumor / heart	6.8 ± 1.4	3.9 ± 1.1
tumor / lungs	6.3 ± 2.2	3.5 ± 1.1
tumor / stomach	19.4 ± 3.9	13.5 ± 4.4
tumor / pancreas	17.1 ± 3.6	8.7 ± 2.5
tumor / spleen	6.1 ± 2.1	3.7 ± 1.1
tumor / liver	4.8 ± 0.9	2.6 ± 1.5
tumor / s. intestine	29.8 ± 7.9	15.4 ± 4.6
tumor / l. intestine	34.1 ± 12.4	18.9 ± 7.2
tumor / kidneys	9.0 ± 2.5	4.6 ± 1.6
tumor / muscle	20.8 ± 8.7	12.7 ± 5.4
tumor / bone	5.7 ± 1.5	4.1 ± 1.3

PET imaging studies of directly radiolabeled [^{89}Zr]Zr-DFO-M5A

The PET imaging study of the [^{89}Zr]Zr-DFO-M5A included one cohort of BxPC3 xenografted nude mice (n=4). The mice received the [^{89}Zr]Zr-DFO-M5A (7.4 MBq/nmol, 5.9-6.7 MBq) in PBS (pH 7.4, V=150 μL) via tail vein injection. The molar activity of the control cohort doses was adjusted to the desired antibody concentration using unmodified M5A. The mice were imaged 72 h post radiotracer injection with a static 15 min scan with the energy and time coincides windows of 350 – 650 keV and 3.438 ns. The imaging was performed on a small animal Siemens Inveon PET/CT. Data from all possible lines of response (LOR) were saved in the list mode raw data format. The raw data was then binned into 3D sinograms with a span of 3 and ring difference of 79. The images were

reconstructed into transaxial slices (128 x 128 x 159) with voxel sizes of 0.0815 x 0.0815 x 0.0796 cm^3, using the MAP algorithm with 16 subsets and 18 iterations at a beta value of 0.0043. The mice were anesthetized by inhalation of 2% isoflurane with 1.5 mL/min flow for approximately 5 minutes and the mice were kept under anesthesia during the imaging. The maximum intensity projection images of all four mice are presented in the **Figure S6.**

Biodistribution of the pretargeted [^{68}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc (1)

The experimental groups including three cohorts of BxPC3 tumor bearing nude mice (n=3-4 per group) were administrated CB7-M5A (150 μg, 1 nmol) in PBS via tail vein injection. 72 hours later the mice received [^{68}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc (6.29-7.88 MBq/nmol, 1.5 nmol, 9.44-11.8 MBq) in PBS via tail vein injection. Mice were sacrificed at 1, 2 or 4 hours after the radioligand injection. Mice that were sacrificed 4 hours post radioligand injection were anesthetized with isoflurane and imaged on a Siemens Inveon PET/CT at 2 hours and 4 hours post injection, prior to euthanizing. In addition, a fourth cohort of BxPC3 tumor bearing mice (n=3) received only the [^{68}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc radioligand dose (6.29-7.88 MBq/nmol, 1.5 nmol, 9.44-11.8 MBq). This control cohort was sacrificed 2 hours after the radioligand injection. The relevant organs were harvested and their mass and activity measured using an automated gamma counter to determine the %ID/g of each organ.

Table S5. Activity biodistribution data of female nude mice with subcutaneous BxPC3 xenografts. The mice were injected with either CB7-M5A and [^{68}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc with a 72 h interval time (pretargeted) or with only the [^{68}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc radioligand (non-pretargeted). The three cohorts of mice which received the pretargeted radioligand were sacrificed at different timepoints (1, 2 or 4 h). The mice in the control group were sacrificed 2 h post radioligand injection.

