Published in final edited form as:

Nucl Med Biol. 2016 September ; 43(9): 566–576. doi:10.1016/j.nucmedbio.2016.06.004.

A comparative evaluation of the chelators H₄octapa and CHX-A''-DTPA with the therapeutic radiometal ⁹⁰Y★

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Abstract

Objectives—To compare the radiolabeling performance, stability, and practical efficacy of the chelators CHX-A''-DTPA and H₄octapa with the therapeutic radiometal ⁹⁰Y.

Methods—The bifunctional chelators *p*-SCN-Bn-H₄octapa and *p*-SCN-Bn-CHX-A''-DTPA were conjugated to the HER2-targeting antibody trastuzumab. The resulting immunoconjugates were radiolabeled with ⁹⁰Y to compare radiolabeling efficiency, *in vitro* and *in vivo* stability, and *in vivo* performance in a murine model of ovarian cancer.

Results—High radiochemical yields (>95%) were obtained with ⁹⁰Y-CHX-A'-DTPA-trastuzumab and ⁹⁰Y-octapa-trastuzumab after 15 min at room temperature. Both ⁹⁰Y-CHX-A''-DTPA-trastuzumab and ⁹⁰Y-octapa-trastuzumab exhibited excellent *in vitro* and *in vivo* stability. Furthermore, the radioimmunoconjugates displayed high tumoral uptake values (42.3 ± 4.0%ID/g for ⁹⁰Y-CHX-A''-DTPA-trastuzumab and 30.1 ± 7.4%ID/g for ⁹⁰Y-octapa-trastuzumab at 72 h post-injection) in mice bearing HER2-expressing SKOV3 ovarian cancer xenografts. Finally, ⁹⁰Y radioimmunotherapy studies performed in tumor-bearing mice demonstrated that ⁹⁰Y-CHX-A''-DTPA-trastuzumab and ⁹⁰Y-octapa-trastuzumab are equally effective therapeutic agents, as

★These authors declare no competing financial interests.

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Ethical approval All applicable institutional and national guidelines for the care and use of animals were followed.

Appendix A. Supplementary data Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nucmedbio.2016.06.004>.

treatment with both radioimmunoconjugates yielded substantially decreased tumor growth compared to controls.

Conclusions—Ultimately, this work demonstrates that the acyclic chelators CHX-A''-DTPA and H₄octapa have comparable radiolabeling, stability, and *in vivo* performance, making them both suitable choices for applications requiring ⁹⁰Y.

Keywords

Yttrium-90; Trastuzumab; CHX-A''-DTPA; H₄octapa; Radiometal; Radioimmunotherapy

1. Introduction

The β^- -emitting radiometal ⁹⁰Y is commonly used for peptide receptor radionuclide therapy (PRRT, e.g., ⁹⁰Y-DOTATOC) and radioimmunotherapy (e.g., ⁹⁰Y-DOTA-trastuzumab) to deliver a therapeutic dose of radiation to tumor cells [1,2]. One advantage of ⁹⁰Y ($t_{1/2}$ =64.1 h, β^- =72%, E_{β^-} =2.288 MeV) compared to other therapeutic isotopes is that it can treat relatively large and poorly vascularized tumors due to its long *in vivo* β^- range of ~12 mm [3–5]. This relatively long particle range means that tumor cells up to ~550 cell diameters away from the radiopharmaceutical will also receive therapeutic irradiation, a phenomenon known as the “crossfire effect” [3–5]. While the crossfire effect is generally regarded as positive due to the ability to treat tumors of larger sizes, an inevitable disadvantage of the long β^- range is the potential irradiation of healthy tissue near the tumor.

Critically, the use of ⁹⁰Y for PRRT and radioimmunotherapy necessitates the use of a pre-therapy scout imaging scan to perform dosimetry, as ⁹⁰Y itself emits no particles or gamma rays suitable for imaging [6,7]. In this type of scout imaging, ⁹⁰Y is replaced with an isotope appropriate for imaging: for example, ¹¹¹In-DOTATOC will be used as an imaging counterpart to ⁹⁰Y-DOTATOC [6,7]. A number of isotopes – including ¹¹¹In, ⁶⁴Cu, ⁸⁹Zr, and ⁶⁸Ga – have been used for dosimetry imaging scans prior ⁹⁰Y-based radiotherapy. However, these approaches leave much to be desired, as labeling a targeting vector (e.g., DOTATOC) with different radiometals can drastically change its *in vivo* properties [6–8]. Indeed, changes in the coordination environment, charge, or stability a radiometal–chelator complex [e.g., ⁹⁰Y(DOTA)¹⁻ vs. ⁶⁴Cu(DOTA)²⁻] can cause significant discrepancies in tumor uptake and organ distribution [9].

For this reason, the use of the positron-emitting radiometal ⁸⁶Y (⁸⁶Y, $t_{1/2}$ = 14.7 h, β^+ = 33%, E_{β^+} =640 keV) for PET imaging prior to ⁹⁰Y-based therapy would appear to be ideal, as the ⁸⁶Y- and ⁹⁰Y-labeled radiopharmaceuticals would be chemically identical isotopologues [10]. Imaging with ⁸⁶Y is a more attractive option than imaging with the commonly-used surrogate isotope ¹¹¹In due to the former's suitability for PET (rather than SPECT) and its identical aqueous coordination chemistry to ⁹⁰Y³⁺. However, the use of ⁸⁶Y³⁺ nonetheless has its fair share of drawbacks. First, when compared to ⁹⁰Y ($t_{1/2}$ ~ 64.1 h), ⁸⁶Y possesses a shorter half-life (14.7 h), which can preclude imaging studies at later time points. In addition, ⁸⁶Y emits a relatively high-energy β^+ (E_{mean} = 640 keV), meaning that the positrons have a long mean-free path length (R_{mean} = 2.5 mm) compared to

other popular positron-emitting radiometals such as ^{64}Cu ($E_{\text{mean}} = 278.2$ keV, $R_{\text{mean}} = 0.56$ mm) and ^{89}Zr ($E_{\text{mean}} = 402.7$ keV, $R_{\text{mean}} = 1.27$ mm) [11]. As a result of this higher β^+ energy and an abundance of gamma emissions (1077, 1153, 1854, 1921 keV), the image quality of ^{86}Y -PET scans is reported as being sub-optimal. However, the subtraction of some of these prompt gamma coincidences can improve image quality and useful images can be obtained from ^{86}Y , despite the shortcomings described above [12]. Another concern that arises from this abundance of high-energy gamma emissions from ^{86}Y is the radiation dose to anyone handling it. As a result, substantial radioactive shielding is required during radiolabeling, and the quantity of ^{86}Y that can be transported in one container is limited [5]. For these reasons – as well as its limited availability in North America and the low purity of the available ^{86}Y – experiments with ^{86}Y were not included in this study. Although ^{86}Y has previously been used successfully, the issues encountered with the available ^{86}Y during this study prevented its inclusion [6,10,13–22].

Regardless of the identity of the metal, any radiopharmaceutical labeled with a metallic isotope must have a suitable chelator attached to the targeting vector (e.g., peptide, antibody) to facilitate the stable coordination of the radiometal ion [9,23–25]. The acyclic chelating ligand H_4octa (Fig. 1) has been previously synthesized and studied with the radiometals ^{111}In ($t_{1/2} \sim 2.8$ days) and ^{177}Lu ($t_{1/2} \sim 6.6$ days) and was observed to possess rapid radiolabeling kinetics and excellent *in vitro* and *in vivo* stability with both radiometals [26–29]. A popular class of targeting vectors to use with longer half-life radiometals such as ^{111}In , ^{177}Lu , and ^{90}Y are antibodies, proteins that possess exquisite selectivity and binding affinity to their target antigens but are also sensitive to elevated temperatures (>40 °C). As a result, a chelator must be used that can coordinate radiometals at relatively low temperatures (e.g., 25–40 °C) so that the structural integrity of the antibody is not compromised. The acyclic chelator H_4octa was demonstrated to radiolabel with ^{111}In and ^{177}Lu in less than 15 min at room temperature. This suggests that radiolabeling performance with the chemically similar tricationic radiometals $^{86}\text{Y}^{3+}$ and $^{90}\text{Y}^{3+}$ should also be favorable.

