Design, Synthesis, and Characterization of a Dual Modality Positron Emission Tomography and Fluorescence Imaging Agent for Monoclonal Antibody Tumor-Targeted Imaging

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A novel lysine-based trifunctional chelate **3** was designed, synthesized, and characterized and bears both a chelating moiety (CHX-A″) for sequestering radiometals (^{86}Y or ^{111}In) and the near-infrared dye Cy5.5 for dual modality PET (or SPECT) and fluorescence imaging, respectively. Successful conjugation of **3** to the monoclonal antibody trastuzumab (Herceptin) was achieved by efficient thiol-maleimide chemistry, thereby yielding immunoconjugate **2**. Analysis of **2** by flow cytometry and competitive binding assay demonstrates that immunoconjugate **2** binds to SKOV3 tumor cells comparably to native trastuzumab and, thus, may be used as a tumor-targeted monoclonal antibody probe for multimodality imaging.

Introduction

Multimodality imaging is becoming more common as a primary clinical tool for imaging human diseases, especially cancers.[1] Dual labeled imaging probes allow the same target to be evaluated with two different modalities, such as positron emission tomography (PET[a]), computed tomography (CT),[2] magnetic resonance imaging (MRI), near-infrared spectroscopy (NIR) optical imaging (OI).[3] This allows the strengths of each modality to be combined in a single imaging session, thereby improving diagnostic accuracy. The multimodality imaging approach is widely considered to provide a more precise, multiparametric description of a disease process such as its location, extent, metabolic activity, blood flow, and function of target tissue, resulting in better characterization of disease processes. However, the development of imaging agents for multimodality imaging is more challenging than single modality agents, requiring more complex design, multistep synthesis, and careful selection of nuclear and optical tracers to avoid physical−chemical interference between molecular components. Few examples of multimodality agents have been reported in the literature and even fewer have been carried forward for investigation and evaluation in vivo.[4−7] For example, Meade et al. reported a class of dual imaging agents in which multiple copies of both Gd(III)-DTPA and a fluorescent dye were covalently attached to a macromolecular framework of either polylysine or dextran.[4] Meijer et al. labeled a targeting cyclic peptide cNGR with both Gd(III)-DTPA and Oregon Green 488 for potential use in imaging angiogenesis.[5] Bornhop and co-workers coupled a Gd(III)-chelated peripheral-type benzodiazepine receptor ligand to cyclen-based fluorophores, resulting in an agent that was both strongly fluorescent and readily detected by MRI.[6] In our laboratory, amino-terminated PAMAM G6 dendrimers have been employed to carry both Gd(III) and near-infrared dye Cy5.5 moieties.[7] The resulting dual modality dendrimer-based imaging agent demonstrated efficient visualization of sentinel lymph nodes in mice by both MR and fluorescence imaging.[7]

Tumor-specific monoclonal antibodies (mAbs) have been widely used as delivery vectors to transport radiometal ions for cancer imaging and therapy.[8,9] The targeted nature of radiolabeled mAb imaging and therapies offers the promise of greater efficacy, less toxicity, and potentially greater treatment success. The humanized mAb trastuzumab (Herceptin) targets the cell surface antigen HER2, which is overexpressed in a variety of epithelial tumors. Radiolabeling of trastuzumab using isothiocyanate derivatives of the bifunctional chelating agents 1B4M (2-(4-aminobenzyl)-6- methyl-diethylenetriaminepentaacetic acid) and CHX-A″ (*N*-[(*R*)-2-Amino-3-(*p*-aminophenyl)propyl]-*trans*-(*S,S*)-cyclohexane-1,2-diamine-*N,N,N′,N″,N″*-pentaacetic acid) has been thoroughly investigated for tumor targeting and cancer therapy in our laboratory (for example, see trastuzumab conjugate **1** in Figure 1).[9−11] These immunoconjugates have efficient tumor targeting, excellent toleration of the modification, and normal whole body clearance patterns. Therefore, we hypothesized that conjugation of a dual imaging agent to trastuzumab would exploit the proven vector capabilities of monoclonal antibodies to perform multimodality imaging of tumors.

Herein, we report a modular synthetic approach to assemble radioactive metal chelating agents and optical dyes into a "single" trifunctional (tumor targeting, radionuclide, and OI) agent for conjugation to antibodies, peptides, or other potential delivery vectors. In this approach, the near-infrared dye Cy 5.5 and a radiometal chelator, for example, CHX-A″ DTPA (^{86}Y(III) for PET, ^{111}In for SPECT), were introduced to the α-NH$_2$ and