	CB7-M5A & [^{68}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc			**[^{68}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc**
	1 h (%ID/g)	**2 h (%ID/g)**	**4 h (%ID/g)**	**2 h (%ID/g)**
blood	1.3 ± 0.5	0.6 ± 0.2	0.3 ± 0.1	0.2 ± 0.0
tumor	2.4 ± 1.0	3.6 ± 0.7	2.1 ± 0.6	0.2 ± 0.1
heart	0.5 ± 0.2	0.3 ± 0.1	0.1 ± 0.0	0.0 ± 0.0
lungs	0.7 ± 0.2	0.4 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
stomach	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.1
pancreas	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0
spleen	0.5 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
liver	3.4 ± 1.4	1.3 ± 0.1	0.8 ± 0.2	1.0 ± 0.1
s. intestine	5.2 ± 2.9	4.2 ± 2.2	0.6 ± 0.3	1.7 ± 0.3
l. intestine	0.4 ± 0.2	8.7 ± 1.7	5.9 ± 3.6	10.8 ± 1.8
kidneys	4.3 ± 1.2	2.1 ± 0.5	1.4 ± 0.1	1.5 ± 0.2
muscle	0.5 ± 0.3	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0
bone	1.1 ± 1.0	0.8 ± 0.4	0.3 ± 0.0	0.2 ± 0.0

Table S6. Tumor to organ ratios of the BxPC3 tumor bearing nude mice at 1, 2 and 4 h post pretargeted [^{68}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc radioligand injection and 2 h post non-pretargeted [^{68}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc radioligand injection (control).

	CB7-M5A & [^{68}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc			**[^{68}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc**
	1 h (%ID/g)	**2 h (%ID/g)**	**4 h (%ID/g)**	**2 h (%ID/g)**
tumor / blood	1.9 ± 1.1	5.6 ± 2.0	7.5 ± 2.0	1.3 ± 0.4
tumor / heart	5.2 ± 2.7	12.5 ± 4.7	15.1 ± 4.0	5.7 ± 4.2
tumor / lungs	3.4 ± 1.7	9.0 ± 2.9	11.7 ± 2.9	2.0 ± 0.5
tumor / stomach	9.8 ± 4.4	23.9 ± 5.7	30.9 ± 14.8	1.2 ± 1.1
tumor / pancreas	13.7 ± 7.4	26.2 ± 18.1	25.7 ± 7.7	10.0 ± 10.2
tumor / spleen	5.3 ± 2.5	9.3 ± 2.8	8.3 ± 2.4	1.0 ± 0.3
tumor / liver	0.7 ± 0.4	2.7 ± 0.6	2.6 ± 0.8	0.2 ± 0.1
tumor / s. intestine	0.5 ± 0.3	0.9 ± 0.5	3.4 ± 1.7	0.1 ± 0.0
tumor / l. intestine	6.3 ± 4.0	0.4 ± 0.1	0.4 ± 0.2	0.0 ± 0.0
tumor / kidneys	0.6 ± 0.3	1.7 ± 0.5	1.6 ± 0.4	0.2 ± 0.0
tumor / muscle	4.5 ± 3.2	17.7 ± 12.4	12.0 ± 6.0	3.0 ± 1.2
tumor / bone	2.3 ± 2.3	4.6 ± 2.5	6.5 ± 1.9	1.3 ± 0.4

Pretargeted PET imaging studies

Mice were anesthetized by inhalation of 2% isoflurane with 2.5 mL/min flow for approximately 3 minutes and kept under anesthesia during the imaging. The imaging was performed on a small animal PET/CT scanner (Siemens Inveon PET/CT). The scanning was performed as a static scan for either 15 minutes (2 h timepoint) or 20 minutes (4 h timepoint, **Figure S7**), where the energy and time coincides windows were 350 – 650 keV and 3.438 ns. Data from all possible lines of response (LOR) were saved in the list mode raw data format. The raw data was then binned into 3D sinograms with a span of 3 and ring difference of 47. The images were reconstructed into transaxial slices (128 x 128 x

159) with voxel sizes of 0.0815 x 0.0815 x 0.0796 cm^3, using the MAP algorithm with 16 subsets and 18 iterations at a beta value of 0.047.

Statistical analysis

Statistical analysis for all of the biodistribution data was performed with 2-tailed unpaired t tests using GraphPad Prism (GraphPad Software, Inc.). A p-value between two groups of $p<0.05$ was considered significant.

REFERENCES

1. Scasnár, V., van Lier JE. (1993) The use of SEP-PAK SI catridges for the preparation of gallium chloride form the citrate solution. Eur. J. Nucl. Med. 20, 273.

2. Lindmo, T., Bunn Jr, P.A. (1986) Determination of the true immunoreactive fraction of monoclonal antibodies after radiolabeling. Method. enzymol. 121, 678-691. DOI: 10.1016/0076-6879(86)21067-8.