One of the more common chelators used for $^{86}\text{Y}/^{90}\text{Y}$ is the macrocycle 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA; Fig. 1), which typically requires elevated temperatures of 60–90 °C over a period of 30–60 min for radiometallation [26–29]. The high temperatures required for radiolabeling make DOTA a very poor choice for use with antibodies. A newer and more suitable chelator for this radiometal pair is $\text{CHX-A}''$ -DTPA, which – in contrast to DOTA – can be effectively radiolabeled at ambient temperatures within 15–30 min [26–31]. Although DOTA and $\text{CHX-A}''$ -DTPA are the most established Y^{3+} chelators, a new NOTA derivative 3p-C-NETA has also been used with ^{177}Lu , ^{86}Y , and ^{90}Y and gives high radiolabeling yields in only 5 min at room temperature [32–34].

H_4octa was previously studied with non-radioactive yttrium, with which it formed a complex with thermodynamic stability and solution chemistry comparable to the H_4octa complexes with Lu^{3+} and In^{3+} [26,28,29]. These results with non-radioactive yttrium suggested that testing H_4octa with ^{90}Y should yield fast radiolabeling kinetics and robust stability, as was found with ^{111}In and ^{177}Lu . To determine the suitability of H_4octa for use

with isotopes of Y^{3+} , the bifunctional chelators *p*-SCN-Bn-H₄octapa and *p*-SCN-Bn-CHX-A''-DTPA were conjugated to the antibody trastuzumab and radiolabeled with ^{90}Y , and the resulting radioimmunoconjugates were evaluated *in vitro* and *in vivo*. The stability of the ^{90}Y chelate complexes were evaluated *in vitro* by serum stability and *in vivo* by measuring the uptake of ^{90}Y in organs in which “free” $^{90}Y^{3+}$ is known to accumulate (e.g., bone, liver, kidneys).

2. Materials and methods

2.1. General materials

All solvents and reagents were purchased from commercial suppliers (Sigma Aldrich, St. Louis, MO; TCI America, Portland, OR; Fisher Scientific, Waltham, MA) and were used as received unless otherwise indicated. DMSO used for chelator stock solutions was of molecular biology grade (>99.9%: Sigma, D8418). The bifunctional chelator *p*-SCN-Bn-H₄octapa was synthesized as previously reported [28], and [(*R*)-2-amino-3-(4-isothiocyanatophenyl)propyl]-trans-(*S,S*)-cyclohexane-1,2-diamine-pentaacetic acid (*p*-SCN-Bn-CHX-A''-DTPA) and 1-(4-isothiocyanatophenyl)-3-[6,17-dihydroxy-7,10,18,21-tetraoxo-27-(*N*-acetylhydroxylamino)-6,11,17,22-tetraazaheptaicosine] thiourea (*p*-SCN-Bn-DFO) were purchased from Macrocytics, Inc. (Dallas, TX). UV-Vis measurements for determining antibody stock solution concentrations were taken on a Thermo Scientific Nanodrop 2000 spectrophotometer (Wilmington, DE), using the full-sized antibody (150 kDa) molar extinction coefficient of 210,000 L mol⁻¹. $^{90}YCl_3$ (0.5 M HCl) was purchased from PerkinElmer. ^{89}Zr was produced at Memorial Sloan Kettering Cancer Center using an EBCO TR19/9 variable-beam energy cyclotron (Ebco Industries Inc., British Columbia, Canada) *via* the $^{89}Y(p,n)^{89}Zr$ reaction. ^{89}Zr was purified in accordance with previously reported methods to create ^{89}Zr with a specific activity of 5.3–13.4 mCi/μg (195–497 MBq/μg) [35]. Labeling reactions were monitored using silica-gel impregnated glass-microfiber instant thin layer chromatography paper (iTLC-SG, iTLC-SA, Varian, Lake Forest, CA) and analyzed on a Bioscan AR-2000 radio-TLC plate reader using Winscan Radio-TLC software (Bioscan Inc., Washington, DC). All radiolabeling chemistries were performed with ultra-pure water (>18.2 MΩ cm⁻¹ at 25 °C, Milli-Q, Millipore, Billerica, MA) that had been stirred with Chelex-100 resin (1.2 g/L, 24 h) (BioRad Laboratories, Hercules, CA). Radioactivity in samples was measured using a Capintec CRC-15R dose calibrator (Capintec, Ramsey, NJ). For ^{89}Zr biodistribution studies, a PerkinElmer (Waltham, MA) Automated Wizard Gamma Counter was used for counting radioactivity in organ samples and creating calibration curves for ^{89}Zr . For ^{90}Y biodistribution studies, a PerkinElmer (Waltham, MA) Automated Tri-Carb® 2910 TR liquid scintillation counter was used with samples that had been treated with Solvable™ (PerkinElmer) and Ultima Gold™ high flash-point universal liquid scintillation-cocktail (PerkinElmer).

2.2. Antibody modification

Trastuzumab (purchased commercially as Herceptin; Genentech, San Francisco, CA) was purified using PD10 size-exclusion columns [with phosphate buffered saline (PBS, pH 7.4), 3 times], followed by centrifugal filter units with a 50,000 molecular weight cut off (Amicon® ultra centrifuge filters, Ultracel®-50: regenerated cellulose, Millipore Corp.,

Billerica, MA) (PBS, pH 7.4) to remove α - α -trehalose dihydrate, *L*-histidine, and polysorbate 20 additives. After purification, the antibody was taken up in PBS pH 7.4 and kept in the fridge at 4 °C as a stock solution (12.8 mg/mL). Subsequently, 150 μ L of antibody solution (~1.92 mg antibody) was combined with 850 μ L PBS (pH 7.4), the pH of the resulting solution was adjusted to 8.8–9.0 with 0.1 M Na₂CO₃ (~30 μ L), and 6 equivalents of *p*-SCN-Bn-H₄octapa or *p*-SCN-Bn-CHX-A''-DTPA were added in 10–15 μ L DMSO. The reactions were incubated at 37 °C for 1 h, followed by PD10 purification (PD10, Sephadex G-25 M, PD10 column, GE Healthcare; dead volume = 2.5 mL, product eluted with 2.0 mL fractions of sterile saline), and centrifugal filtration (Amicon 50 kDa) to purify the resultant antibody conjugate. The final immunoconjugate stock solutions were stored in PBS (pH 7.4) at 4 °C.

2.3. ⁹⁰Y-radiolabeling of H₄octapa- and CHX-A''-DTPA-trastuzumab

Aliquots of chelate-modified antibody (200 μ g for ⁹⁰Y) bearing either H₄octapa or CHX-A''-DTPA were transferred to 2 mL microcentrifuge tubes and made up to 0.5 mL with ammonium acetate buffer (pH 5.5, 200 mM, treated with Chelex-100 resin at 1.2 g/L overnight before use). Aliquots of ⁹⁰Y (~0.35 mCi, ~12.9 MBq) were added. The radiolabeling mixtures were allowed to react at 37 °C (500 rpm, thermomixer) for 60 min and then analyzed *via* iTLC-SA (acidic) with an eluent of 50 mM DTPA (pH 2.5). Crude radiochemical yields for ⁹⁰Y-CHX-A''-DTPA-trastuzumab were ~96% and for ⁹⁰Y-octapa-trastuzumab were ~99%. After 60 min, EDTA solution was added to quench the reactions (20 μ L EDTA, 50 mM, pH 5.5), and were then purified using size-exclusion chromatography (PD10), followed by centrifugal filtration (Amicon® ultra 50 kDa, rinsed 3 times with saline). The radiochemical purity of the final purified radiolabeled bioconjugate was assayed by radio-iTLC-SA (DTPA mobile phase, 50 mM, pH 2.5). For ⁹⁰Y reactions, crude radiochemical yields of both compounds were >95%, and purified yields were all >99%. In the iTLC experiments, ⁹⁰Y-octapa-trastuzumab and ⁹⁰Y-CHX-A''-DTPA-trastuzumab remained at the baseline, while ⁹⁰Y³⁺ ions eluted with or near the solvent front (~100–150 mm) with the DTPA mobile phase (50 mM, pH 2.5).

2.4. ⁸⁹Zr-DFO-trastuzumab radiolabeling

⁸⁹Zr-DFO-trastuzumab was prepared as previously published, and *p*-SCN-Bn-DFO was conjugated to trastuzumab using the same method as described above for *p*-SCN-Bn-H₄octapa and *p*-SCN-Bn-CHX-A''-DTPA.[36,37] Briefly, aliquots of ⁸⁹Zr oxalate were neutralized to ~pH 7.0–7.4 using sodium carbonate (1 M), and subsequently mixed with DFO-trastuzumab (~500 μ g, 0.5 mL PBS, pH 7.4) and reacted for 60 min at 37 °C. Radiochemical yields obtained were >99% after 1 h, and ⁸⁹Zr-DFO-trastuzumab was purified as described above for the ⁹⁰Y-labeled radioimmunoconjugates.