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[a] Abbreviations: PET, positron emission computed tomography; CT, computed tomography; MRI, magnetic resonance imaging; NIR, near-infrared spectroscopy; OI, optical imaging; SPECT, single photon emission computed tomography; DTPA, diethylenetriaminepentaacetic acid; PAMAM, polyaminonamido; G6, generation 6; Cy, cyanine dyes; mAbs, monoclonal antibodies; 1B4M, 2-(4-isothiocyanatobenzyl)-6-methyl-diethylenetriamine pentaacetic acid; CHX-A″, *N*-[(*R*)-2-amino-3-(*p*-aminophenyl)propyl]-*trans*-(*S,S*)-cyclohexane- 1,2-diamine-*N,N,N′,N″,N″*-pentaacetic acid; Boc, *tert*-butyl carbamate; Z, benzyloxy carbonyl; TFA, trifluoroacetic acid; TIS, triisopropylsilane; PBS, phosphate-buffered saline; RIA, radioimmunoassay; SMCC, succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate; NHS, *N*-hydroxysuccinimide; EDCI, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole.
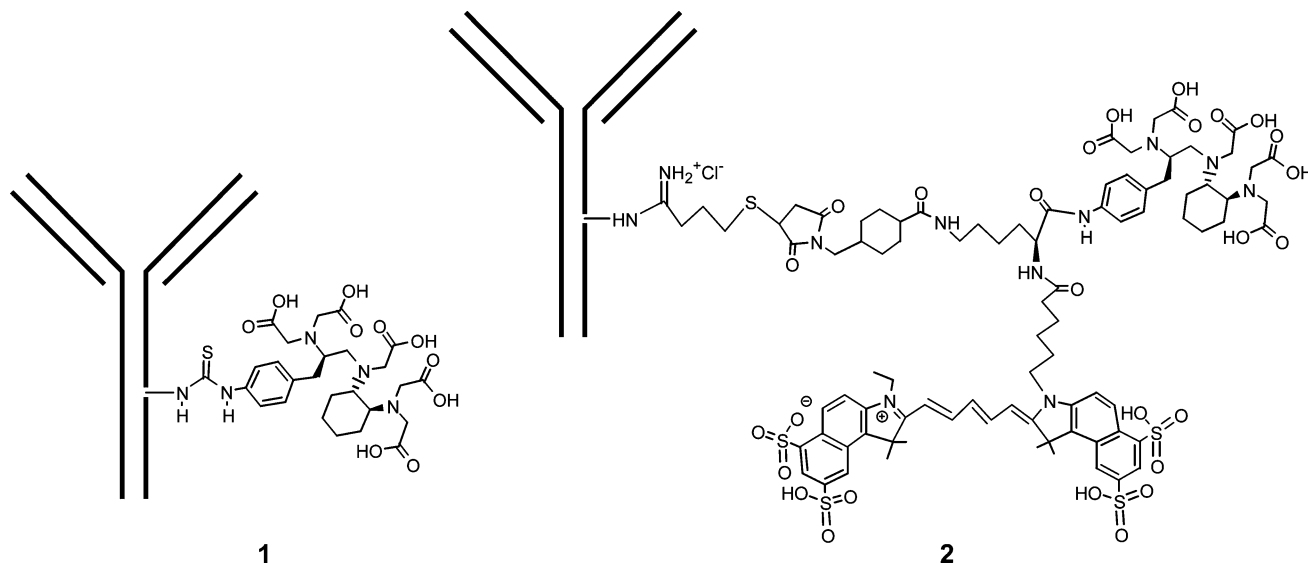
Figure 1. A schematic presentation of CHX-A″ conjugated trastuzumab **1** and trifunctional agent **3** conjugated to trastuzumab to form **2**.

α-COOH of L-lysine, respectively, while the ε-NH₂ was used to introduce a maleimide moiety, thereby permitting conjugation of the novel agent **3** to targeting vectors of interest, in this case, trastuzumab. In addition to the synthesis and characterization of **3**, we demonstrate the successful conjugation of this trifunctional agent **3** to trastuzumab. The resulting radiolabeled immunoconjugate **2** (Figure 1) showed excellent tumor cell binding capabilities comparable with trastuzumab and, thus, may be used as a tumor-targeting probe for dual modality PET (or SPECT) and fluorescence imaging.

Results and Discussion

In current clinical practice, multimodality imaging usually utilizes different imaging agents for each modality. For example, in PET/CT, ^{18}F-deoxyglucose is used for the PET component and iodinated contrast media is used for the CT. If the same imaging agent possessed the signaling components for more than one imaging modality it would be advantageous as it would require a single injection and exact colocalization of pathology based on two or more different parameters. By providing the imaging agents on the same vector, differences in the distribution of the agents would be minimized if not eliminated. This is one of the advantages of multimodality imaging agents such as the trifunctional agent, **3**, presented in this study.

For the synthesis of **3**, we chose to take advantage of the selectively protected lysine derivative, *N*-α-Boc-*N*-ε-benzyloxy-carbonyl-L-lysine, which possesses a carboxylic group and two NH₂ groups with distinguishable reactivity.[12] Other diaminocar-boxylate compounds were considered as starting material for the core of **3**, however, this particular lysine derivative was readily available. The synthetic route for preparing **3** is shown in Figure 2. In brief, *p*-amino functionalized CHX-A″ **4** was reacted with lysine derivative **5** using standard peptide coupling conditions to provide adduct **6** (84%). The ε-NH₂ benzyl carbamate (Z)-protecting group on **6** was quantitatively cleaved by catalytic hydrogenation as monitored by TLC. The liberated ε-NH₂ group was reacted with succinimidyl 4-[*N*-maleimidom-ethyl]cyclohexane-1-carboxylate (SMCC) to yield maleimide **8** (70%). Incorporation of a maleimide function in this step provides a highly reactive group toward thiol groups either extant or introduced into proteins or peptides. The efficient maleimide−thiol coupling chemistry can be performed for the