3. Lindmo, T., Boven, E., Cuttitta, F., Fedorko, J., Bunn Jr, P.A. (1984) Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. J. Immunol. Methods. 72, 77-89. DOI: 10.1016/0022-1759(84)90435-6.

4. Girgis, M.D., Olafsen, T., Kenanova, V., McCabe, K.E., Wu, A.M., Tomlinson, J.S. (2011) Targeting CEA in Pancreas Cancer Xenografts with a Mutated scFv-Fc Antibody Fragment. EJNMMI Res. 1. DOI: 10.1186/2191-219X-1-24.

FIGURES

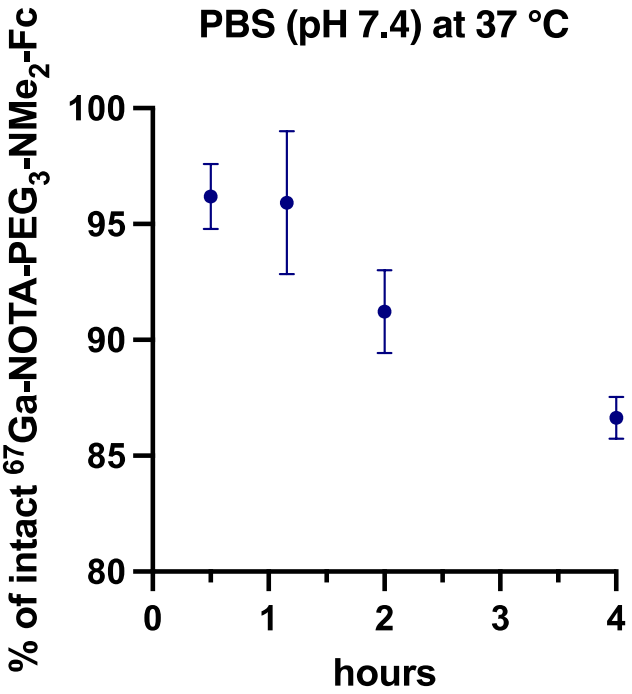

FIGURE S1. *In vitro* stability of the radioligand. % of intact [^{67}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc in PBS (pH 7.4) at 37 °C over a 4 hour monitoring period.

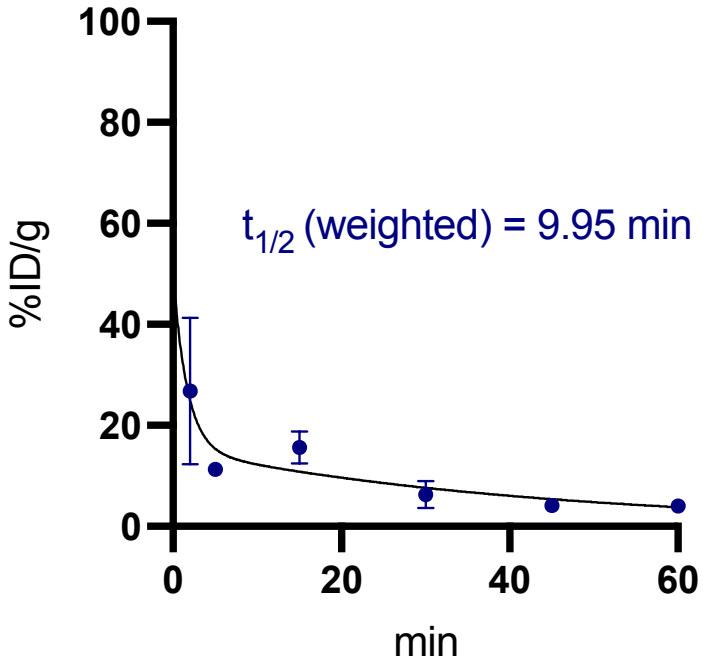

FIGURE S2. Blood half-life the radioligand. [^{67}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc was injected via tail vein in healthy male nude mice and blood was collected from the saphenous vein to study the presence of the radioligand in the blood pool. Based on the activity and the mass of the collected blood, %ID/g is presented as a function of time post radioligand injection.

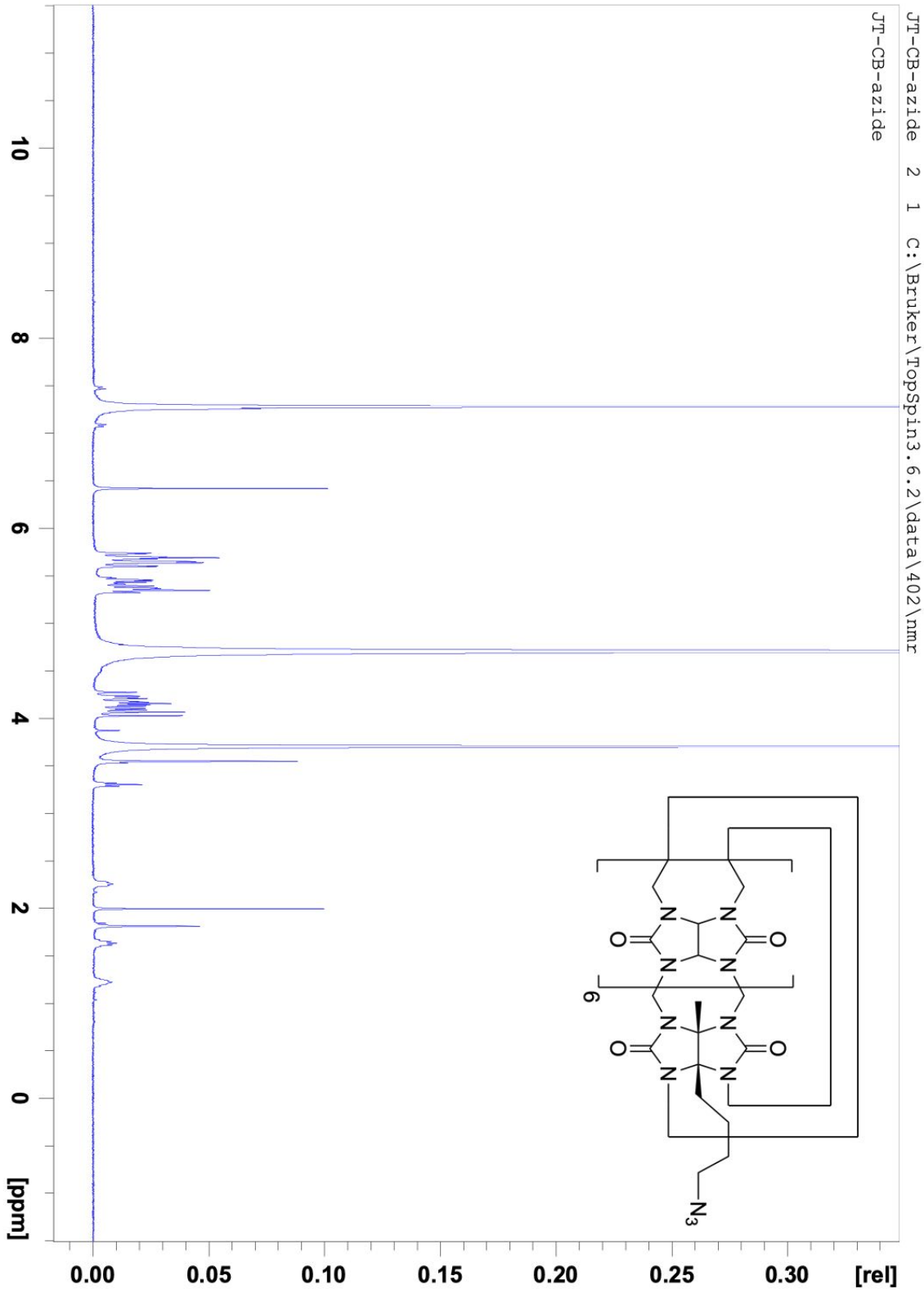

FIGURE S3. 1H-NMR spectrum of the purified CB7-azide precursor.

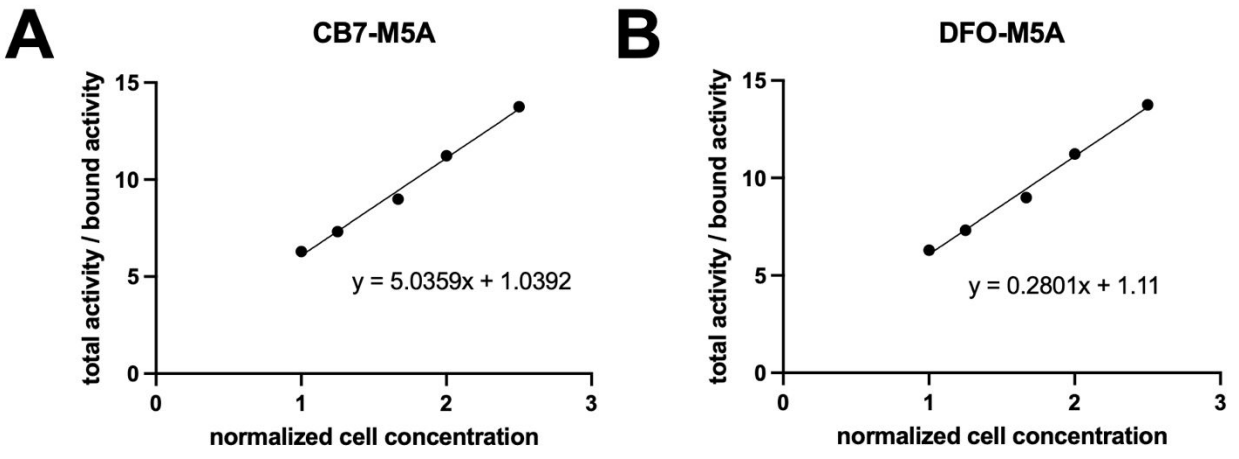

FIGURE S4. The determination of the immunoreactivity of the modified antibodies. The data from the cellular binding assay (Lindmo) is presented in total activity/bound activity as a function of normalized cell concentration. The y_0 value represents the total activity/bound activity at a theoretical infinite number of cells which allows the determination of the immunoreactive fraction of the modified antibody. **S4A** and **S4B** represent the result of Lindmo assays performed on CB7 and DFO modified M5A antibodies.

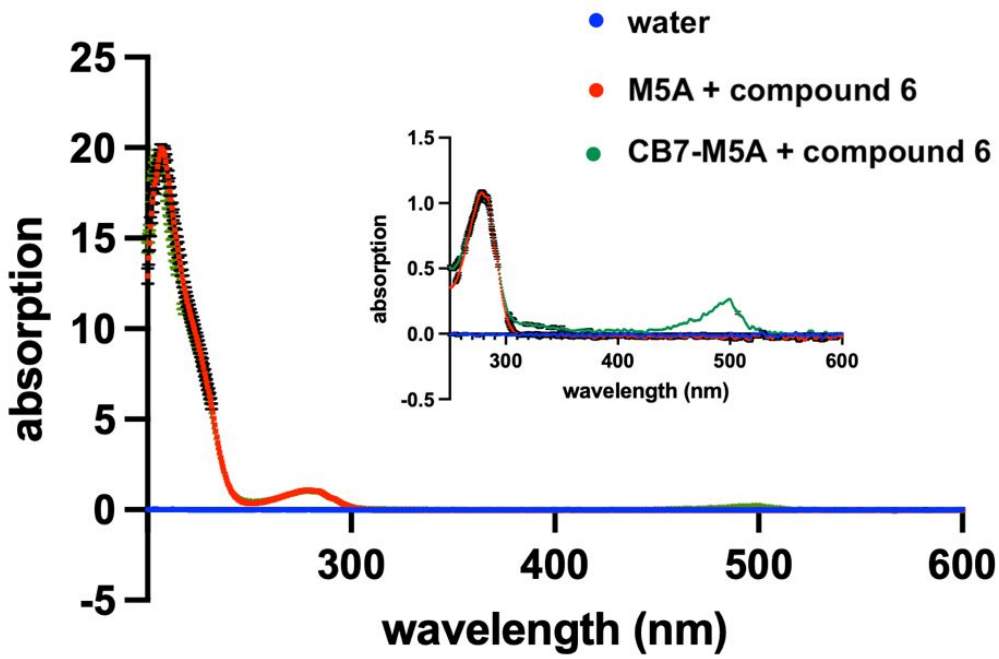

FIGURE S5. Absorption spectrum of the purified CB7-M5A bound adamantane-fluorescein (compound 6, green). To monitor the non-specific binding of the compound 6 to the M5A, a sample containing M5A and compound 6 was prepared and purified and the absorption spectrum of the purified eluate was measured (red). The absorption at two wavelengths, 280 nm and 495 nm were used to determine the number of CB7 moieties per M5A antibody.

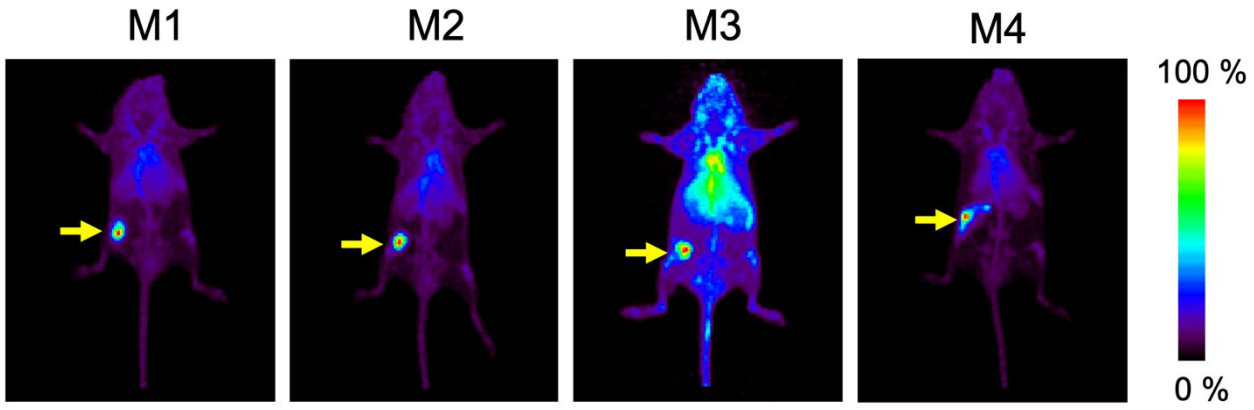

FIGURE S6. Maximum projection images of BxPC3 tumor bearing nude male mice (M1-M4) 72 h post [^{89}Zr]Zr-DFO-M5A injection. The location of the tumor is indicated with a yellow arrow.

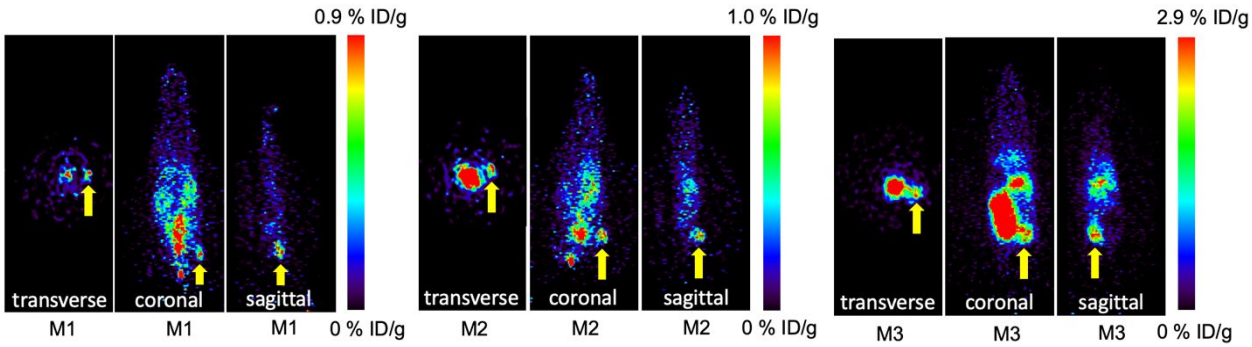

FIGURE S7. Planar images of BxPC3 tumor bearing nude female mice (M1-M3) 4 h post [^{68}Ga]Ga-NOTA-PEG$_3$-NMe$_2$-Fc radioligand injection. Mice were administered primary pretargeting agent, CB7-M5A, 72 h prior to the radioligand injection. The planar images are dissecting the tumor whose location is indicated with a yellow arrow.