2.5. MALDI-TOF MS/MS analysis to determine the number of chelates per antibody

The number of benzylthiourea-linked H₄octapa or CHX-A''-DTPA chelates conjugated to trastuzumab was determined using MALDI-TOF MS/MS (Alberta Proteomics and Mass Spectrometry Facility, University of Alberta, Canada). All experiments were performed in triplicate, and all samples were run along with standard samples of unmodified trastuzumab (run on the same day). 1 μ L of each sample (1 mg/mL) was mixed with 1 μ L of sinapic acid

(10 mg/mL in 50% acetonitrile:water and 0.1% trifluoroacetic acid). 1 μ L of the sample/matrix solution was then spotted onto a stainless steel target plate and allowed to air dry. All mass spectra were obtained using a Bruker Ultraflex MALDI-TOF/TOF (Bruker Daltonics GmbH). Ions were analyzed in positive mode, and external calibration was performed using a standard protein mixture (bovine serum albumin). The mass signals ($M^{+2}/2$) at half the molecular weight of the antibody were taken from each chromatogram and averaged ($n = 3$), and the molecular weight of the unmodified trastuzumab was subtracted from the molecular weight of the modified antibody to determine the mass contributed by the conjugated chelators. The mass difference was divided by the molecular weight of the attached bifunctional chelator (*p*-SCN-Bn-H₄octapa, *p*-SCN-Bn-CHX-A''-DTPA), and the error in each triplicate set of measurements (unmodified trastuzumab vs. modified trastuzumab) was propagated to the final value of the number of chelates per antibody. The MALDI-TOF spectra are available in the supporting information Figs. S1–S9.

2.6. Determination of immunoreactivity

The immunoreactivity of ⁹⁰Y-CHX-A''-DTPA-trastuzumab and ⁹⁰Y-octapa-trastuzumab was determined using specific radioactive cellular-binding assays in which an excess of cells was used (~10 million per microcentrifuge tube). This quantity of cells was assumed to contain effectively “infinite antigen” relative to the quantity of antibody added to each (~6 ng of radiolabeled antibody). To this end, 10 million SKOV3 cells were suspended in microcentrifuge tubes in ~0.2 mL of media in microcentrifuge tubes. Aliquots of the bioconjugates – ⁹⁰Y-octapa-trastuzumab and ⁹⁰Y-CHX-A''-DTPA-trastuzumab (20 μ L of a stock solution containing ~10–20 μ Ci, ~0.37–0.73 MBq, ~3 μ g of radioimmunoconjugate, in 10 mL of 1% bovine serum albumin in PBS pH 7.4) – were added to each tube and mixed by pipette ($n = 4$ each immunoconjugate; final volume: ~220 μ L). The samples were then left to sit on ice for 60 min. Subsequently, the treated cells were pelleted *via* centrifugation (600 g, 4 $^{\circ}$ C, for 2 min), and the media was transferred by pipette to clean microcentrifuge tubes. The cell pellets were not re-suspended and were washed three times with ice-cold PBS (1 mL) and centrifuged (600g, 4 $^{\circ}$ C, 2 min) between each wash. Each wash was collected into an empty microcentrifuge tube (1 mL). Each tube (cell pellet, media, and 3 PBS washes) was measured in a scintillation counter (other isotopes can be counted using an automated gamma counter). Each tube was rinsed with 0.5 mL of PBS and transferred to scintillation vials (5 mL), 4.5 mL of Ultima gold scintillation fluid was added, samples were vortex mixed for 30 s, and then measured on an automated scintillation counter (PerkinElmer). The activity data were background-corrected, and the amount of activity (antibody) bound to the cells was compared to the total amount of activity present in the media and washes. The immunoreactivity was determined by dividing the amount of radioactivity bound to the cell pellet by the total amount of radioactivity present in the cell pellet, the media, and the washes.

2.7. ⁹⁰Y-octapa-trastuzumab and ⁹⁰Y-CHX-A''-DTPA-trastuzumab blood serum competition experiments

Frozen human blood serum was thawed for 30 min, and 500 μ L aliquots were transferred to 2.0 mL Corning microcentrifuge vials. A portion of radiolabeled immunoconjugate (~150 μ Ci, ~50–75 μ g, 500 μ L, purified) was transferred to the blood serum ($n=3$ for each

chelator). Serum competition samples were then incubated at 37 °C with gentle agitation (500 rpm, Eppendorf thermomixer) and analyzed *via* iTLC-SA elution with a DTPA eluent (50 mM, pH 2.5) followed by scanning (Bioscan AR-2000) at time points of 0, 1, 24, 48, 72, and 96 h.

2.8. Cell culture

Human ovarian cancer cell line SKOV3 was obtained from the American Tissue Culture Collection (ATCC, Bethesda, MD) and maintained in a 1:1 mixture of Dulbecco's modified Eagle medium: F-12 medium, supplemented with 10% heat-inactivated fetal calf serum (Omega Scientific, Tarzana, CA), 2.0 mM glutamine, nonessential amino acids, 100 units/mL penicillin, and 100 units/mL streptomycin in a 37 °C environment containing 5% CO₂. Cell lines were harvested and passaged weekly using a formulation of 0.25% trypsin/0.53 mM EDTA in Hank's Buffered Salt Solution without calcium and magnesium.

2.9. Subcutaneous SKOV3 xenografts

All experiments were performed under an Institutional Animal Care and Use Committee-approved (IACUC) protocol, and the experiments followed institutional guidelines for the proper and humane use of animals in research. Six- to eight-week-old athymic nu/nu female mice (NCRNUM) were obtained from Charles River Laboratories (Kingston, NY). Animals were housed in ventilated cages, were given food and water *ad libitum*, and were allowed to acclimatize for approximately 1 week prior to treatment. SKOV3 tumors were induced on the left shoulder by a subcutaneous injection of 5.0×10^6 cells in a 100 μ L cell suspension of a 1:1 mixture of fresh media/BD Matrigel (BD Biosciences, Bedford, Ma). Experiments were performed 3–4 weeks following the injection of the cancer cells.

2.10. ⁹⁰Y-octapa-trastuzumab and ⁹⁰Y-CHX-A''-DTPA-trastuzumab biodistribution studies

The purified ⁹⁰Y immunoconjugates (radiolabeled and purified as described above) were suspended in sterile saline (0.9% NaCl) to a concentration of ~20–25 μ Ci (1.1 MBq, ~15–20 μ g) in 200 μ L. The specific activity of both ⁹⁰Y-octapa-trastuzumab and ⁹⁰Y-CHX-A''-DTPA-trastuzumab was ~1.8 mCi/mg. Biodistribution experiments could not be performed using a gamma counter for ⁹⁰Y, as the strictly β^- -emitting isotope requires organ digestion, bleaching, and scintillation counting. After doses were drawn in syringes, they were weighed before and after injection to obtain the total mass of injected antibody solution. This weight was then used with a set of standards to determine the total injected dose in each mouse in scintillation counts (in counts per mg of injected dose; see below).

Each mouse was injected with ⁹⁰Y-trastuzumab *via* the tail vein and euthanized by CO₂ (g) asphyxiation at time points of 24 and 72 h after the administration of the radioimmunoconjugate (n = 4 per time point). After sacrifice, the blood, tumor, heart, lungs, liver, spleen, pancreas, kidneys, large intestine, small intestine, muscle, bone (femur), and skin (ear) were collected. All organs were rinsed in water after removal and air-dried for 5 min. The organs were collected into pre-weighed glass scintillation vials (20 mL), and samples of well-vascularized tissues with higher blood content – including the blood, spleen, and liver – were obtained in small quantities (50–100 mg) to minimize color quenching in the final digested organ solutions. Subsequently, tissues were weighed and then digested for

scintillation counting. Full tables of biodistribution data for ^{90}Y (%ID/g values), and experimental details for organ digestion are available in the supporting information Tables S1–S4.

A control biodistribution experiment using unbound $^{90}\text{YCl}_3$ was performed as well. In this experiment, an aliquot of pure $^{90}\text{YCl}_3$ (~100 μCi) was transferred to sterile saline (1.0 mL), and doses were drawn (~20 μCi in 200 μL) for injection ($n = 3$). In this case, the biodistribution experiment was performed only a 72 h post-injection. The organ samples were weighed and measured by scintillation counting as described above.

Standards for the ^{90}Y biodistribution experiments were prepared by transferring a small volume of the same injected ^{90}Y -antibody or $^{90}\text{YCl}_3$ doses in saline (in the same formulations as were injected into mice, ~20 μL , ~2 μCi) into pre-weighed glass scintillation vials (20 mL vials, same as used for biodistributions, $n = 5$) and then weighing the mass of the 20 μL transferred mixture. These standards were then processed in the same manner as described above for organ samples and counted on the scintillation counter at the same time as the organ samples. The average number of scintillation counts per milligram of standard injected dose was calculated from these standards (counts per milligram), and the mass of injected dose for each biodistribution (obtained by subtracting weight of empty syringes from full syringes after injection) was then multiplied by this standard factor to obtain a value for the total injected dose for each mouse in scintillation counts.

2.11. ^{90}Y -CHX-A''-DTPA-trastuzumab and ^{90}Y -octapa-trastuzumab preliminary radioimmunotherapy pilot study

Female nude mice ($n = 24$) were xenografted with SKOV3 cells (subcutaneous, left shoulder) as described above. Tumor growth and volume were monitored by caliper measurements, in which the length (L) was taken as the longest dimension, and the width (W) as the shortest. Tumor volume (V) was calculated using the formula [$V = L \times (W/2)^2$]. Once the tumors grew to 50–100 mm^3 , the mice with the most consistent tumors were arranged in cages of 4 with approximately equal average tumor volumes amongst all mice. Mice were arranged into 3 groups ($n = 4$ each) using ear tags and ear punching as methods of sorting and identification. Tumors in several mice did not take, and some tumor sizes were very inconsistent, so smaller groups of $n = 4$ were used for this preliminary radioimmunotherapy pilot study ($n = 5$ for controls). Groups were injected with ^{90}Y -octapa-trastuzumab ($n = 4$, ~100 μCi , 200 μL saline, ~30 μg antibody, ~3.3 mCi/mg SA), ^{90}Y -CHX-A''-DTPA-trastuzumab ($n = 4$, ~100 μCi , 200 μL saline, ~30 μg antibody, ~3.3 mCi/mg), and controls ($n = 5$, 200 μL saline, ~30 μg trastuzumab antibody). New batches of CHX-A''-DTPA-trastuzumab and octapa-trastuzumab were synthesized to preclude aggregation and abnormal spleen uptake similar to that observed in the biodistribution of ^{90}Y -octapa-trastuzumab. Mice were assessed *via* tumor caliper measurements and bodyweight twice per week for the duration of the experiment, which was terminated when tumors approached the size limit of 1000 mm^3 . At the termination of the ^{90}Y therapy study (day 36), mice were injected with ^{89}Zr -DFO-trastuzumab (~130 μCi , ~30 μg) to allow for PET imaging, biodistribution, and autoradiography/histology experiments. These experiments could not be

performed with the strictly β^- -emitting isotope ^{90}Y (due both to the 36 day therapy study and ^{90}Y 's lack of analytically useful gamma or positron emissions).

2.12. Cerenkov luminescence imaging (CLI)

Optical images of Cerenkov radiation (CR) were obtained using an IVIS 200 (Caliper Life Sciences) optical imaging machine. This system uses a cryo-cooled charge-coupled device for high-sensitivity detection of low-intensity sources. The mice that had been injected with ^{90}Y -CHX-A''-DTPA-trastuzumab and ^{90}Y -octapa-trastuzumab for the radioimmunotherapy experiments ($n = 4$ per group; $\sim 100 \mu\text{Ci}/\text{mouse}$) were imaged using two-minute exposures 72 h after the administration of the therapeutic radioimmunoconjugates.

2.13. ^{89}Zr -DFO-trastuzumab PET imaging and biodistribution studies

Mice from the ^{90}Y therapy study (36 days post-injection of ^{90}Y or control) were imaged *via* PET with ^{89}Zr -DFO-trastuzumab ($n = 4$ from each therapy group). To this end, ^{89}Zr -DFO-trastuzumab was radiolabeled, purified, and prepared as described above. PET imaging was performed using a micro-PET R4 rodent scanner (Focus 120, Concord Microsystems). Mice were administered radiolabeled antibody ($\sim 30 \mu\text{g}$, $\sim 130 \mu\text{Ci}$, $\sim 4.8 \text{ MBq}$) in 200 μL sterile saline (0.9% NaCl) *via* intravenous tail vein injection. Approximately 5 min prior to PET image acquisition, mice were anesthetized *via* inhalation of 2% isoflurane/oxygen gas mixture (Baxter Healthcare, Deerfield, IL) and placed on the scanner bed where anesthesia was maintained. PET images were acquired at 72 h, with PET data being recorded via static scans with a minimum of 15 million coincident events ($\sim 30 \text{ min}$). An energy window of 350–700 keV and a coincidence timing window of 6 ns was used. Data were sorted into 2D histograms by Fourier rebinning, and transverse images were reconstructed by filtered back-projection (FBP) into a $128 \times 128 \times 63$ ($0.72 \times 0.72 \times 1.3 \text{ mm}^3$) matrix. The image data were normalized to correct for non-uniformity of response of the PET, dead-time count losses, positron branching ratio, and physical decay to the time of injection, but no attenuation, scatter, or partial-volume averaging correction was applied. The counting rates in the reconstructed images were converted to activity concentrations (percentage injected dose [%ID] per gram of tissue) by use of a system calibration factor derived from the imaging of a mouse-sized water-equivalent phantom containing ^{89}Zr . Images were analyzed using ASIPro VM software (Concorde Microsystems).

These ^{89}Zr -DFO-trastuzumab-treated mice were euthanized for the biodistribution experiments immediately following the single 72 h PET imaging time point. Biodistribution experiments with ^{89}Zr -DFO-trastuzumab did not require organ digestion or scintillation counting, as the gamma emissions from ^{89}Zr could be quantified using an automated gamma counter. The organs from ^{89}Zr biodistribution experiments were placed in pre-weighed test tubes, weighed, and then the amount of radioactivity present was determined using automated gamma counting (PerkinElmer). The counts were background- and decay-corrected from the time of injection and then converted to the percentage of injected dose (%ID) per gram of organ tissue (%ID/g). The radioactivity counts measured in each organ were converted to activity (μCi) using a calibration curve created from known standards

of ^{89}Zr (serial dilution from $\sim 3 \mu\text{Ci } ^{89}\text{Zr}$). Biodistribution data from mice injected with ^{89}Zr -DFO-trastuzumab can be found in the supporting information (Tables S5–S7).

2.14. Autoradiography and histology of ^{89}Zr -DFO-trastuzumab in ^{90}Y treated tumors

Following PET imaging, tumors were excised and embedded in optimal-cutting-temperature mounting medium (OCT, Sakura Finetek) and frozen on dry ice. Series of $10 \mu\text{m}$ frozen sections were then cut. To determine radiotracer distribution, digital autoradiography was performed by placing tissue sections in a film cassette against a phosphor imaging plate (Fujifilm BAS-MS2325; Fuji Photo Film) for an appropriate exposure period at -20°C . Phosphor imaging plates were read at a pixel resolution of $25 \mu\text{m}$ with a Typhoon 7000 IP plate reader (GE Healthcare). After autoradiographic exposure, the same frozen sections were then used for immunohistochemical (IHC) staining and microscopy. The immunohistochemistry detection of Androgen Receptor was performed using a Discovery XT processor (Ventana Medical Systems). A rabbit polyclonal C-erbB2 antibody (Enzo, cat.# ALX-810-227) was used at $10 \mu\text{g/mL}$ concentration. The tissue sections were fixed in 4% paraformaldehyde and blocked for 30 min in 10% normal goat serum, 2% BSA in PBS. The incubation with the primary antibody was done for 5 h, followed by 16 min incubation with biotinylated goat anti-rabbit IgG (Vector labs, cat#:PK6101) in 1:200 dilution. Blocker D, streptavidin–HRP and DAB detection kit (Ventana Medical Systems) were used according to the manufacturer instructions. Sequential sections were stained with H&E. Whole mount images were acquired at $\times 100$ magnification using a BX60 microscope (Olympus America, Inc.) equipped with a motorized stage (Prior Scientific Instruments Ltd.) and DP80 camera (Olympus). Whole-tumor montage images were obtained by acquiring multiple fields at $\times 40$ magnification, followed by alignment using MicroSuite Biologic Suite (version 2.7; Olympus). IHC and autoradiographic images were registered using Adobe Photoshop (CS6) as previously described [38].

3. Results and discussion

3.1. H_4octa pa coordination chemistry

Solution nuclear magnetic resonance (NMR) data and density functional theory (DFT) calculations from a previous study exploring the solution chemistry of H_4octa pa with Y^{3+} suggested that this metal–ligand pair forms complexes similar to those of H_4octa pa with In^{3+} and Lu^{3+} [26]. More specifically, the structures of $[\text{Y}(\text{octa})]^-$ and $[\text{Lu}(\text{octa})]^-$ were predicted for both 8-coordinate (ligand only) and 9-coordinate (ligand and one H_2O) architectures and showed striking similarities. Furthermore, the molecular electrostatic potentials (MEP) of the $[\text{Y}(\text{octa})]^-$ and $[\text{Lu}(\text{octa})]^-$ complexes were mapped onto the surface of the DFT-calculated structures, suggesting very similar charge distributions between the two complexes [26,28]. Potentiometric titrations determined H_4octa pa to have a formation constant ($\log K_{\text{ML}}$) of 18.3 ± 0.1 with Y^{3+} and 20.08 ± 0.9 with Lu^{3+} , revealing high thermodynamic stability [26,28]. Another previous study compared the *in vivo* performance of H_4octa pa-trastuzumab and DOTA-trastuzumab with the radiometals ^{111}In and ^{177}Lu , highlighting the improved radiolabeling properties of H_4octa pa compared to DOTA [28]. This preliminary *in vivo* work demonstrated that H_4octa pa is a strong chelator for the radiometals ^{111}In and ^{177}Lu , and the similarities in non-radioactive characterization

between the $[Y(octapa)]^-$ and $[Lu(octapa)]^-$ complexes suggest that $H_4octapa$ may be an excellent ligand for radiopharmaceutical applications with ^{86}Y and ^{90}Y . The chelator $H_4octapa$ presents an alternative to the “gold standard” chelator CHX-A''-DTPA for radiometals such as ^{111}In , ^{177}Lu , and $^{86}Y/^{90}Y$.

3.2. Bioconjugation, radiolabeling, and in vitro characterization

The purpose of this study was to evaluate the *in vitro* and *in vivo* performance of the chelator $H_4octapa$ with the radiometal ^{90}Y and compare it with the existing ‘gold standard’ yttrium chelator CHX-A''-DTPA. Although DOTA forms superbly stable complexes with radiometals such as ^{111}In , ^{177}Lu , and $^{86}Y/^{90}Y$, its radiolabeling kinetics are very slow, and it the high temperature conditions that it requires for radiolabeling are not compatible with antibodies. To begin, trastuzumab (HER2-targeting antibody, ~150 kDa) was incubated under slightly basic conditions (pH 8.5–9.0) with 6 equivalents of *p*-SCN-Bn- $H_4octapa$ or *p*-SCN-Bn-CHX-A''-DTPA and purified *via* size exclusion chromatography (2 × PD10) and spin filtration (2 × Amicon Ultra 50 kDa). After purification, aliquots of trastuzumab immunoconjugates (10 µL, 1 µg/µL, n = 3) were frozen and sent for MALDI-TOF analysis. Mass spectrometry results indicated that these modifications yielded 1.5 ± 0.1 chelates per antibody in the case of *p*-SCN-Bn- $H_4octapa$ and 1.0 ± 0.6 chelates per antibody in the case of *p*-SCN-Bn-CHX-A''-DTPA.

$H_4octapa$ -trastuzumab and CHX-A''-DTPA-trastuzumab were radiolabeled with $^{90}Y^{3+}$ in NH_4OAc buffer (pH 5.5, 200 mM, treated with Chelex-100 resin) for 60 min at 37 °C. Radiolabeling yields were evaluated using iTLC-SA (acidic) silica-embedded paper strips, and a mobile phase of DTPA (pH 2.5, 50 mM) was used to resolve “free” $^{90}Y^{3+}$ from antibody-bound radiometal (Fig. 2). High radiochemical yields were easily achieved with $^{90}Y^{3+}$ (>95% radiochemical yield crude, >99% purity after purification). Both $H_4octapa$ -trastuzumab and CHX-A''-DTPA-trastuzumab (50 µg) were also radiolabeled with ^{90}Y at room temperature to compare their kinetics. In these experiments, the radiochemical yields were evaluated after 15 min, with both immunoconjugates obtaining crude radiochemical yields of >95% (by iTLC), revealing equally facile radiolabeling kinetics with ^{90}Y .

The radiolabeled immunoconjugates were evaluated by a cell-binding assay to determine their immunoreactivity (Table 1, % of native binding affinity). This *in vitro* assay used the same SKOV3 cancer cells used for *in vivo* experiments. Briefly, the antigen over-expressing cells (10 million SKOV3 per microcentrifuge tube [39]) were mixed with a small quantity of radioimmunoconjugate (~6 ng), and the percent radioimmunoconjugate bound to cells after washing was determined as the % immunoreactivity. The immunoreactivity values for ^{90}Y -CHX-A''-DTPA-trastuzumab and ^{90}Y -octapa-trastuzumab were $93.9 \pm 0.9\%$ and $91.2 \pm 0.8\%$, respectively. These values indicate that chelator conjugation and radiolabeling yielded the final radioimmunoconjugates with minimal antibody degradation or radiolysis, and did not disrupt their ability to bind their target antigen. In order to test the stability of the radioimmunoconjugates under biologically relevant conditions, both constructs were incubated in human serum for 3 days at 37 °C. At the end of the experiment (96 h), the stability of the ^{90}Y -CHX-A''-DTPA-trastuzumab and ^{90}Y -octapa-trastuzumab conjugates

were found to be excellent: $87.1 \pm 0.9\%$ and $94.8 \pm 0.6\%$, respectively (Table 1, Fig. 3). In summary, the radiolabeling yields, radiochemical purity, immunoreactivity, and serum stability results were excellent for both ^{90}Y -CHX-A''-DTPA-trastuzumab and ^{90}Y -octapa-trastuzumab. Despite the problems encountered with ^{86}Y that precluded its inclusion in this study, ^{86}Y remains an attractive and potentially useful isotope for PET imaging applications.

3.3. Biodistribution studies of ^{90}Y -CHX-A''-DTPA-trastuzumab and ^{90}Y -octapa-trastuzumab

To expand further our comparison of the new chelator H_4octapa to the incumbent CHX-A''-DTPA, the *in vivo* performance of the ^{90}Y -labeled immunoconjugates was assessed *via* biodistribution experiments in a model system using HER2-expressing SKOV3 ovarian cancer cells. Each of the radiolabeled antibodies – ^{90}Y -CHX-A''-DTPA-trastuzumab and ^{90}Y -octapa-trastuzumab – was injected *via* tail vein into female nude athymic mice bearing subcutaneous SKOV3 ovarian cancer xenografts on the left shoulder ($\sim 20\text{--}30\ \mu\text{Ci}$, $0.74\text{--}1.1\ \text{MBq}$, $\sim 15\text{--}20\ \mu\text{g}$, in $200\text{--}250\ \mu\text{L}$ of sterile saline; tumor volume $\sim 100\text{--}150\ \text{mm}^3$). After 24 and 72 h ($n = 4$ per time point), the mice were euthanized *via* CO_2 (g) asphyxiation, and 14 organs including the SKOV3 tumors were removed, weighed, and assayed for radioactivity content on a scintillation counter (digested and bleached organ samples). A control biodistribution experiment was also performed with $^{90}\text{YCl}_3$, which illustrated that “free” $^{90}\text{Y}^{3+}$ accumulates primarily in the lungs, liver, spleen, kidneys, and bones.

The biodistribution profiles of both ^{90}Y -labeled immunoconjugates were nearly identical with the exception of higher uptake of ^{90}Y -octapa-trastuzumab in the spleen and slightly higher tumor uptake with ^{90}Y -CHX-A''-DTPA-trastuzumab. Higher spleen uptake is typically a result of antibody aggregation, which can occur during the storage, radiolabeling, purification, or formulation of radiolabeled antibodies. The slightly lower tumor uptake observed for ^{90}Y -octapa-trastuzumab could be because part of the injected dose was trapped in the spleen (possibly due to aggregation) instead of being free to accumulate in the tumor. The uptake of both radioimmunoconjugates was identical (within error) in organs that are known to accumulate free $^{90}\text{Y}^{3+}$ (e.g., kidneys, liver, bone), which suggests that the higher spleen uptake observed for ^{90}Y -octapa-trastuzumab was not a result of chelate instability. The control group of $^{90}\text{YCl}_3$ clearly demonstrates which organs “free” ^{90}Y accumulates in, suggesting that both chelators offer equivalent and impressive *in vivo* stability (Fig. 4). Importantly, subsequent radioimmunotherapy experiments performed with ^{90}Y -CHX-A''-DTPA-trastuzumab and ^{90}Y -octapa-trastuzumab (*vide infra*) showed no signs of abnormal spleen uptake for either tracer. Antibody aggregation sometimes occurs during radiolabeling, purification, and handling, and it appears that it did not occur for this ^{90}Y therapy study. This comparison suggests that both H_4octapa and CHX-A''-DTPA are highly stable and appropriate choices for ^{90}Y chelation for *in vivo* use.

3.4. ^{90}Y Radioimmunotherapy and Cerenkov luminescence imaging using ^{90}Y -CHX-A''-DTPA-trastuzumab and ^{90}Y -octapa-trastuzumab in mice bearing subcutaneous SKOV3 xenografts

After the successful radiolabeling and biodistribution results with ^{90}Y described above, it was deemed prudent to test these two radioimmunoconjugates for their practical ability to deliver therapeutic doses of ^{90}Y to target tissue. To this end, therapeutic doses ($100\ \mu\text{Ci}$, 3.7

MBq) of both ^{90}Y -labeled radioimmunoconjugates were injected into cohorts of mice bearing subcutaneous SKOV3 xenografts. More specifically, three cohorts of mice were prepared ($n = 4$ each treatment, $n = 5$ control): ^{90}Y -CHX-A''-DTPA-trastuzumab (30 μg , 100 μCi , 3.7 MBq), ^{90}Y -octapa-trastuzumab (30 μg , 100 μCi , 3.7 MBq), and controls (30 μg non-radiolabeled trastuzumab). Cerenkov luminescence imaging (CLI) was performed 3 days after the injection of the radioimmunoconjugates, which provided visual confirmation of tumor uptake that could not be obtained by PET or SPECT imaging with ^{90}Y (Fig. 5). To ensure that no abnormal uptake in the spleen was observed for either antibody (typically the result of antibody aggregation during radiolabeling and the formulation of doses), the mice were also imaged by CLI on the left side (the side opposite the subcutaneous SKOV3 xenografts). The spleen sits close to the skin on the left side of mice, and therefore high spleen uptake should be observable by CLI. No accumulation in the spleen could be observed by CLI in this experiment, suggesting no abnormal uptake in the spleen.

The tumor volumes of the ^{90}Y -treated mice were monitored twice per week using caliper measurements, and the general health of the mice was observed by appearance and body weight. This preliminary radioimmunotherapy pilot study was started with larger cohorts of mice bearing SKOV3 xenografts ($n=8$ per group) to account for tumors that did not take and for tumors that were much larger or smaller than average. This approach yielded cohorts of $n=4$ with similarly sized tumors for each therapy group and $n=5$ for the control group. By day 36, the control tumors were approaching the 1000 mm^3 limit of the study, so this time was chosen as the end point for the study. A plot of the normalized tumor growth vs. time is displayed in Fig. 6 and clearly shows nearly identical treatment efficacy for the ^{90}Y -CHX-A''-DTPA-trastuzumab and ^{90}Y -octapa-trastuzumab groups, with substantial reductions in tumor growth rate being compared to the control group. This demonstrates that the serum stability and biodistribution experiments – which suggested comparable stability and *in vivo* performance of ^{90}Y -CHX-A''-DTPA-trastuzumab and ^{90}Y -octapa-trastuzumab – did indeed translate to equivalent radioimmunotherapeutic efficacy as measured by tumor growth response. The slightly lower tumor uptake observed in the biodistribution study of ^{90}Y -octapa-trastuzumab could have been a result of higher spleen uptake due to a minor amount of aggregation of the antibody during radiolabeling, which was not observed during the ^{90}Y therapy study. These results confirm that the chelators H_4octapa and CHX-A''-DTPA are effective choices for ^{90}Y in terms of radiolabeling kinetics, stability, and *in vivo* performance.

3.5. Post-radioimmunotherapy ^{89}Zr -DFO-trastuzumab PET imaging, biodistribution, and autoradiography in mice following radioimmunotherapy

PET scans are commonly used in the clinic to monitor the response of cancer patients to therapy. However, in the case of this radioimmunotherapy study, the therapeutic isotope (^{90}Y) does not emit any particles suitable for imaging. Furthermore, 36 days after the administration of the ^{90}Y -labeled radioimmunoconjugates in this study, too little radioactive ^{90}Y was left to perform autoradiography experiments. At the conclusion of the therapy study, the treated mice were injected with ^{89}Zr -DFO-trastuzumab to facilitate PET imaging and autoradiography of tumor sections. To this end, at the end of the ^{90}Y radioimmunotherapy study (day 36), the mice from each group ($n = 4$) were injected

with ^{89}Zr -DFO-trastuzumab ($\sim 130\ \mu\text{Ci}$, $\sim 4.8\ \text{MBq}$, $\sim 30\text{--}35\ \mu\text{g}$) and imaged after 72 h. The PET images in Fig. 7 illustrate substantial uptake of ^{89}Zr -DFO-trastuzumab in the SKOV tumors of the mice from the ^{90}Y -CHX-A''-DTPA-trastuzumab and ^{90}Y -octapa-trastuzumab radioimmunotherapy therapy cohorts. Not surprisingly, these images provide a telling picture of the substantially larger tumors of the animals in the control group. Furthermore, lower activity concentrations of ^{89}Zr -DFO-trastuzumab were observed in tumors of the control group, most likely due to the large size of the tumors ($\sim 1000\ \text{mm}^3$) and the presence of poorly-perfused necrotic regions.

The localization of ^{89}Zr -DFO-trastuzumab in the mice from the therapy study was evaluated *via* biodistribution, which showed similar tumor uptake and tissue distribution in both therapy groups. Tumor uptake in control mice was lower than therapy groups as a result of the substantially larger tumors (expressed as %ID/g). During the biodistribution necropsy (Fig. 8), pieces of SKOV3 tumor were taken from the ^{90}Y treated and control mice that had been injected with ^{89}Zr -DFO-trastuzumab. Autoradiography and histology (hematoxylin and eosin as well as HER2 staining) were performed on sections of tumors from each therapy group (Fig. 9). Results confirm high expression of HER2 in SKOV3 tumors, with the uptake of ^{89}Zr -DFO-trastuzumab mimicking the distribution that would be expected from the ^{90}Y -labeled radioimmunoconjugates. Interestingly, the distribution of ^{89}Zr -DFO-trastuzumab in the tumors of the ^{90}Y -octapa-trastuzumab therapy group (Fig. 9, row 2) shows high concentrations of ^{89}Zr in a region with low HER2 expression. This is most likely a result of fluid pooling in a necrotic region of the tumor, which likely formed as a result of radioimmunotherapy. ^{89}Zr -DFO-trastuzumab showed similar uptake in both ^{90}Y -CHX-A''-DTPA-trastuzumab and ^{90}Y -octapa-trastuzumab groups (Figs. 7 and 8).

4. Conclusions

The principal benefit of acyclic chelators such as H₄octapa and CHX-A''-DTPA over macrocyclic chelators such as DOTA lies in the ability of the former to be radiolabeled with rapid kinetics without significant heating. Radiolabeling experiments revealed that both CHX-A''-DTPA-trastuzumab and H₄octapa-trastuzumab could be labeled with $^{90}\text{Y}^{3+}$ in very high radiochemical purity after only 15 min at room temperature. Both ^{90}Y -CHX-A''-DTPA-trastuzumab and ^{90}Y -octapa-trastuzumab exhibited excellent *in vitro* and *in vivo* stability, high immunoreactivity, and comparable biodistribution profiles in mice bearing HER2-expressing SKOV3 ovarian cancer xenografts. Furthermore, both ^{90}Y -labeled radioimmunoconjugates displayed identical therapeutic efficacy in radioimmunotherapy studies in mice bearing SKOV3 tumors. In these experiments, 36 days after the administration of the radioimmunoconjugates, the average tumor volume in both treatment groups was $\sim 300\ \text{mm}^3$, a remarkable shift compared to the average volume of $\sim 1000\ \text{mm}^3$ for the tumors of the control group. In the end, these data clearly suggest that H₄octapa and CHX-A''-DTPA are both suitable chelators for use with ^{90}Y .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Eric W. Price was supported by a Natural Sciences and Engineering Research Council (NSERC) postdoctoral fellowship (PDF, 2014–2016). The MSKCC Small-Animal Imaging Core Facility, supported in part by NIH Small-Animal Imaging Research Program (SAIRP) Grant No. R24 CA83084 and NIH Center Grant No. P30 CA08748, is gratefully acknowledged. The Radiochemistry and Molecular Imaging Probe core is also supported in part by NIH Center grant N0P30CA08748. The authors also thank the NIH (Award 4R00 CA178205-2, B.M.Z.).

References

- [1]. Forrer F, Valkema R, Kwekkeboom DJ, de Jong M, Krenning EP. Peptide receptor radionuclide therapy. *Best Pract Res Clin Endocrinol Metab.* 2007; 21:111–29. [PubMed: 17382268]
- [2]. Larson SM, Carrasquillo JA, Cheung N-KV, Press OW. Radioimmunotherapy of human tumours. *Nat Rev Cancer.* 2015; 15:347–60. [PubMed: 25998714]
- [3]. Wadas TJ, Wong EH, Weisman GR, Anderson CJ. Coordinating Radiometals of copper, gallium, indium, yttrium, and zirconium for PET and SPECT imaging of disease. *Chem Rev.* 2010; 110:2858–902. [PubMed: 20415480]
- [4]. Forrer F, Waldherr C, Maecke HR, Mueller-Brand J. Targeted radionuclide therapy with ^{90}Y -DOTATOC in patients with neuroendocrine tumors. *Anticancer Res.* 2006; 26:703–7. [PubMed: 16739341]
- [5]. Holland JP, Williamson MJ, Lewis JS. Unconventional nuclides for radiopharmaceuticals. *Mol Imaging.* 2010; 9:1–20. [PubMed: 20128994]
- [6]. Walrand S, Flux G, Konijnenberg M, Valkema R, Krenning E, Lhommel R, et al. Dosimetry of yttrium-labelled radiopharmaceuticals for internal therapy: ^{86}Y or ^{90}Y imaging? *Eur J Nucl Med Mol Imaging.* 2011; 38:57–68.
- [7]. Pauwels S, Barone R, Walrand S, Borson-Chazot F, Valkema R, Kvols LK, et al. Practical dosimetry of peptide receptor radionuclide therapy with ^{90}Y -labeled Somatostatin analogs. *J Nucl Med.* 2005; 46:92S–8S. [PubMed: 15653657]
- [8]. Nayak TK, Brechbiel MW. (^{86}Y) based PET radiopharmaceuticals: radiochemistry and biological applications. *Med Chem.* 2011; 7:380–8. [PubMed: 21711222]
- [9]. Price EW, Orvig C. Matching chelators to radiometals for radiopharmaceuticals. *Chem Soc Rev.* 2014; 43:260–90. [PubMed: 24173525]
- [10]. Banerjee SR, Foss CA, Pullambhatla M, Wang Y, Srinivasan S, Hobbs R, et al. Preclinical evaluation of ^{86}Y -labeled inhibitors of prostate-specific membrane antigen for dosimetry estimates. *J Nucl Med.* 2015
- [11]. Jødal L, Loirec CL, Champion C. Positron range in PET imaging: non-conventional isotopes. *Phys Med Biol.* 2014; 59:7419. [PubMed: 25386999]
- [12]. Lubberink M, Herzog H. Quantitative imaging of ^{124}I and ^{86}Y with PET. *Eur J Nucl Med Mol Imaging.* 2011; 38:10–8.
- [13]. Nayak TK, Garmestani K, Milenic DE, Baidoo KE, Brechbiel MW. HER1-targeted ^{86}Y -Panitumumab possesses superior targeting characteristics than ^{86}Y -Cetuximab for PET imaging of human malignant mesothelioma tumors xenografts. *PLoS One.* 2011; 6:e18198. [PubMed: 21464917]
- [14]. Nayak TK, Garmestani K, Baidoo KE, Milenic DE, Brechbiel MW. PET imaging of tumor angiogenesis in mice with VEGF-A-targeted ^{86}Y -CHX-A''-DTPA-bevacizumab. *Int J Cancer.* 2011; 128:920–6. [PubMed: 20473899]
- [15]. Nayak T, Regino C, Wong K, Milenic D, Garmestani K, Baidoo K, et al. PET imaging of HER1-expressing xenografts in mice with ^{86}Y -CHX-A''-DTPA-cetuximab. *Eur J Nucl Med Mol Imaging.* 2010; 37:1368–76. [PubMed: 20155263]
- [16]. Nayak TK, Garmestani K, Baidoo KE, Milenic DE, Brechbiel MW. PET imaging of tumor angiogenesis in mice with VEGF-A-targeted ^{86}Y -CHX-A''-DTPA-bevacizumab. *Int J Cancer.* 2010; 128:920–6. [PubMed: 20473899]

- [17]. Milenic DE, Wong KJ, Baidoo KE, Nayak TK, Regino CAS, Garmestani K, et al. Targeting HER2: a report on the *in vitro* and *in vivo* pre-clinical data supporting trastuzumab as a radioimmunoconjugate for clinical trials. *MAbs*. 2010; 2:550–64. [PubMed: 20716957]
- [18]. Schneider DW, Heitner T, Alicke B, Light DR, McLean K, Satozawa N, et al. *In vivo* Biodistribution, PET imaging, and tumor accumulation of ^{86}Y - and ^{111}In -Antimindin/RG-1, engineered antibody fragments in LNCaP tumor-bearing nude mice. *J Nucl Med*. 2009; 50:435–43. [PubMed: 19223400]
- [19]. Wei L, Zhang X, Gallazzi F, Miao Y, Jin X, Brechbiel MW, et al. Melanoma imaging using ^{111}In -, ^{86}Y - and ^{68}Ga -labeled CHX-A''-Re(Arg11)CCMSH. *Nucl Med Biol*. 2009; 36:345–54. [PubMed: 19423001]
- [20]. McQuade P, Miao Y, Yoo J, Quinn TP, Welch MJ, Lewis JS. Imaging of melanoma using ^{64}Cu - and ^{86}Y -DOTA-ReCCMSH(Arg11), a cyclized peptide analogue of α -MSH. *J Med Chem*. 2005; 48:2985–92. [PubMed: 15828837]
- [21]. Palm S, Enmon RM, Matei C, Kolbert KS, Xu S, Zanzonico PB, et al. Pharmacokinetics and Biodistribution of ^{86}Y -trastuzumab for ^{90}Y dosimetry in an ovarian carcinoma model: correlative MicroPET and MRI. *J Nucl Med*. 2003; 44:1148–55. [PubMed: 12843231]
- [22]. Rösch F, Herzog H, Stolz B, Brockmann J, Köhle M, Mühlensiepen H, et al. Uptake kinetics of the somatostatin receptor ligand [^{86}Y]DOTA-d Phe1-Tyr3-octreotide ([^{86}Y]SMT487) using positron emission tomography in non-human primates and calculation of radiation doses of the ^{90}Y -labelled analogue. *Eur J Nucl Med Mol Imaging*. 1999; 26:358–66.
- [23]. Zeglis, B.; Holland, J.; Lebedev, A.; Cantorias, M.; Lewis, J. Radiopharmaceuticals for imaging in oncology with special emphasis on positron-emitting agents. In: Strauss, HW.; Mariani, G.; Volterrani, D.; Larson, SM., editors. *Nuclear oncology*. New York: Springer: 2013. p. 35-78.
- [24]. Zeglis BM, Lewis JS. A practical guide to the construction of radiometallated bioconjugates for positron emission tomography. *Dalton Trans*. 2011; 40:6168–95. [PubMed: 21442098]
- [25]. Ramogida CF, Orvig C. Tumour targeting with radiometals for diagnosis and therapy. *Chem Commun*. 2013; 49:4720–39.
- [26]. Price EW, Cawthray JF, Adam MJ, Orvig C. Modular syntheses of H_4octa and H_2dedpa , and yttrium coordination chemistry relevant to $^{86}\text{Y}/^{90}\text{Y}$ radiopharmaceuticals. *Dalton Trans*. 2014; 43:7176–90. [PubMed: 24676528]
- [27]. Price EW, Zeglis BM, Cawthray JF, Lewis JS, Adam MJ, Orvig C. What a difference a carbon makes: H_4octa vs. $\text{H}_4\text{C}_3\text{octa}$, ligands for In-111 and Lu-177 radiochemistry. *Inorg Chem*. 2014; 53:10412–31. [PubMed: 25192223]
- [28]. Price EW, Zeglis BM, Cawthray JF, Ramogida CF, Ramos N, Lewis JS, et al. H_4octa -trastuzumab: versatile acyclic chelate system for ^{111}In and ^{177}Lu imaging and therapy. *J Am Chem Soc*. 2013; 135:12707–21. [PubMed: 23901833]
- [29]. Price EW, Cawthray JF, Bailey GA, Ferreira CL, Boros E, Adam MJ, et al. H_4octa : an acyclic Chelator for ^{111}In radiopharmaceuticals. *J Am Chem Soc*. 2012; 134:8670–83. [PubMed: 22540281]
- [30]. Camera L, Kinuya S, Garmestani K, Wu C, Brechbiel MW, Pai LH, et al. Evaluation of the serum stability and *in vivo* biodistribution of CHX-DTPA and other ligands for yttrium labeling of monoclonal antibodies. *J Nucl Med*. 1994; 35:882–9. [PubMed: 8176477]
- [31]. Harrison A, Walker CA, Parker D, Jankowski KJ, Cox JPL, Craig AS, et al. The *in vivo* release of ^{90}Y from cyclic and acyclic ligand–antibody conjugates. *Int J Rad Appl Instrum B*. 1991; 18:469–76. [PubMed: 1917516]
- [32]. Chong, H-s; Garmestani, K.; Ma, D.; Milenic, DE.; Overstreet, T.; Brechbiel, MW. Synthesis and biological evaluation of novel macrocyclic ligands with pendent donor groups as potential yttrium chelators for radioimmunotherapy with improved complex formation kinetics. *J Med Chem*. 2002; 45:3458–64. [PubMed: 12139456]
- [33]. Chong H-S, Song HA, Ma X, Milenic DE, Brady ED, Lim S, et al. Novel bimodal bifunctional ligands for radioimmunotherapy and targeted MRI. *Bioconjug Chem*. 2008; 19:1439–47. [PubMed: 18564868]

- [34]. Kang CS, Sun X, Jia F, Song HA, Chen Y, Lewis M, et al. Synthesis and preclinical evaluation of bifunctional ligands for improved chelation chemistry of ^{90}Y and ^{177}Lu for targeted radioimmunotherapy. *Bioconjug Chem.* 2012; 23:1775–82. [PubMed: 22881720]
- [35]. Holland JP, Sheh Y, Lewis JS. Standardized methods for the production of high specific-activity zirconium-89. *Nucl Med Biol.* 2009; 36:729–39. [PubMed: 19720285]
- [36]. Zeglis BM, Mohindra P, Weissmann GI, Divilov V, Hilderbrand SA, Weissleder R, et al. Modular strategy for the construction of radiometalated antibodies for positron emission tomography based on inverse electron demand Diels–Alder click chemistry. *Bioconjug Chem.* 2011; 22:2048–59. [PubMed: 21877749]
- [37]. Holland JP, Divilov V, Bander NH, Smith-Jones PM, Larson SM, Lewis JS. ^{89}Zr -DFO-J591 for immunoPET of prostate-specific membrane antigen expression *in vivo*. *J Nucl Med.* 2010; 51:1293–300. [PubMed: 20660376]
- [38]. Carlin S, Zhang H, Reese M, Ramos NN, Chen Q, Ricketts S-A. A comparison of the imaging characteristics and microregional distribution of 4 hypoxia PET tracers. *J Nucl Med.* 2014; 55:515–21. [PubMed: 24491409]
- [39]. DeFazio-Eli L, Strommen K, Dao-Pick T, Parry G, Goodman L, Winslow J. Quantitative assays for the measurement of HER1–HER2 heterodimerization and phosphorylation in cell lines and breast tumors: applications for diagnostics and targeted drug mechanism of action. *Breast Cancer Res.* 2011; 13:R44. [PubMed: 21496232]

Fig. 1.

The bifunctional chelators discussed in this work: *p*-SCN-Bn- H_4 octapa, *p*-SCN-Bn-CHX-A''-DTPA, and *p*-SCN-Bn-DOTA (C-DOTA).

Fig. 2.

Radiolabeling yields with ⁹⁰Y as determined by iTLC (iTLC-SA acidic strips, mobile phase 50 mM DTPA, pH 2.5). Un-coordinated ⁹⁰Y³⁺ elutes with the solvent front of the iTLC-SA strip (~100–125 mm), while the ⁹⁰Y-labeled radioimmunoconjugate remains at the baseline (~25 mm).

Fig. 3. Stability comparison of ^{90}Y -CHX-A''-DTPA-trastuzumab and ^{90}Y -octapa-trastuzumab in human blood serum from 0 to 96 h (37 °C, 500 rpm mixing), showing excellent stability for both radioimmunoconjugates ($87.1 \pm 0.9\%$ and $94.6 \pm 0.3\%$ at 96 h, respectively).

Fig. 4. Biodistribution data showing a comparison of the stability and performance of ^{90}Y -CHX-A''-DTPA-trastuzumab and ^{90}Y -octapa-trastuzumab (S.I.=small intestine, L.I.=large intestine), including a control group inject with $^{90}\text{YCl}_3$ used to demonstrate the primary organs in which “free” $^{90}\text{Y}^{3+}$ accumulates.

Fig. 5. Cerenkov luminescence imaging of ^{90}Y therapy mice 72 h after the administration of ^{90}Y -CHX-A''-DTPA-trastuzumab or ^{90}Y -octapa-trastuzumab (100 μCi per mouse, ~ 30 μg trastuzumab, 2-min imaging time), anesthetized with isoflurane, imaged for 2 min, showing substantial accumulation in the HER2-expressing SKOV3 tumors.

Fig. 6. Normalized tumor growth during the 36 day therapy experiment, showing substantial reductions in tumor growth in the cohorts treated with ^{90}Y -CHX-A''-DTPA-trastuzumab and ^{90}Y -octapa-trastuzumab compared to the control group that was treated with trastuzumab only.

Fig. 7. ^{89}Zr -DFO-trastuzumab PET imaging of representative mice from the ^{90}Y radioimmunotherapy experiment. The mice were injected with ^{89}Zr -DFO-trastuzumab ($\sim 30 \mu\text{g}$, $\sim 130 \mu\text{Ci}$, $\sim 4.8 \text{ MBq}$) on day 36 of the radioimmunotherapy study and imaged 72 h later. The images clearly show higher uptake of ^{89}Zr -DFO-trastuzumab in the tumors of the mice that had been treated with ^{90}Y -CHX-A''-DTPA-trastuzumab and ^{90}Y -octapa-trastuzumab and lower uptake in the much larger tumors of the mice from the control group.

Fig. 8.

Biodistribution of ^{89}Zr -DFO-trastuzumab in the mice from the ^{90}Y radioimmunotherapy experiment. The mice were injected with ^{89}Zr -DFO-trastuzumab on day 36 of the radioimmunotherapy trial and euthanized for biodistribution 72 h after the administration of the imaging agent.

Fig. 9.

Sections of tumors from the ^{89}Zr -trastuzumab PET/biodistribution obtained at the conclusion of the ^{90}Y radioimmunotherapy trial, showing representative SKOV3 tumors from the (1) ^{90}Y -CHX-A''-DTPA-trastuzumab group, (2) ^{90}Y -octapa-trastuzumab group, and (3) control (trastuzumab only) group. Panels display (A) hematoxylin- and eosin (H&E)-stained tumor sections outlining the architecture of the tumor; (B) immunofluorescence staining of HER2 expression; (C) digital autoradiography demonstrating the distribution of ^{89}Zr -DFO-trastuzumab within the tumors; and (D) overlay of the digital autoradiograph with the H&E and HER2 staining.

Table 1

Chemical and *in vitro* biological characterization data for ^{90}Y -CHX-A''-DTPA-trastuzumab and ^{90}Y -octapa-trastuzumab radioimmunoconjugates.

Chelator	Isotope	Radiolabeling yields (37 °C, 60 min)	Chelates/mAb ^a	Specific activity (mCi/mg)	Immunoreactive fraction (%) ^b	Serum stability 96 h (%) ^c
H ₄ octapa	^{90}Y	Crude = 99%, Pure = 99%	1.5 ± 0.1	1.8	91.2 ± 0.8	94.8 ± 0.6
CHX-A''-DTPA	^{90}Y	Crude = 96%, Pure = 99%	1.0 ± 0.6	1.8	93.9 ± 0.9	87.1 ± 0.9

^aMALDI-TOF MS/MS mass spectrometry (n = 3) spectra in Figs. S1–S9.

^bDetermined by saturation binding cell assay (10×10^6 SKOV3 cells per vial, n = 4).

^cDetermined by incubation in human serum (37 °C for 96 h; n = 3).