convenient synthesis of dye-chelate-mAb immunoconjugates. Cleavage of the *tert*-butyl ester on the CHX-A″ moiety and the *tert*-butyl carbamate (Boc) on the α-NH₂ of the lysine were cleanly achieved in one step by treatment of **8** with TFA/H₂O/ TIS (95:2.5:2.5). Finally, NIR dye Cy5.5 mono NHS ester was reacted with the α-NH₂ group of **9** in DMSO. Purification by reverse-phase HPLC using a C18 column gave **3** in ~23% yield. It is noteworthy that the free α-NH₂ group in **9** provides almost limitless possibilities of dye options that can be introduced and that, in principle, any dye bearing active ester or isothiocyanate moieties might be chosen. By our design, the NIR dye was intended to be coupled to the lysine core in the final step of the synthesis primarily because quantum yields of Cy dyes are significantly decreased after treatment with strong acids (e.g., TFA). Another reason for incorporating Cy dyes last is related to the high cost(s) of NIR dyes. Compound **3** was fully characterized by ^{1}H NMR, ES-MS, and HPLC. In the ^{1}H NMR spectra, the aromatic protons of **3** are consistent with those of the published NMR data of Cy 5.5 dye, except two additional doublet peaks from the CHX-A″ fragment. Negative ion ES-MS of **3** gave a *m/z* of 1797.6 and 897.8 for [M − H]⁻ and [M − 2H]²⁻, respectively, which confirmed its identity. The HPLC spectra of **3** showed a single, symmetric peak, supportive of its high purity (see Supporting Information).

Metal ion complexation of ^{111}In was demonstrated by reverse-phase HPLC of the radiolabeled **3** (^{111}In-**3**) under neutral pH conditions. Time-resolved elution of **3** (15.0 min) and ^{111}In-**3** (16.8 min) was observed following a no-carrier-added com-plexation reaction, evident from a nearly 2 min separation between the radiometric and the UV peaks, respectively. An analogous carrier-added (with ^{nat}In) radiolabeling resulted in convergence of the UV peak (16.9 min) and the radiometric peak for ^{111}In-**3** (17.1 min). To conjugate **3** to trastuzumab, the mAb was first dialyzed into the thiolation buffer (50 mM NaHCO₃, 150 mM NaCl, and 10 mM EDTA, pH 8.6) and then reacted with 15 equiv of Traut's agent using the standard procedure.[13] Excess reagents were removed by passing the reaction solution through a PD-10 column. The resulting −SH groups on trastuzumab were quantitated by Ellman's reagent.[14,15] In our hands, ~3.5 −SH groups per trastuzumab were intro-duced as calculated based on the molar absorptivity at 412 nm. The thiolated trastuzumab was then reacted with 5 equiv of **3**
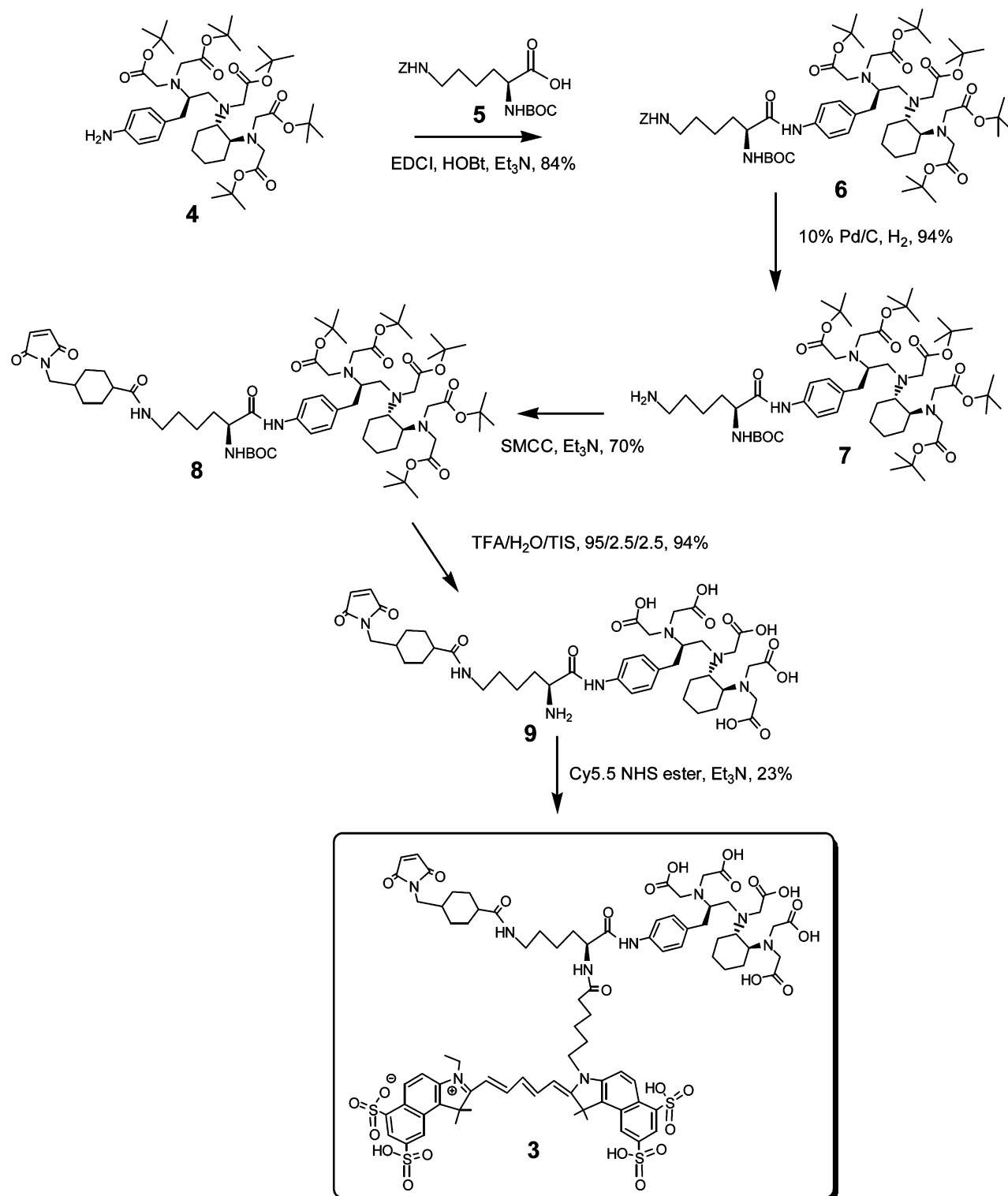
Figure 2. Synthesis of trifunctional chelate Cy5.5-Lys(SMCC)-CHX-A″, **3**.

in the dark at room temperature (RT) for 1 h to produce compound **2**. The unreacted thiols were capped with iodoacetamide to minimize cross-linking of antibody product and to permit a longer shelf life for the immunoconjugate. Finally, the reaction mixture was dialyzed into phosphate-buffered saline (PBS; pH 7.2) at 4 °C, with four buffer changes over 48 h. The final protein concentration was 0.901 mg/mL. The number of Cy5.5 dye moieties per trastuzumab were calculated to be ~2.4 based on UV absorption at 675 nm, which corresponds to the same number of CHX-A″ chelates on trastuzumab according

to the 1:1 ratio of Cy5.5 and CHX-A″ within **3**. Thus, not only does incorporation of Cy5.5 provide an NIR imaging modality in addition to the potential of PET or SPECT imaging, but also provides a direct readout of the degree of protein modification in much the same way that a ^{14}C-labeled chelate would provide.

The immunoreactivity of **2** was found to be retained using three in vitro analytical methods. Analysis by flow cytometry (Figure 3) demonstrates the immunoconjugate's ability to bind HER2; 99.2% of the cells were positive with a mean fluorescence intensity of 106. The competition radioimmunoassay
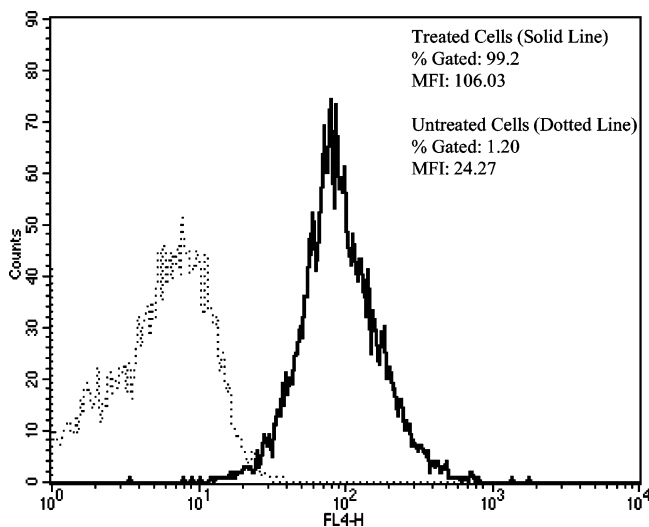
Figure 3. Conjugate **2** was evaluated by flow cytometric analysis. Cells incubated with **2** (solid line) were 99.2% positive, with a MFI of 106.0, while those in buffer (dotted line) were only 1.2% positive, with a MFI of 24.3.

(RIA) demonstrates that modification of trastuzumab with the trifunctional chelate did not affect immunoreactivity as illustrated in Figure 4. The amount of trastuzumab modified with the trifunctional agent **3** required for 50% inhibition was comparable to the amount of native trastuzumab (10 ng vs 9 ng).

Immunoconjugate **2** was radiolabeled efficiently (>79%) with the SPECT radionuclide ^{111}In within 30 min at RT. Figure 5 shows the size exclusion HPLC profile of the labeled product.

The immunoreactivity of radiolabeled **2** (^{111}In-**2**) was then assessed in a direct binding RIA and compared to radiolabeled trastuzumab modified with just the CHX-A″-DTPA chelate. The percent binding of the ^{111}In-**2** and ^{111}In-trastuzumab was 67.7 and 57.9%, respectively. In the presence of excess unlabeled **2**, the values were 17.4% for the ^{111}In-**2** and 8.4% for ^{111}Indium-trastuzumab. These values were comparable to those obtained, 13.8% and 7.8%, when excess unmodified trastuzumab was added in the presence of ^{111}In-**2** and ^{111}Indium-trastuzumab, respectively.

Radiolabeling of the trifunctional chelating agent and its conjugates was facile, and this should be readily applicable to other radionuclides such as ^{86}Y for PET imaging. The ability to label the same vector with both SPECT/PET and OI agents has the advantage of obtaining both a quantitative gross deep tissue assessment and microcellular distribution using the same molecule. Products of the trifunctional chelate would also potentially be amenable for use in endoscopy and intraoperative procedures to aid in mapping the borders of tumors during surgery. Last, we also note that the chemistry described herein is highly flexible. Variations of both chelating chemistry to adjust for other radionuclides of interest combined with the vast array of available dyes suitable for in vivo imaging open a broad spectrum of imaging application for such agents when conjugated to suitable targeting vectors such as monoclonal antibodies, peptides, and possibly to small molecular weight cell surface receptor agents.

In addition, through this modular strategy of synthesizing the trifunctional agent **3** and its conjugates, potent cytotoxic chemotherapeutic agents, such as geldanamycin or doxorubicin, in principle, can also be chemically modified and then attached with either a chelating moiety CHX-A″ or a fluorescent dye Cy5.5, generating a single agent for both therapeutic and imaging and tracking purposes. Therefore, our synthetic ap-

proach opens an extensive range of possibilities for combination of different modality imaging agents and/or therapeutic compounds for cancer imaging and therapy.

Ongoing studies are evaluating the combination of ^{86}Y PET imaging with Cy5.5 NIR OI in both subcutaneous and intraperitoneal xenograft tumor targeting model systems. Results will be forthcoming in the appropriate venue.

Materials and Methods

N-[(*R*)-2-Amino-3-(*p*-aminophenyl)propyl]-*trans*-(*S*,*S*)-cyclohexane-1,2-diamine-*N*,*N*,*N*′,*N*″,*N*″ -penta-*tert*-butylacetate (**4**) was prepared by the previously described procedure.[16] Boc-Lys(Z)-OH (**5**) was purchased from Novabiochem (San Diego, CA). SMCC was purchased from Pierce Biotechnology (Rockford, IL). Cy5.5 mono NHS ester was obtained from GE Healthcare (Piscataway, NJ). *N*-hydroxysuccinimide (NHS), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI), peptide sequence grade TFA were acquired from Aldrich/Sigma Chemical Company (St. Louis, MO) and used as received. All experiments with moisture- and air-sensitive compounds were carried out under a dried N$_2$ or Ar atmosphere. For column chromatography, Merck 60 Silica Gel was used (70−230 mesh). Thin-layer chromatography (TLC) was performed on silica gel 60 F-254 plates from EM Reagents. All water used was purified using a Hydro Ultrapure Water Purification system (Rockville, MD).

Proton and ^{13}C NMR data were obtained using a Varian Gemini 300 MHz instrument and chemical shifts are reported in ppm on the δ scale relative to TMS, TSP or residual solvent. Proton chemical shifts are annotated as follows: ppm (multiplicity, coupling constant (Hz), integration). Low- and high-resolution mass spectra (HRMS) were obtained on a Waters' LCT Premier time-of-flight mass spectrometer using electrospray ionization (ESI/TOF/MS) in positive ion mode operated at a resolution of 10 000. The electrospray capillary voltage was 3 kV and the sample cone voltage was 60 V. Desolvation temperature was 225 °C and the desolvation gas was nitrogen at 300 L/hr. Accurate masses were obtained using the lock spray mode with Leu-enkephalin as the external reference compound.

Trifunctional chelate **3** was purified by reverse-phase HPLC (RP-HPLC) using a Gilson system equipped with Model 811B solvent mixer and Knauer variable wavelength monitor controlled by Gilson Unipoint System Software. RP-HPLC was performed on a Vydac 5 μm C$_{18}$ reverse-phase 10 mm × 25 cm column equilibrated with 15 mM NH$_4$OAc (pH 7). A gradient of CH$_3$CN that increased from 0% at 0 min to 50% at 30 min was employed.

Boc-Lys(Z)-CHX-A″ Penta-*tert*-butyl Ester (6). Boc-Lys(Z)-OH **5** (0.50 g, 1.32 mmol), EDCI (0.51 g, 2.64 mmol), HOBt (0.36 g, 2.64 mmol), and DIPEA (0.23 mL, 1.32 mmol) were added to a stirred solution of **4** (1.00 g, 1.20 mmol) in DMF (15 mL). The mixture was stirred for 24 h at RT and then concentrated under reduced pressure, diluted with CH$_2$Cl$_2$ (100 mL), and washed successively with water (2 × 100 mL), 5% NaHCO$_3$ (2 × 100 mL), and water (1 × 100 mL). The organic layer was concentrated and the residue was chromatographed on silica gel eluted with EtOAc−EtOH (9:1) to afford **6** (1.20 g, 84%). ^{1}H NMR (DMSO-d_6, 300 Hz) δ 9.77 (s, 1H), 7.45 (d, *J* = 8.7 Hz, 2H), 7.33 (m, 5H), 7.23 (t, *J* = 6.9 Hz, 1H), 7.16 (d, *J* = 8.4 Hz, 2H), 6.92 (d, *J* = 8.1 Hz, 1H), 4.99 (s, 2H), 4.03 (m, 1H), 3.43 (s, 2H), 3.32 (s, 8H), 2.96 (m, 4H), 2.59 (m, 2H), 1.91 (m, 2H), 1.70−1.20 (m, 10H), 1.37 (s, 54H), 1.03 (m, 4H); ^{13}C NMR 172.1, 171.9, 171.8, 171.6, 171.5, 170.1, 156.7, 156.2, 137.9, 136.6, 135.4, 130.0, 128.6, 128.2, 119.5, 80.4, 80.3, 80.25, 80.2, 66.7, 64.1, 63.2, 62.7, 54.0, 53.6, 53.1, 52.2, 40.3, 36.2, 31.4, 29.5, 29.3, 28.5, 28.4, 28.1, 27.1, 26.1, 25.9, 22.6; HRMS calcd for C$_{64}$H$_{103}$N$_6$O$_{15}$ [M + H]$^+$, 1195.7481; found, 1195.7506; Anal. Calcd. for C$_{64}$H$_{102}$N$_6$O$_{15}$•0.5H$_2$O: C, 63.82; H, 8.62; N, 6.98. Found: C, 63.67; H, 8.57; N, 6.88.

Boc-Lys-CHX-A″ Penta-*tert*-butyl Ester (7). A solution of **6** (0.39 g, 0.33 mmol) in MeOH (15 mL) was treated with 10% Pd/C (50 mg) and stirred under a hydrogen atmosphere overnight (normal pressure, supply of hydrogen from a balloon). The mixture was
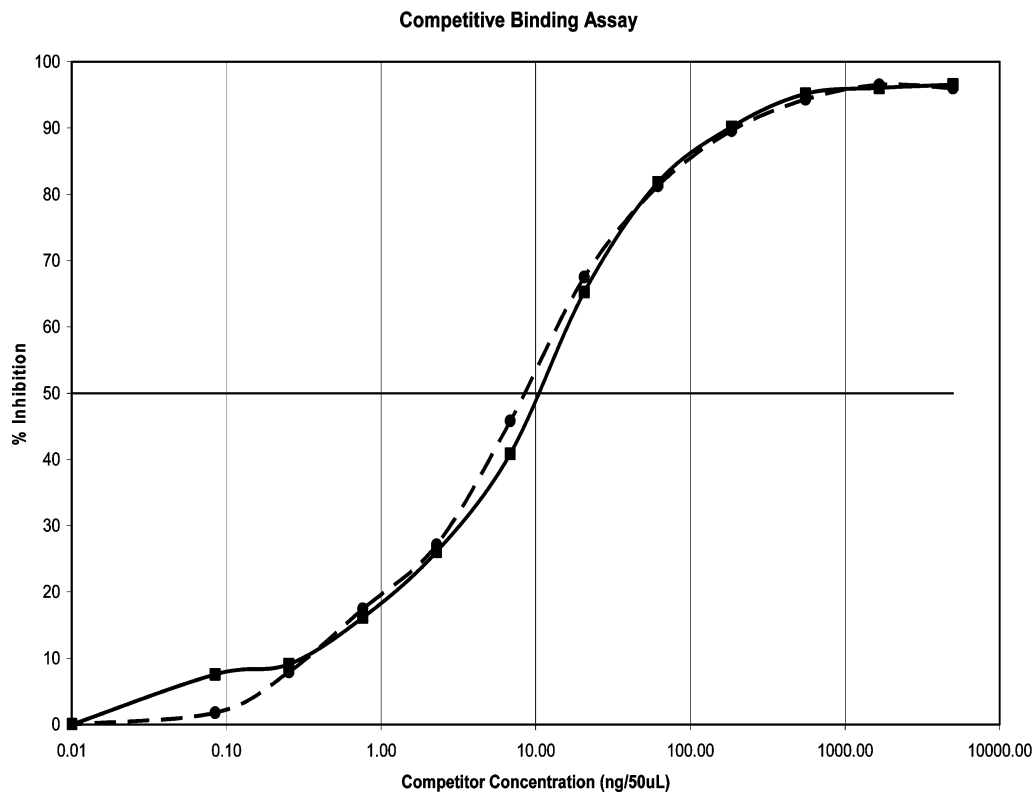
Competitive Binding Assay

Figure 4. Competition radioimmunoassay was conducted to demonstrate that modification with **3** did not alter immunoreactivity of trastuzumab. The ability of **2** (solid line) to react with HER2 expressing SKOV-3 cells and to displace ^{111}In-trastuzumab was compared to unmodified trastuzumab (dashed line).
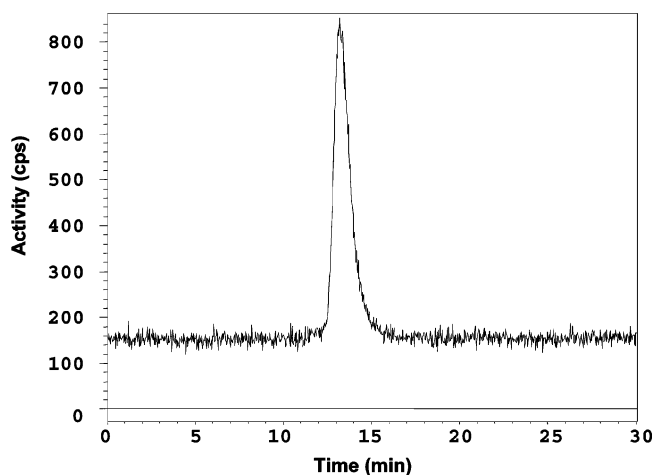

Figure 5. SE-HPLC chromatograph of ^{111}In-**2**.

filtered through Celite 535 (Fluka) and concentrated. The residue was dried under high vacuum to give amine **7** (0.33 g, 94%), which was used without further purification. ^{1}H NMR (DMSO-d_6) δ 9.81 (s, 1H), 7.45 (d, $J = 8.4$ Hz, 2H), 7.16 (d, $J = 8.1$ Hz, 2H), 6.95 (d, $J = 7.0$ Hz, 1H), 4.12 (m, 1H), 3.44 (s, 2H), 3.33 (s, 8H), 2.96 (m, 2H), 2.80−2.40 (m, 6H), 1.89 (m, 2H), 1.59 (m, 4H), 1.39 (s, 54H), 1.50−1.20 (m, 6H), 1.03 (m, 4H); HRMS calcd for $C_{56}H_{97}N_6O_{13}$ [M + H]$^+$, 1061.7114; found, 1061.7140; Anal. Calcd. for $C_{56}H_{96}N_6O_{13}\cdot H_2O$: C, 62.31; H, 9.15; N, 7.79. Found: C, 62.09; H, 9.00; N, 8.09.

Boc-Lys(SMCC)-CHX-A″ Penta-*tert*-butyl Ester (8). To a solution of amine **7** (0.33 g, 0.31 mmol) in DMF (10 mL) was added SMCC (0.10 g, 0.30 mmol) and Et$_3$N (0.04 mL, 0.31 mmol). The mixture was stirred under N$_2$ atmosphere at RT for 18 h. Afterward, the solution twas concentrated and the residue was diluted with CH$_2$Cl$_2$ (100 mL) and washed successively with water (1 × 100 mL), 5% NaHCO$_3$ (1 × 100 mL), and water (1 × 100 mL). The organic layer was concentrated and chromatographed on silica

gel eluted with EtOAc/EtOH (10:0−9:1) to afford **8** as a white solid (0.27 g, 70%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 9.78 (s, 1H), 7.64 (t, $J = 6.0$ Hz, 1H), 7.44 (d, $J = 7.8$ Hz, 2H), 7.16 (d, $J = 8.4$ Hz, 2H), 7.02 (s, 2H), 6.88 (d, $J = 7.0$ Hz, 1H), 4.01 (m, 1H), 3.43 (s, 2H), 3.32 (s, 8H), 3.23 (d, $J = 6.3$ Hz, 2H), 2.97 (m, 4H), 2.59 (m, 4H), 2.01(m, 2H), 1.80−1.00 (3 × m, 22H), 1.37 (s, 54H); HRMS calcd for $C_{68}H_{110}N_7O_{16}$ [M + H]$^+$, 1280.8014; found, 1280.8040; Anal. Calcd. for $C_{68}H_{109}N_7O_{14}\cdot 1.1H_2O$: C, 62.80; H, 8.62; N, 7.54. Found: C, 62.46; H, 8.15; N, 7.44.

NH$_2$-Lys(SMCC)-CHX-A″ (9). Lysine derivative **8** (0.25 g, 0.20 mmol) was stirred with 10 mL of TFA/TIS/H$_2$O (9.5/0.25/0.25) for 4 h. The reaction mixture was concentrated in vacuum to afford amine **9** (0.17 g, 94%). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 10.60 (s, 1H), 7.76 (t, $J = 6.0$ Hz, 1H), 7.63 (d, $J = 7.8$ Hz, 2H), 7.37 (d, $J = 8.4$ Hz, 2H), 7.11 (s, 2H), 4.05 (m, 1H), 3.80−2.60 (m, 20H), 1.80−0.90 (m, 25H), HRMS calcd for $C_{43}H_{62}N_7O_{14}$ [M + H]$^+$, 900.4355; found, 900.4331.

Cy5.5-Lys(SMCC)-CHX-A″ (3). To a solution of amine **9** (0.84 mg, 0.94 μmol) in DMSO (2 mL) was added Cy5.5 mono NHS ester (1.0 mg, 0.88 μmol) and Et$_3$N (20 μL). The reaction mixture was stirred under N$_2$ atmosphere at RT for 18 h and then diluted with diethyl ether (20 mL). The precipitated product was purified by reverse-phase (RP) analytical HPLC on a C$_{18}$ column using 0−50% CH$_3$CN in 15 mM NH$_4$OAc in a 30 min run followed by lyophilization of the solvent from the relevant fraction to yield trifunctional chelate **3** (0.36 mg, 22.7%). ^{1}H NMR (D$_2$O, 300 MHz) δ 8.85 (d, $J = 9.0$ Hz, 2H), 8.80 (s, 2H), 8.36 (m, 2H), 8.25 (d, $J = 9.0$ Hz, 2H), 7.83 (d, $J = 9.3$ Hz, 1H), 7.81 (d, $J = 9.6$ Hz, 1H), 7.43 (d, $J = 8.1$ Hz, 2H), 7.29 (d, $J = 8.1$ Hz, 2H), 6.67 (s, 2H), 4.20 (br triplet, 4H), 3.80−2.60 (m, 22H), 2.34 (t, $J = 6.0$ Hz 2H), 2.15−1.00 (m, 46H); ES-MS *m/z* calcd for $C_{84}H_{104}N_9O_{27}S_4$ [M − H]$^-$, [M − 2H]$^{2-}$ 1796.6, 897.8; found, 1797.6, 897.8.

Radiosynthesis and Characterization of Cy5.5-Lys(SMCC)-^{111}In-CHX-A″ (^{111}In-3). A 200 μCi portion of ^{111}In (Perkin-Elmer, Wellesley, MA) in 0.05 N HCl was added to Cy5.5-Lys(SMCC)-CHX-A″ dissolved in 0.15 M NH$_4$OAc, pH 7. The reaction mixture was incubated at 37 °C for 30 min. An aliquot of the resulting

solution was analyzed by RP-HPLC using a Vydac Protein & Peptide C_{18} column equilibrated with 0.015 M NH_4OAc (pH 7). A 0.0 to 50% gradient of increasing CH_3CN over 30 min was employed, followed by an additional 10 min plateau at 50% CH_3-CN. A UV detector and radiometric detector were coupled to measure absorbance at 254 nm and radioactivity, respectively.

Conjugation of 3 to Trastuzumab (General Procedure). Trastuzumab was dialyzed into thiolation buffer (50 mM $NaHCO_3$, 150 mM NaCl, and 10 mM EDTA, pH 8.6) and reacted with Traut's reagent[13] (Sigma Chemical Co., St. Louis, MO) at a 1:15 molar ratio for 45 min at RT. These conditions were empirically determined to yield ~3.5 −SH groups per trastuzumab molecule. Excess Traut's reagent was removed by passage of the reaction solution through a PD-10 column eluted with the thiolation buffer. The −SH concentration was measured using Ellman's reagent.[15] Just prior to protein conjugation, **3** was dissolved in thiolation buffer and then added dropwise to the mAb solution to achieve a molar reaction ratio of 5:1 (**3**/trastuzumab) and gently vortexed. The solution was gently agitated in the dark at 25 °C for 1 h. Excess free SH groups were capped with iodoacetamide solution (2.0 mM). Finally, the reaction mixture was dialyzed into PBS buffer at 4 °C with 4 buffer changes over 48 h.

^{111}In Labeling of the Immunoconjugates. The trifunctionalized immunoconjugate, **2**, and CHX-A″-herceptin, **1**, were labeled with ^{111}In.[17] The pH of a solution of $^{111}InCl_3$ in 0.05 M HCl (0.85 mCi, 2.5 μL) in a vial was adjusted to 4.5−5.0 with aqueous NH_4OAc (150 μL, 0.15 M, pH 7.0). Solutions of the respective immuno-conjugates in PBS (50 μg, 8−50 μL) were brought up to 100 μL with NH_4OAc (0.15 M, pH 7.0) and then added to the ^{111}In solution, mixed, and incubated at RT for 30 min. The reaction was quenched by the addition of a solution of DTPA (10 μL, 0.001 M, pH 6.5). Protein bound ^{111}In was separated from unbound ^{111}In using a PD-10 column pre-equilibrated and eluted with PBS. The labeling efficiency ranged from 79 to 96%. Figure 5 shows the size exclusion HPLC profile of the ^{111}In-**2**.

Characterization of Trastuzumab−Trifunctional Chelate Conjugate. The final protein concentration of the immunoconjugate was quantified by the Lowry method[18] with a bovine serum albumin (BSA) standard. To assess the final Cy5.5 dye concentration of the trastuzumab−trifunctional chelate conjugate, approximately 1.5 mL of the final product was added to a cuvette and the absorbance was measured at 675 nm in a Beckman-Coulter DU 520 spectro-photometer (Beckman-Coulter, Inc., Fullerton, CA).

Cell Culture. The ovarian adenocarcinoma cell line SKOV3, obtained from ATCC (Manassas, VA), was used for experiments assessing immunoreactivity. SKOV-3 has been shown to express at least 5×10^5 HER2 molecules per cell.[19] The cells were grown in McCoy's 5A medium (Quality Biological, Inc., Gaithersburg, MD) containing 10% Fetalplex (Gemini Bioproducts, Woodland, CA) and 1% nonessential amino acids (BioWhittaker, Walkersville, MD) in a humidified incubator at 37°C in 5% CO_2.

Immunoreactivity Studies. The immunoreactivity of **2** to SKOV3 cells was determined by flow cytometry, competition RIA, and RIA.

Flow Cytometry. Reactivity of **2** was confirmed with HER2 expressing SKOV3 cells using flow cytometric methods as previously described. The cells were incubated with 1.25 ug of trastuzumab for 1 h at 4°C in PBS containing 1% BSA, pH 7.2 (BSA-PBS). Following three washes, the cells were then resuspended in 1 mL of PBS. The cells (10 000 events collected) were analyzed using a FACSCalibur flow cytometer with CellQuest software (Becton-Dickinson, Franklin Lakes, NJ).

Competition Radioimmunoassay. In preparation for the competition RIA, SKOV3 cells (~3×10^5 cells/well) were plated on a 96-well plate and incubated for 6 d. The wells were washed with cold PBS, and 200 μL of a cold 80% MeOH solution was added to each well. Following an overnight incubation at 4 °C, the wells were washed three times with 200 μL of PBS and 200 μL of 1% BSA-PBS was added. After 30 min at RT, the wells were aspirated and serial dilutions of **2** (5000−0.08 ng) were then added to the wells in triplicate (50 μL) followed by the addition of ~33 nCi

(50 μL in BSA-PBS) of ^{111}In-trastuzumab. The RIA was incubated, overnight, at RT. On the following day, the wells were washed three times with 200 μL of BSA-PBS. The radioactivity was removed from the wells by adding 100 μL of 0.2 M NaOH, gently shaking the plate for 10 min and adsorbing the solution to filters (Molecular Device Corp., Sunnyvale, CA). The filters were placed in 12×75 mm polypropylene tubes, and the radioactivity was counted in a γ-scintillation counter (WizardOne, Perkin-Elmer, Shelton, CT). The assay included unmodified trastuzumab as well as HuM195, an anti-CD33 humanized mAb (a generous gift from D. Scheinberg, Memorial Sloan Kettering Cancer Center),[20] served as a negative control. The assay also included wells to which no competitor was added. The percent inhibition was calculated and plotted.

Radioimmunoassay. The immunoreactivity of the ^{111}In-**2** was assessed using methanol-fixed SKOV3 cells. Briefly, the cells were trypsinized, pelleted, and resuspended in 5 mL of PBS. Cold methanol (20 mL) was then added dropwise while vortexing to yield a final solution of 80% methanol. After sitting overnight at 4 °C, the cells were washed with PBS, pelleted, resuspended in BSA-PBS, and aliquoted (2×10^6 in 50 μL) into 12×75 mm polypropylene tubes. Serial dilutions (~84 to ~2 nCi) of ^{111}In-**2** or ^{111}In-trastuzumab (for comparison) were added to the cells in duplicate and gently vortexed. The cells were washed with BSA-PBS following an overnight incubation at RT, pelleted, and the radioactivity was measured in a γ-scintillation counter (WizardOne). The percent binding was calculated for each dilution and the values presented represent an average of the serial dilutions. To confirm the specific reactivity of the ^{111}In-**2**, additional cells were incubated with ~84 nCi of the ^{111}In-**2** along with an excess (10 μg) of either unlabeled trastuzumab or unlabeled **2**.

Supporting Information Available: Analytical data and spectra of intermediate compounds **3** and **6−9** are presented. This material is available free of charge via the Internet at http://pubs.acs.org.

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