Comparison of bifunctional chelates for $^{64}$Cu antibody imaging

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Abstract

Purpose Improved bifunctional chelates (BFCs) are needed to facilitate efficient $^{64}$Cu radiolabeling of monoclonal antibodies (mAbs) under mild conditions and to yield stable, target-specific agents. The utility of two novel BFCs, 1-Oxa-4,7,10-triazacyclododecane-5-$\text{S}$-(4-isothiocyanatobenzyl)-4,7,10-triacetic acid ($p$-SCN-Bn-Oxo-DO3A) and 3,6,9,15-tetraazaazicyclo[9.3.1]pentadeca-1(15),11,13-triene-4-$\text{S}$-(4-isothiocyanatobenzyl)-3,6,9-triacetic acid ($p$-SCN-Bn-PCTA), for mAb imaging with $^{64}$Cu were compared to the commonly used $^{64}$Cu-DOTA conjugate.

Methods The BFCs were conjugated to trastuzumab, which targets the HER2/neu receptor. $^{64}$Cu radiolabeling of the conjugates was optimized. Receptor binding was analyzed using flow cytometry and radioassays. Finally, PET imaging and biodistribution studies were done in mice bearing either HER2/neu-positive or HER2/neu-negative tumors.

Results $^{64}$Cu-Oxo-DO3A- and PCTA-trastuzumab were prepared at room temperature in >95% radiochemical yield (RCY) in <30 min, compared to only 88% RCY after 2 h for the preparation of $^{64}$Cu-DOTA-trastuzumab under the same conditions. Cell studies confirmed that the immunoreactivity of the mAb was retained for each of the bioconjugates. In vivo studies showed that $^{64}$Cu-Oxo-DO3A- and PCTA-trastuzumab had higher uptake than the $^{64}$Cu-DOTA-trastuzumab at 24 h in HER2/neu-positive tumors, resulting in higher tumor to background ratios and better tumor images. By 40 h all three of the $^{64}$Cu-BFC-trastuzumab conjugates allowed for clear visualization of the HER2/neu-positive tumors but not the negative control tumor.

Conclusion The antibody conjugates of PCTA and Oxo-DO3A were shown to have superior $^{64}$Cu radiolabeling efficiency and stability compared to the analogous DOTA conjugate. In addition, $^{64}$Cu-PCTA and Oxo-DO3A antibody conjugates may facilitate earlier imaging with greater target to background ratios than the analogous $^{64}$Cu-DOTA antibody conjugates.

Keywords Antibody · $^{64}$Cu · