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An Efficient Bifunctional Decadentate Ligand 3p-*C*-DEPA for Targeted Alpha Radioimmunotherapy Applications

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Abstract

A new bifunctional ligand 3p-*C*-DEPA was synthesized and evaluated for use in targeted alpha radioimmunotherapy. 3p-*C*-DEPA was efficiently prepared via regiospecific ring opening of an aziridinium ion and conjugated with trastuzumab. The 3p-*C*-DEPA-trastuzumab conjugate was extremely rapid in binding $^{205/6}$Bi, and the corresponding $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab complex was stable in human serum. Biodistribution studies were performed to evaluate *in vivo* stability and tumor targeting of $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab conjugate in tumor bearing athymic mice. $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab conjugate displayed excellent *in vivo* stability and targeting as evidenced by low organ uptake and high tumor uptake. The results of the *in vitro* and *in vivo* studies indicate that 3p-*C*-DEPA is a promising chelator for radioimmunotherapy of ^{212}Bi and ^{213}Bi.

Introduction

The α-emitting radioisotopes, ^{212}Bi ($t_{1/2}$ = 60.6 m) and ^{213}Bi ($t_{1/2}$ = 45.6 m) have proven to be effective for radioimmunotherapy (RIT) of cancers.[1] The α-emitters with high alpha energy (5–8 MeV) and a short emission path length (50–80 μm) can be closely deposited in the target tumor cells resulting in a minimum damage to normal tissues.[2] The radionuclides, ^{212}Bi and ^{213}Bi, with very short half-lives decay ultimately to stable bismuth nuclides and are considered to be suitable for convenient out-patient RIT.[3] The therapeutic efficacy of ^{212}Bi and ^{213}Bi has been demonstrated in numerous pre-clinical and clinical trials involving cancer patients with leukemia, melanoma, and glioblastoma.[4–7]

Three optimal components, a bifunctional ligand, an antibody, and a radioisotope are required for a successful RIT. An effective bifunctional ligand that can rapidly form a stable complex with the radionuclide should be employed to minimize toxic side effects related to biological deposition of the radionuclide if it becomes dissociated from the radiolabeled ligand-antibody conjugate during RIT.[8] Research efforts have been directed towards improving chelation chemistry for RIT. *C*-DOTA and *C*-DTPA (Figure 1) analogues are two bifunctional ligands that are frequently explored for RIT applications.[3] *C*-DOTA forms a kinetically inert and stable complex with Bi(III), however the slow complex formation

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Supporting material. HPLC chromatograms for assessment of radiolabeling reaction kinetics and serum stability and *in vivo* biodistribution data. This material is available free of charge via the Internet at http://pubs.acs.org/BC.

kinetics render this chelator unacceptable for use in RIT when employing short-lived radionuclides such as ^{213}Bi and ^{212}Bi.[9–10] The acyclic bifunctional ligand, *C*-DTPA, displayed rapid and high yield radiolabeling with Bi(III) radioisotopes.[3] However, *C*-DTPA produced a less stable Bi(III)-*C*-DTPA complex than that of Bi(III)-*C*-DOTA,[3,11,12]

We recently reported the synthesis and evaluation of DEPA (Figure 1, 7-[2-(Bis-carboxymethylamino)-ethyl]-4,10-bis-carboxymethyl-1,4,7,10-tetraaza-cyclododec-1-yl-acetic acid).[10] DEPA is a decadentate ligand in a hybridized form of the macrocyclic DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetracarboxylic acid) and the acyclic DTPA (diethylenetriamine pentaacetic acid). This novel bimodal ligand DEPA was hypothesized to rapidly form a stable complex with a metal based on cooperative coordination of the macrocyclic and acyclic binding moieties. DEPA radiolabeled with $^{205/6}$Bi was indeed found to be stable in human serum without any loss of the radioactivity for two weeks and displayed excellent *in vivo* stability in mice.[10]

With the promising complexation kinetics and stability data for DEPA, we sought preparation of an effective bifunctional version of DEPA for RIT. In this paper, we report the synthesis and evaluation of a bifunctional DEPA analogue, 3p-*C*-DEPA (Figure 1, 2-[(carboxymethyl)][5-(4-nitrophenyl-1-[4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl]pentan-2-yl)amino]acetic acid) which contains the parent DEPA backbone and the isothiocyanate (NCS) group for conjugation to an antibody or a peptide. 3p-*C*-DEPA was synthesized, characterized, and conjugated to trastuzumab. Trastuzumab is a HER2 (human epidermal growth factor receptor 2) targeting antibody which has been reported to selectively target the HER2 protein overproduced in various tumors, including 90% of colorectal carcinomas.[13–14] The corresponding 3p-*C*-DEPA-trastuzumab conjugate was evaluated for its radiolabeling reaction kinetics with $^{205/6}$Bi ($t_{1/2}$ = 15.3 d for ^{205}Bi; $t_{1/2}$ = 6.24 d for ^{206}Bi), a γ-emitting surrogate of ^{212}Bi and ^{213}Bi. *In vitro* analysis of the 3p-*C*-DEPA-trastuzumab conjugate radiolabeled with $^{205/6}$Bi included assessment of its stability in human serum and retention of reactivity with HER2 using a radioimmunoassay. Finally, the *in vivo* biodistribution and tumor uptake of the $^{205/6}$Bi-labeled 3p-*C*-DEPA-trastuzumab was assessed in mice bearing s.c. tumor (LS-174T) xenografts. For comparison and as a reference standard, the *C*-DOTA-trastuzumab conjugate was evaluated in the same *in vitro* and *in vivo* studies.

Experimental Procedure

Instruments and methods

^{1}H and ^{13}C NMR spectra were obtained using a Bruker 300 instrument and chemical shifts are reported in ppm on the δ scale relative to TMS or solvent. Electrospray iodization (ESI) high resolution mass spectra (HRMS) were obtained on JEOL double sector JMS-AX505HA mass spectrometer (University of Notre Dame, IN). Analytical HPLC was performed on an Agilent 1200 (Agilent, Santa Clara, CA) equipped with a diode array detector (λ = 254 and 280 nm), a thermostat set at 35 °C, and a Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 80Å, Agilent, Santa Clara, CA). The mobile phase of a binary gradient (0–100% B/40 min; solvent A, 0.05M AcOH/Et_3N, pH 6.0; solvent B, CH_3OH for method 1 or 0–50% B/30 min; solvent A, 0.05M AcOH/Et_3N, pH 6.0; solvent B, CH_3OH for method 2) at a flow rate of 1 mL/min was used for analytical HPLC. Semi-prep HPLC was performed on an Agilent 1200 equipped with a diode array detector (λ= 254 and 280 nm), a thermostat set at 35 °C, and a Zorbax Eclipse XDB-C18 column (9.4 × 250 mm, 80Å). The mobile phase of a binary gradient (0–100%B/160 min; solvent A = 0.05 M AcOH/Et_3N, pH 6.0; solvent B = MeOH for method 3 at a flow rate of 2 mL/min was used for semi-prep HPLC. Size-exclusion HPLC (SE-HPLC) chromatograms were obtained on Agilent 1200 equipped with a diode array detector and an in-line IN/US γ-Ram Model 2

radiodetector (Tampa, FL), fitted with Bio-Silect SEC 250-5 column (Biorad, Hercules, CA) or a TSKgel G3000PW column (Tosoh Biosep, Montgomeryville, PA).

Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) and used as received unless otherwise noted. Trastuzumab (Herceptin; Genetech, South San Francisco, Calif) was obtained through the Veterinary Resources Program (National Institutes of Health, Bethesda, Md). ^{205,6}Bi was produced using a CS30 cyclotron (PET Dept, Clinical Center, NIH) and purified as described previously.[15] *C*-DOTA was purchased from Macrocyclics (Dallas, TX).

tert-Butyl 2,2',2''-(10-(2-(bis(2-*tert*-butoxy-2-oxoethyl)amino)-5-(4-nitrophenyl)-pentyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (4)

To a solution of **1**[16] (547.7 mg, 1.06 mmol) and DIPEA (410.9 mg, 3.18 mmol) in CH_3CN (10 mL) was added tri-substituted cyclen **3** (546.6 mg, 1.06 mmol). The resulting mixture was stirred for 4 weeks at room temperature while monitoring the reaction progress using TLC. The reaction mixture containing the starting materials and the product **4** was concentrated to dryness. The residue was purified via column chromatography on silica gel (220–440 mesh) eluted with 3% CH_3OH in CH_2Cl_2. The fractions containing the product **4** along with the starting material **3** as impurity were combined. After evaporating of the solvents, ether (20 mL) was added to the residue, and the starting material **3** formed a slurry which was removed by filtration. The filtrate containing the product **4** was washed with DI water (2 × 10 mL). The ether layer was dried over $MgSO_4$, filtered, and concentrated *in vacuo* to provide pure **4** (610 mg, 61%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.37–1.45 (m, 45 H), 1.60–1.78 (m, 2 H), 1.81–1.95 (m, 1 H), 2.02–2.19 (m, 1 H), 2.39–2.50 (m, 3H), 2.56–2.83 (m, 18 H), 3.21 (s, 4H), 3.26 (s, 2 H), 3.36 (dd, J = 16.9, 21.9 Hz, 4 H), 7.36 (d, J = 8.6 Hz, 2 H), 8.09 (d, J = 8.7 Hz, 2 H); ^{13}C NMR ($CDCl_3$, 300MHz) δ 27.89 (t), 28.11 (q), 28.22 (q), 30.98 (t), 35.91 (t), 52.04 (t), 52.11 (t), 52.20 (t), 53.09 (t), 53.19 (t), 56.34 (t), 56.44 (t), 58.38 (t), 60.08 (d), 80.41 (s), 80.57 (s), 123.43 (d), 129.30 (d), 146.18 (s), 151.17 (s), 170.98 (s), 171.10 (s), 171.41 (s). HRMS (Positive ion FAB) Calcd for $C_{49}H_{85}N_6O_{12}$ $[M + H]^+$ *m/z* 949.6225 Found: $[M + H]^+$ *m/z* 949.6256.; Analytical HPLC (t_R = 42 min, method 1).

Tri-*tert*-butyl 10-(2-(*tert*-butoxycarbonyl)-5-(4-nitrophenyl)pentyl)-1,4,7,10-tetraazacyclododecane-1,4,7-tricarboxylate (7)

To a mixture of **5** (1.24 g, 3.85 mmol) and **6** (1.82 g, 3.85 mmol) in 1,2-dichloroethane (30 mL) was added portionwise sodium triacetoxyborohydride (1.14 g, 5.4 mmol). The mixture was stirred at room temperature for 24 h. The reaction mixture was quenched with saturated $NaHCO_3$ (40 mL), and the product was extracted while washing with CH_2Cl_2. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by silica gel (60–230 mesh) column chromatography eluted with 25% EtOAc in hexanes to provide 7 (1.90 g, 63%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.03–1.51 (m, 36 H), 1.51–2.01 (m, 4 H), 2.50–3.99 (m, 21 H), 7.30 (d, J = 8.5 Hz, 2 H), 8.08 (d, J = 8.4 Hz, 2H); ^{13}C NMR ($CDCl_3$, 300 MHz) 24.69 (t), 27.16 (t), 28.57 (q), 28.69 (q), 35.13 (t), 35.68, (t), 36.64 (t), 47.49 (t), 48.46 (d), 50.54 (t), 51.20 (t), 58.18 (t), 58.64 (t), 79.18 (s), 79.28 (s), 79.57 (s), 123.56 (d), 129.19 (d), 146.27 (s), 150.39 (s), 155.14 (s), 155.73 (s), 155.95 (s), 156.72 (s). HRMS (Positive ion ESI) Calcd for $C_{39}H_{67}N_6O_{10}$ $[M + H]^+$ *m/z* 779.4913 Found: $[M + H]^+$ *m/z* 779.4890.

5-(4-Nitrophenyl)-1-(1,4,7,10-tetraazacyclododecan-1-yl)pentan-2-amine (8)

Compound 7 (1.84 g, 2.36 mmol) at 0–5 °C was treated dropwise with 4M HCl (g) in 1,4-dioxane (18 mL) over 20 min. The resulting mixture was warmed to room temperature and stirred for 22 h. Ether (100 mL) was added and stirred for 10 min. The resulting mixture was capped and placed in the freezer for 1 h. The slurry formed was filtered, washed with ether, and quickly dissolved in deionized (DI) water. The aqueous solution was concentrated *in vacuo* to provide **8** as an acidic salt (1.17 g, 89%). ^{1}H NMR (D_2O, 300 MHz) δ 1.50–1.71 (m, 4 H), 2.50–2.80 (m, 6 H), 2.82–3.40 (m, 15 H), 7.30 (d, *J* = 8.7 Hz, 2 H), 8.00 (d, *J* = 8.7 Hz, 2 H); ^{13}C NMR (D_2O, 300 MHz) δ 25.65 (t), 30.63 (t), 34.46 (t), 41.30 (t), 42.13 (t), 44.21 (t), 48.56 (t), 48.57 (d), 56.81 (t), 123.66 (d), 129.40 (d), 145.90 (s), 150.21 (s). HRMS (Positive ion ESI) Calcd for $C_{19}H_{35}N_6O_2$ $[M + H]^+$ *m/z* 379.2816 Found: $[M + H]^+$ *m/z* 379.2804.

A solution of the acidic salt **8** (670 mg, 1.7 mmol) in DI water (5 mL) was neutralized using 0.5 M NaOH. The aqueous layer was then extracted with $CHCl_3$ (2 × 25 mL). The aqueous layer was further adjusted to pH 10 and re-extracted with $CHCl_3$ (2 × 25 mL). The organic layers extracted from both neutral (pH 7) and basic (pH 10) solutions were combined, dried over $MgSO_4$, filtered, and concentrated *in vacuo* to provide free amine **8** (452 mg, 100%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.20–1.49 (m, 2 H), 1.60–1.89 (m, 2 H), 2.15–3.00 (m, 21 H), 7.33 (d, *J* = 8.6 Hz, 2 H), 8.14 (d, *J* = 8.6 Hz, 2 H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 27.26 (t), 35.00 (t), 35.64 (t), 45.16 (t), 46.01 (t), 46.95 (t), 48.71 (d), 52.10 (t), 62.56 (t), 123.27 (d), 129.00 (d), 145.94 (s), 150.24 (s).

Synthesis of 4 from compound 8

To a solution of **8** (170.3 mg, 0.45 mmol) in CH_3CN (5 mL) was added *tert*-butyl bromoacetate (438.9 mg, 2.25 mmol) and K_2CO_3 (311.0 mg, 2.25 mmol). The resulting mixture was heated at 65 °C and stirred for 13 h while monitoring the reaction progress by analytical HPLC. The reaction mixture was cooled to room temperature, and the solvent was evaporated. The residue was purified by semi-prep HPLC (method 3, 138–143 min) to afford **4** (37 mg, 9%).

2-[(Carboxymethyl)][5-(4-nitrophenyl-1-[4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl]Pentan-2-yl)amino]acetic acid (9)

Compound **4** (77.0 mg, 0.08 mmol) at 0–5 °C was treated dropwise with 4M HCl (g) in 1,4-dioxane (15 mL) over 20 min. The resulting mixture was allowed to warm to room temperature for 22 h. Ether (~20 mL) was added and continuously stirred for 10 min. The resulting mixture was capped and placed in the freezer for 1 h. The solid formed was filtered, washed with ether, and quickly dissolved in DI water. Evaporation of the aqueous solution gave **9** (68.0 mg, 97%) as an off-white solid. ^{1}H NMR (D_2O, 300 MHz) δ 1.26–1.40 (m, 1H), 1.48–1.70 (m, 3H), 2.52–2.78 (m, 2H), 2.90–3.70 (m, 27H), 3.75–3.98 (m, 2H), 7.35 (d, *J* = 8.3 Hz, 2H), 8.06 (d, *J* = 8.2 Hz, 2H); ^{13}C NMR (D_2O, 300MHz) δ 27.08 (t), 34.64 (t), 48.45 (t), 48.82 (t), 50.22 (t), 51.09 (t), 52.40 (t), 53.58 (t), 54.28 (t), 55.46 (t), 59.43 (d), 123.55 (d), 129.40 (d), 145.71 (s), 150.54 (s), 169.54 (s), 172.97 (s), 173.93 (s); HRMS (Positive ion FAB) Calcd for $C_{29}H_{45}N_6O_{12}$ $[M + H]^+$ *m/z* 669.3095 Found: $[M + H]^+$ *m/z* 669.3086.

2,2',2"-(10-(5-(4-Aminophenyl)-2-(bis(carboxymethyl)amino)pentyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (10)

To a solution of **9** (46.0 mg, 68.8 μmol) in DI H_2O (12 mL) was added dry 10% Pd/C (14.0 mg) under Argon. The reaction mixture was subject to hydrogenation in a hydrogenation apparatus set at the constant pressure (15 psi) of H_2 (g) for 19 h. The resulting mixture was

filtered over a Celite bed and washed thoroughly with DI H_2O. The filtrate was concentrated *in vacuo* to provide a light yellow solid **10** (44 mg, 100%). ^{1}H NMR (D_2O, 300 MHz) δ 1.22–1.38 (m, 1H), 1.43–1.68 (m, 3H), 2.45–2.63 (m, 2H), 2.81–3.70 (m, 27H), 3.80–3.98 (m, 2H), 7.17 (d, *J* = 8.5 Hz, 2H), 7.24 (d, *J* = 8.5 Hz, 2H); ^{13}C NMR (D_2O, 300 MHz) δ 27.04 (t), 27.46 (t), 34.18 (t), 48.50 (t), 48.98 (t), 50.28 (t), 51.12 (t), 52.50 (t), 53.69 (t), 54.50 (t), 55.49 (t), 59.40 (d), 123.01 (d), 127.55 (s), 130.10 (d), 143.28 (s), 169.50 (s), 173.13 (s), 174.22 (s); HRMS (Positive ion ESI) Calcd for $C_{29}H_{47}N_6O_{10}$ $[M + H]^+$ *m/z* 639.3348 Found: $[M + H]^+$ *m/z* 639.3342.

2,2',2"-(10-(2-(Bis(carboxymethyl)amino)-5-(4-isothiocyanatophenyl)pentyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (11)

To a solution of **10** (7.0 mg, 8.2 μmol) in water (0.1 mL) was added $CSCl_2$ (7.0 μL, 7.0 μmol) in $CHCl_3$. The resulting mixture was stirred at room temperature for 2 h. The aqueous layer was isolated and concentrated *in vacuo* to give pure 3p-*C*-DEPA-NCS **11** as a solid (8.0 mg, 100%). ^{1}H NMR (D_2O, 300MHz) δ 1.23–1.72 (m, 4H), 2.40–2.58 (m, 2H), 2.80–3.98 (m, 29H), 7.13 (s, 4H). HRMS (Positive ion FAB) Calcd for $C_{30}H_{43}N_6O_{12}S$ $[M + H]^+$ *m/z* 679.2761 Found: $[M + H]^+$ *m/z* 679.2747.

Conjugation of 3p-*C*-DEPA-NCS to trastuzumab

All absorbance measurements were obtained on an Agilent 8453 diode array spectrophotometer equipped with a 8-cell transport system (designed for 1 cm cells). Metal-free stock solutions of all buffers were prepared using Chelex®-100 resin (200–400 mesh, Bio-Rad Lab, Hercules, CA, Cat# 142–2842). Chelex resin (2.5 g) was added into the buffer solution (250 mL) and the mixture was shaken overnight in a shaker and filtered through a Corning filter system (Cat# 430513, pore size 0.2 μm). Disposable PD-10 Sephadex™ G-25M columns (GE Healthcare, #17-0851-01) were rinsed with 25 mL of the appropriate buffer prior to addition of antibody or its ligand conjugates. Amicon centricon C-50 (50,000 MWCO) centrifugal filter devices (Millipore, Cat# UFC805008) were used for purification of trastuzumab conjugate (Bedford, MA). The initial concentration of trastuzumab was determined by UV/Vis spectroscopic method. Phosphate buffered saline (PBS, 1×, 11.9 mM Phosphates, 137 mM NaCl, and 2.7 mM KCl, pH 7.4) was purchased from Fisher and used as received. Conjugation buffer (50 mM HEPES, 150 mM NaCl, pH 8.6) were prepared as 1× solutions, chelexed, and filtered through the Corning filter. Trastuzumab (6.67 mg) was diluted to 1.6 mL using conjugation buffer (1:0.6), and the resulting solution was added to a PD-10 column. Conjugation buffer (10 mL) was added to the PD-10 column to exchange the buffer solution of the antibody and collected in a sterile test tube and checked for the presence of trastuzumab via analysis of the UV/VIS spectrum at 280 nm. To a sterile test tube containing the recovered trastuzumab (6.14 mg) was added a 10-fold excess of 3p-*C*-DEPA-NCS (39.6 μL, 10 mM). The resulting solution was gently agitated for 16 h at room temperature and placed on a Centricon C-50 membrane and spun down to reduce volume. PBS (3 × 2 mL) was added to the remaining solution of the 3p-*C*-DEPA-trastuzumab conjugate, followed by centrifugation in order to remove unreacted ligand. The volume of purified conjugate antibody was brought to 1.0 mL with PBS. To measure [trastuzumab] in the 3p-*C*-DEPA-trastuzumab conjugate, a UV/Vis spectrometer was zeroed against a cuvette filled with 2.0 mL of PBS with a window open from 190 nm to 1100 nm. A 50 μL portion of PBS was removed and discarded, 50 μl of the 3p-*C*-DEPA-trastuzumab conjugate solution was added, and absorbance at 280 nm was noted. Beer's Law was used to calculate [trastuzumab] in the conjugate with molar absorptivity of 1.42. After centrifugation, 2.98 mg (2.02×10^{-5} M, 49.0%) of the trastuzumab remained.

Spectroscopic Determination of Ligand to Protein (L/P) Ratio

A stock solution of the Cu(II)-AAIII reagent was prepared in 0.15 M NH_4OAc, pH 7.0 by adding an aliquot of copper atomic absorption solution (1.55×10^{-2} M) into a 10 μM solution of AAIII to afford a 5 μM solution of Cu(II).[17] This solution was stored in the dark to avoid degradation over time. A UV/Vis spectrometer was zeroed against well-dried blank 8 cuvettes with a window open from 190 nm to 1100 nm. A cuvette was filled with AAIII solution (2 mL), and the other seven cuvettes with the Cu(II)-AAIII solution (2 mL). Cu(II)-AAIII solution (50 μL) in the seven cuvettes was removed and discarded. Milli-Q water (50 μL) was added to the second cuvette, and one to five 10 μL additions of 3p-*C*-DEPA (0.1mM) were added to the five cuvettes to give a series of five different concentrations (2 mL total volume). The solutions in the third to the seventh cuvette were diluted to 2.0 mL by adding an aliquot of milli-Q water. 3p-*C*-DEPA-trastuzumab conjugate (50 μL) was added to the eighth cuvette containing Cu(II)-AAIII reagent (1950 μL). After addition of 3p-*C*-DEPA-trastuzumab conjugate to the Cu(II)-AAIII solution, the resulting solution was equilibrated for 10 min. The absorbance of the resulting solution at 610 nm was monitored every 30 seconds over 6 min. The average of the absorbance of each solution was calculated, and the absorbance data from the Cu(II)-AAIII solutions containing six different concentrations were used to construct a calibration plot of A_{610} versus [3p-*C*-DEPA] by the equation, $Y = 0.0663 - (1.5284 \times 10^4)$[3p-*C*-DEPA] ($R^2 = 0.9969$), wherein $Y = A_{610nm}$. The concentration of 3p-*C*-DEPA in the 3p-*C*-DEPA-trastuzumab conjugate was calculated (3.98×10^{-5} M). The L/P ratio of the 3p-*C*-DEPA-trastuzumab conjugate ([3.98×10^{-5} M]/[2.02×10^{-5} M]) was measured to be 1.97.

Radiolabeling of 3p-*C*-DEPA-trastuzumab or *C*-DOTA-trastuzumab conjugates with $^{205/6}$Bi

All HCl solutions were prepared from ultra-pure HCl (Fisher, Cat# A466–500). For metal-free radiolabeling, plasticware including pipette tips, tubes, and caps was soaked in 0.1M HCl overnight, washed thoroughly with Milli-Q (18.2 MΩ) water, and air-dried overnight. Ultra pure NH_4OAc (Aldrich, #372331) was purchased from Aldrich and used to prepare all NH_4OAc buffer solutions (0.1 M, pH 7). The buffer solutions were treated with Chelex-100 resin (Biorad, #142–2842, 1g/100 ml buffer solution), shaken overnight at room temperature, and filtered through 0.22 μm filter (Corning, #430320) prior to use. To a buffer solution (120 μL, pH 7) in a capped microcentrifuge tube (1.5 mL, Fisher Scientific #05-408-129) was sequentially added a solution of C-DOTA-trastuzumab (30 μg) in PBS (13.5 μL) and $^{205/6}$Bi (0.1 M HI, 60.5 μCi, 12.7 μL) or 3p-*C*-DEPA-trastuzumab (30 μg) in PBS (9.6 μL) and $^{205/6}$Bi (0.1M HI, 60.5 μCi, 12.7 μL). The final volume of the resulting solution was 146.2 μL and 142.3 μL for *C*-DOTA-trastuzumab and 3p-*C*-DEPA-trastuzumab, respectively, and the pH of the resulting reaction mixture was 5.5 for both conjugates. The reaction mixture was agitated on the thermomixer (Eppendorf, #022670549) set at 1,000 rpm at room temperature for 1 h. The labeling efficiency was determined by SE-HPLC (Biorad, Bio-Silect SEC 250-5 column, 7.8 × 30 cm, eluent: PBS, flow rate: 1 mL/min). A solution of radiolabeled mixture (10 μL for *C*-DOTA-trastuzumab and 7 μL for 3p-*C*-DEPA-trastuzumab) was withdrawn at the designated time points (1 min, 5 min, 10 min, 20 min, 30 min, and 60 min), and DTPA solution (10mM, 1 μL or 1.4 μL) was added to the mixture, and the resulting mixture was left for at least 20 min. HPLC samples were prepared and evaluated by SE-HPLC. Peaks for bound and unbound radioisotope appeared around 8.3 min and 11 min, respectively.

In vitro stability of $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab conjugate

$^{205/6}$Bi-3p-*C*-DEPA-trastuzumab was prepared by reaction of 3p-*C*-DEPA-trastuzumab with $^{205/6}$Bi at either RT or 37°C (5.0 M NH_4OAc, pH 5.5). The complex formed was purified from $^{205/6}$Bi by gel filtration chromatography using PD-10 column (Disposable PD-10 Sephadex™ G-25M columns, GE Healthcare, Cat# 17-0851-01) eluted with PBS (pH

7.4, Fisher Scientific, Cat# BP2438-4). The fractions containing $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab (700 μL, 22 μCi) were combined and added into human serum (700 μL, Gemini Bioproducts, #100110) in a microcentrifuge tube (Fisher Scientific, #05-408-129). The stability of the purified $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab was evaluated for 4 days. The serum stability of the radiolabeled complexes was assessed by measuring the transfer of the radionuclide from each complex to serum proteins using SE-HPLC (TSKgel G3000PW column, 7.5 × 30cm, eluent: PBS, flow rate: 1 mL/min). A solution of the radiolabeled complex in serum (20–80 μL) was withdrawn at the designated time point, treated with DTPA (5 mM, 1.0 μL), incubated at room temperature for 20 min and then diluted with PBS (pH 7.4, 50–100 μL) prior to SE-HPLC.

Preparation of $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab and $^{205/6}$Bi-*C*-DOTA-trastuzumab for immunoreactivity and *in vivo* studies

For use in the *in vitro* binding and *in vivo* biodistribution and tumor targeting studies, $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab and $^{205/6}$Bi-*C*-DOTA-trastuzumab with high specific activity were prepared. In this instance, two aliquots of $^{205/6}$Bi solution (100 μL in 0.1 M HI, 1.1 mCi each) were each neutralized to pH 5.5 with NH_4OAc solution (10 μL, 5 M, pH 7). An aliquot of each immunoconjugate containing 50 μg of protein in PBS was added to one or the other $^{205/6}$Bi solution. The mixtures were vortexed briefly and incubated at 37°C. After 30 min EDTA solution (5 μL, 0.1M) was added to each reaction, vortexed briefly and further incubated for 5 min. The products were purified by gel filtration chromatography using disposable PD-10 columns. The labeling efficiencies were 85.8% and 67.3% for $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab and $^{205/6}$Bi-*C*-DOTA-trastuzumab, respectively, as determined by the labeled products collected from the PD-10 columns. The purity of the labeled products as determined by SE-HPLC (TSKgel G3000PW column, PBS eluate at 0.5 mL/min) were >98% for each product. The specific activities were 16 mCi/mg and 13.6 mCi/mg for$^{205/6}$Bi-3p-*C*-DEPA-trastuzumab and $^{205/6}$Bi-*C*-DOTA-trastuzumab, respectively.

Radioimmunoassay

The immunoreactivity of the ^{205}Bi-labeled trastuzumab conjugates was assessed in a radioimmunoassay using purified recombinant human Erb2/Fc chimera (rhErb2/Fc). Briefly, 50 ng of the rhErb2/Fc in PBS with Mg^{+2} and Ca^{+2}(50 μL) was added to each well of a 96-well plate and allowed to adsorb to the well at 4 °C. Following an overnight incubation, the solution was removed and 100 μL of 1% BSA in PBS (BSA/PBS) was added to each well and incubated at room temperature for 30–60 min. Serial dilutions of $^{205/6}$Bi-trastuzumab (~300,000 to ~12,500 cpm in 50 μL of BSA/PBS) were added to the wells in duplicate, covered and incubated at 37 °C for 4 h. The radiolabeled antibody was then removed and the wells were washed three times with PBS. The radioactivity was removed from the wells with a solution of 0.2 M NaOH with 1 mM EDTA (100 μL), adsorbing the solution to cotton filters and transferring the filters to 12 × 75 mm polypropylene tubes. The radioactivity was measured in a γ-scintillation counter (WizardOne, Perkin Elmer, Shelton, CT), and the percentage binding calculated for each dilution. The values presented are an average of the percent bound for the serial dilutions. The specificity of the radiolabeled trastuzumab was confirmed by incubating one set of wells with radiolabeled trastuzumab and 10 μg of unlabeled trastuzumab.

In vivo biodistribution and tumor targeting studies

In vivo studies were conducted using the human colon carcinoma cell line, LS-174T, (kindly provided by Dr. J. Greiner, NCI). The LS-174T cell line was grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10 mM glutamine along with 10% FetalPlex (Gemini Bio-Products, Woodland, CA) and 1 mM non-essential amino acids. Media and

supplements were obtained from Lonza (Walkersville, MD). All *in vivo* studies were performed using 4–6 week old female athymic (nu/nu) mice (Charles River Laboratories, Wilmington, MA). All animal protocols were approved by the National Cancer Institute Animal Care and Use Committee. Mice were injected subcutaneously (s.c.) in the right rear leg with 2×10^6 LS-174T cells in media (200 μL) with 20% Matrigel (BD Biosciences, San Jose, CA). Mice were utilized in studies when the tumor xenografts maximal diameter measured 0.4 – 0.6 cm. Mice (n = 4 per time point) were injected intravenously (i.v.) with the $^{205/6}$Bi-labeled trastuzumab conjugates (~7.5 μCi on 0.6 μg) and sacrificed by exsanguination at 2, 6 and 24 h. The blood, tumor, and major organs were collected, wet-weighed, and counted in a γ-scintillation counter. The percent injected dose per gram (%ID/g) and standard deviation were calculated.

Results and Discussion

The synthesis of the new bifunctional ligand 3p-*C*-DEPA is outlined in Scheme 1. An intramolecular substitution reaction of *N*,*N*-bisubstituted β-amino bromide **1** produced aziridinium ion **2** which was then reacted with tri-substituted cyclen **3** (1,4,7,10-tetraazacyclododecane). Regiospecific ring opening of **2** by nucleophilic attack of bulky cyclen analogue **3**[10] occurred at the less hindered methylene carbon in **2** to provide **4** as the exclusive product (61%). The reaction was carried out at room temperature, although it proceeds extremely slowly at this mild condition (4 weeks). An intramolecular rearrangement product of **1** was obtained when the reaction was under reflux. The desired coupling product **4** was isolated via column chromatography and characterized by ^{1}H and ^{13}C NMR, analytical HPLC, and HRMS. To confirm the regiochemistry observed in the nucelophilic ring opening of the aziridinium ion **2** at the methylene carbon, compound **4** was separately prepared via another synthetic route starting from **5** as shown in Scheme 1. Reductive amination of **5** with **6** using sodium triacetoxyborohydride provided **7** which was subsequently treated with HCl (g) in 1,4-dioxane to afford **8**. A base-promoted reaction of **8** with *tert*-butyl bromoacetate produced compound **4**, and comparison of the spectroscopic data of **4** that was separately produced via the two synthetic routes confirmed regiochemistry in the ring opening of **2**. Synthesis of the bifunctional ligands 3p-*C*-DEPA (**9**) and 3p-*C*-DEPA-NCS (**11**) is shown in Scheme 2. The *tert*-butyl groups in **4** was removed by the reaction of **4** with 4M HCl (g) in 1,4-dioxane to provide 3p-*C*-DEPA (**9**). The nitro group in **9** was transformed into the amino group by hydrogenolysis of **9** on Pd/C to provide **10** which was subsequently reacted with thiophosgene (g) in $CHCl_3$ to the desired bifunctional ligand 3p-*C*-DEPA-NCS (**11**) containing the isothiocyanate group for conjugation to an antibody.

3p-*C*-DEPA-NCS was conjugated to trastuzumab, and the concentration of trastuzumab in 3p-*C*-DEPA-trastuzumab conjugate was quantified by the method of Lowry.[18] The Cu(II)-AAIII based UV-Vis spectrophotometric assay was used for the determination of the number of 3p-*C*-DEPA ligand linked to trastuzumab (L/P ratio).[17] The ligand to protein (L/P) ratio for 3p-*C*-DEPA-trastuzumab conjugate was measured to be 1.97. For comparison, *C*-DOTA-NCS (Macrocyclics, TX) was conjugated to trastuzumab as reported previously.[19]

The purified 3p-*C*-DEPA trastuzumab conjugates (30~50 μg) in 0.25 M NH_4OAc buffer solution at pH 5.5 was radiolabeled with $^{205/6}$Bi (0.1M HI) (60 μCi) at room temperature (RT). During the reaction time (1 h), the radiolabeling kinetics was determined by taking aliquots of the reaction mixture at 6 time points (1 min, 5 min, 10 min, 20 min, 30 min, and 60 min). The components were analyzed using SE-HPLC after challenging the reaction mixture with 10mM DTPA, and the radiolabeling efficiency (%) was determined (Table 1 and the supporting information). The data indicate that $^{205/6}$Bi labeling of 3p-*C*-DEPA-trastuzumab conjugate were extremely rapid (1 min, >93%) at RT. As expected,

radiolabeling of *C*-DOTA with $^{205/6}$Bi was slow and incomplete at 24 h (60.2 ± 8.0%). The $^{205/6}$Bi-3p-*C*-DEPA conjugate was further evaluated for *in vitro* serum stability. The $^{205/6}$Bi-3p-*C*-DEPA conjugate was freshly prepared, purified, and incubated in human serum at 37 °C. At each time point (0 h, 1, 2, 3, 4 d), aliquots of the reaction mixture were analyzed using SE-HPLC after challenging the reaction mixture with 10mM DTPA. $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab was found to be stable in human serum without release of the radioactivity for at least 4 days (the Supporting Information).

The $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab and $^{205/6}$Bi-*C*-DOTA-trastuzumab conjugates were independently prepared at 37 °C and pH 5.5 for specific activity and immunoreactivity evaluations prior to performing comparative *in vivo* biodistribution studies. The respective specific activity of 16.0 mCi/mg, and 13.6 mCi/mg was measured for $^{205/6}$Bi-3p-*C*-DEPA and $^{205/6}$Bi-*C*-DOTA. The radiolabeling yields of 85.8% and 67.3% were determined for $^{205/6}$Bi-3p-*C*-DEPA and $^{205/6}$Bi-*C*-DOTA, respectively. Immunoreactivity of $^{205/6}$Bi-*C*-DOTA-trastuzumab and $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab was assessed in a radioimmunoassay using recombinant human ErbB2/Fc Chimera (R&D Systems, Minneapolis, NM). The respective specific binding of 82.6% and 83.2% was measured for $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab and $^{205/6}$Bi-*C*-DOTA-trastuzumab. In the presence of excess trastuzumab, $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab and $^{205/6}$Bi-*C*-DOTA-trastuzumab exhibited the same non-specific binding (7.6%).

The *in vivo* stability and tumor targeting of $^{205/6}$Bi-3p-*C*-DEPA–trastuzumab was evaluated by performing biodistribution studies in LS174T-bearing athymic mice (i.v. injection, n = 4). $^{205/6}$Bi-*C*-DOTA-trastuzumab conjugate was evaluated for comparison. The mice were euthanized at 2 h, 6 h, and 24 h. Tumors, selected organs, and the blood were harvested, wet weighed, and the radioactivity measured in a γ-counter (Figures 2 and 3). The highest tumor uptake was observed at 24 h for $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab and $^{205/6}$Bi-*C*-DOTA-trastuzumab (27.3% and 25.8%, respectively). Among the organs, the highest accretion of radioactivity was observed in the liver: 7.73% for $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab (6 h) and 8.02% for $^{205/6}$Bi-*C*-DOTA-trastuzumab (24 h). Both of the $^{205/6}$Bi-labeled conjugates exhibited the highest radioactivity level in the blood at 2 h which decreased over time. Renal and liver uptake of $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab peaked at 6 h and decreased by 24 h. $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab and $^{205/6}$Bi-*C*-DOTA-trastuzumab resulted in the respective radioactivity level of 6.27% and 5.52% in the kidney at 24 h. $^{205/6}$Bi-*C*-DOTA-trastuzumab exhibited an upward trend in the accumulation of radioactivity in the liver over the study period. Femur uptake of $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab peaked at 2 h (~3%) and decreased over the rest of the time points, while $^{205/6}$Bi-*C*-DOTA-trastuzumab displayed increased femur uptake from 2.00% at 6 h to 2.49% at 24 h. The respective tumor-to-blood ratio of $^{205/6}$Bi-3p-*C*-DEPA and $^{205/6}$Bi-*C*-DOTA at 24 h was 2.26 and 2.15. Both radioimmuno-conjugates exhibited low blood to tissue ratios (<0.7) in all tissues and higher radioactivity level in tumor as compared to the normal organs over the time points.

In summary, the *in vitro* and *in vivo* experimental results indicate that the new ligand 3p-*C*-DEPA possesses great potential for RIT of ^{212}Bi and ^{213}Bi. Given complete and ongoing numerous preclinical and clinical studies of *C*-DOTA for alpha RIT, this new ligand deserves more extensive evaluations as a chelator of other potent α-emitters with a longer half-life including ^{212}Pb ($t_{1/2}$ = 10.64 h) and ^{225}Ac ($t_{1/2}$ = 10 d). ^{212}Pb is investigated as an *in vivo* generator of ^{212}Bi to abrogate the short half-life of the daughter isotope.[20] ^{225}Ac with the advantage of multiple alpha emissions is a potent α emitter and known to induce apoptosis even with a single particle traversal of the cell.[21] Although encouraging results on the therapeutic efficacy of *C*-DOTA-antibody conjugates radiolabeled with ^{212}Pb or ^{225}Ac as demonstrated in the preclinical studies using tumor bearing mice was reported[22–25], *C*-DOTA-antibody conjugates radiolabeled with ^{203}Pb or ^{225}Ac were less stable in

serum.[21,26,27] The decadentate chelator 3p-*C*-DEPA possesses a larger macrocyclic backbone than *C*-DOTA and may be a suitable ligand for effectively sequestering ^{212}Pb and ^{225}Ac. 3p-C-DEPA will be further evaluated for various alpha RIT applications.

Conclusion

The new bifunctional ligand 3p-*C*-DEPA was efficiently prepared based on the synthetic route including the key reaction step, regiospecific ring opening of aziridinium ion by nucleophilic trisubstituted cyclen. 3p-*C*-DEPA conjugated with trastuzumab was extremely rapid in binding $^{205/6}$Bi, and the corresponding $^{205/6}$Bi-3p-*C*-DEPA-trastuzumab complex was stable in human serum over a 4 day study period, well beyond any concerns of stability with ^{212}Bi and ^{213}Bi. *In vivo* biodistribution data indicate that 3p-*C*-DEPA-trastuzumab radiolabeled with $^{205/6}$Bi displayed excellent *in vivo* stability as evidenced by low organ uptake and excellent tumor targeting that was favorably compared to those of $^{205/6}$Bi-*C*-DOTA-trastuzumab. *In vitro* and *in vivo* data demonstrate that 3p-*C*-DEPA is a promising ligand for RIT applications of ^{212}Bi and ^{213}Bi.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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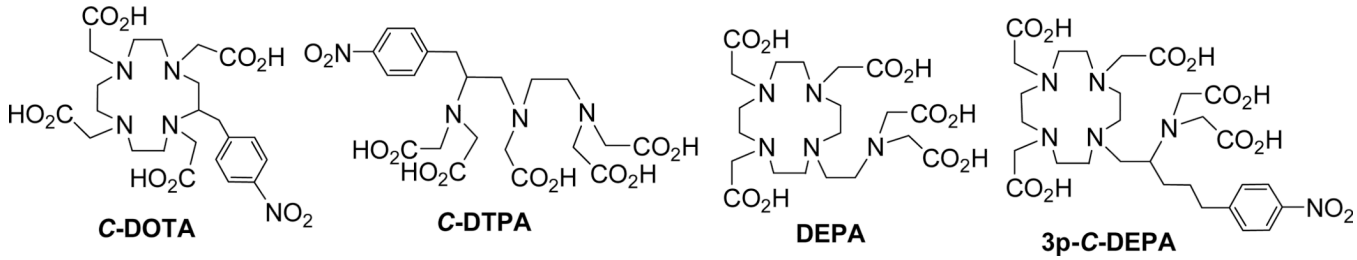


Figure 1.
Ligands in clinical and preclinical use for RIT

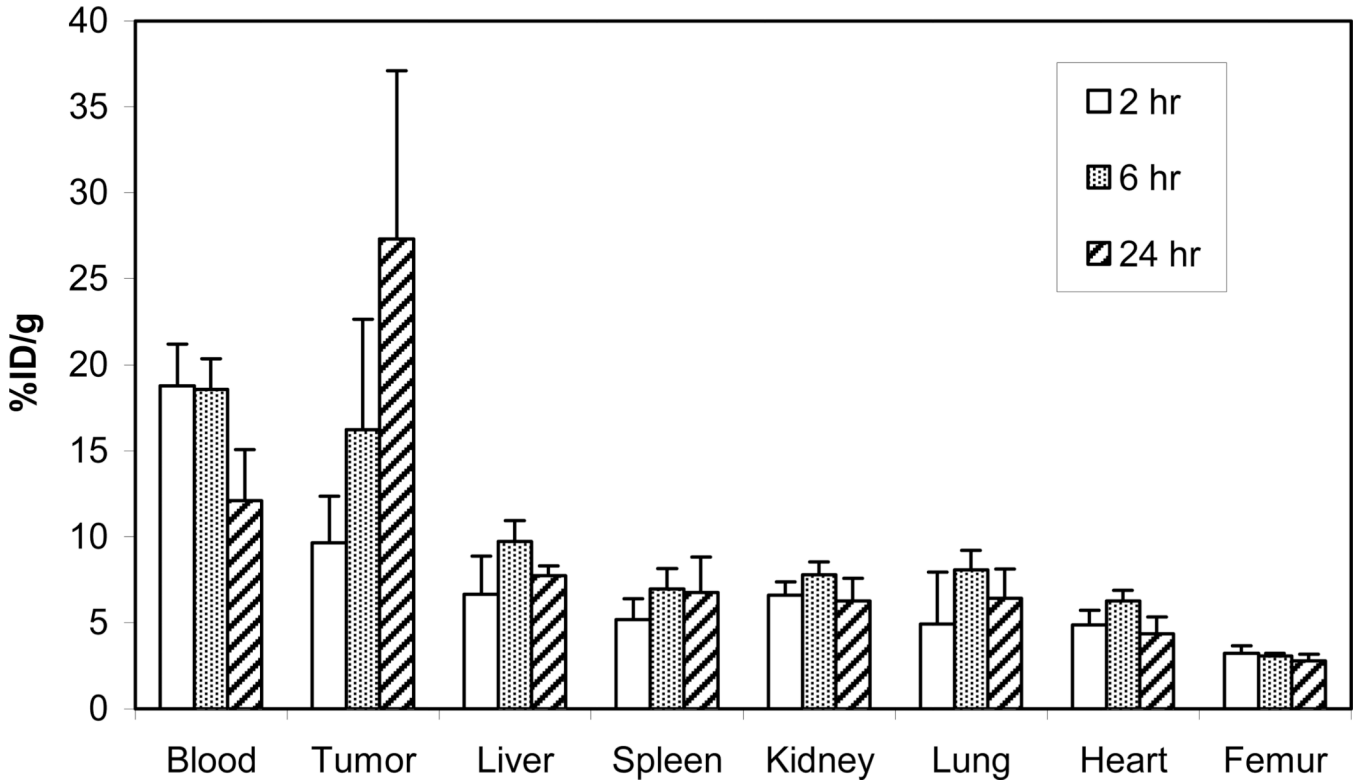


Figure 2.
Biodistribution of $^{205/6}$Bi-3p-*C*-DEPA in athymic mice bearing s.c. LS-174T tumors.

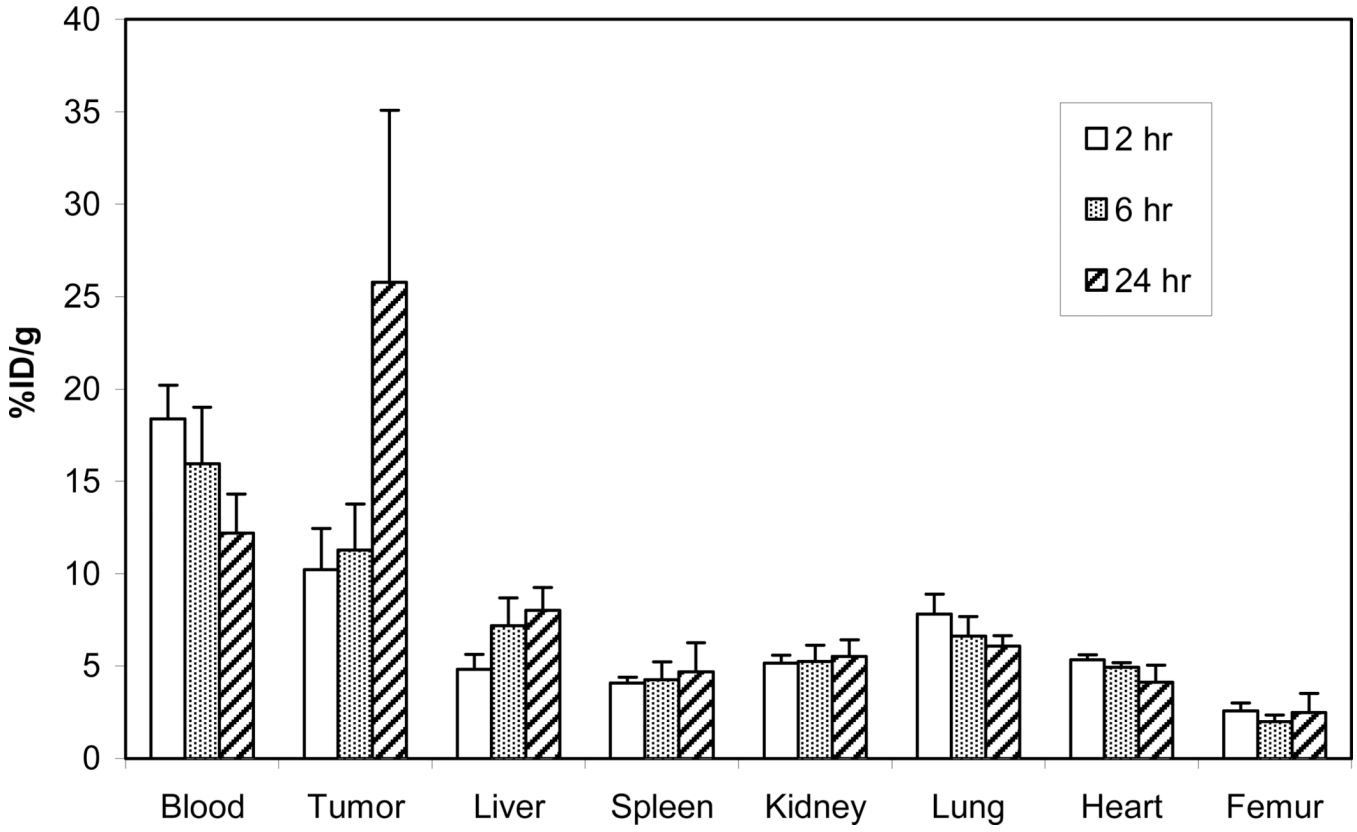
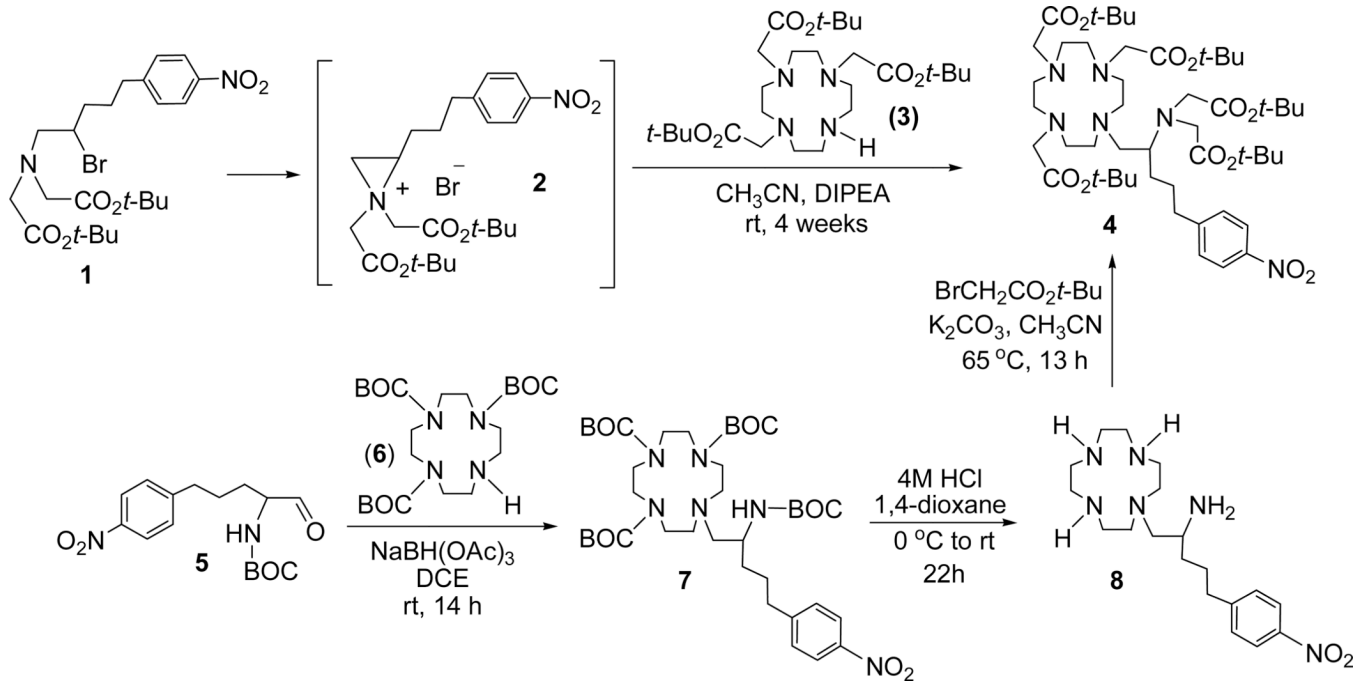
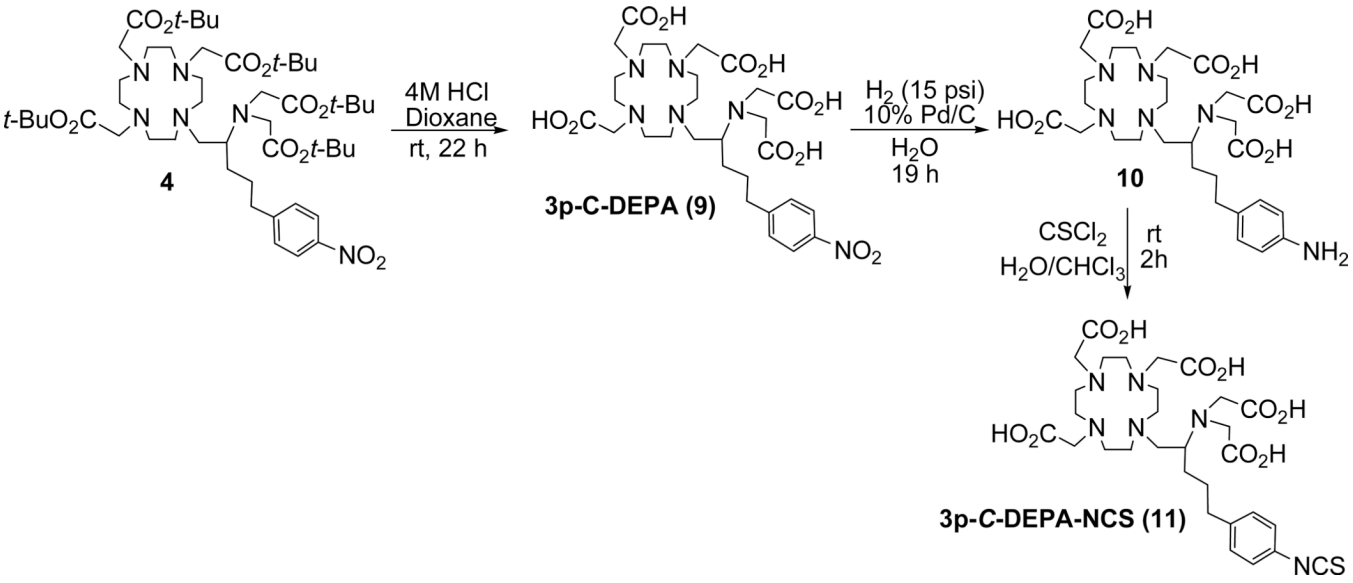


Figure 3.
Biodistribution of $^{205/6}$Bi-*C*-DOTA in athymic mice bearing s.c. LS-174T tumors.



Scheme 1.
Synthesis of Precursor Molecule 4 to 3p-*C*-DEPA



Scheme 2.
Synthesis of 3p-*C*-DEPA (9) and 3p-*C*-DEPA-NCS (11)

Table 1

*Radiolabeling efficiency (%) of bifunctional ligands with $^{205/6}$Bi (0.25 M NH_4OAc, pH 5.5, RT).

Time (min)	3p-*C*-DEPA	*C*-DOTA
1	93.6 ± 0.4	8.4 ± 1.9
5	94.7 ± 0.9	17.2 ± 4.3
10	95.0 ± 0.4	28.7 ± 4.5
20	94.1 ± 0.6	38.0 ± 4.9
30	94.4 ± 1.6	49.7 ± 9.0
60	94.5 ± 0.5	60.2 ± 8.0

*Radiolabeling efficiency (mean ± standard deviation) was measured in triplicate.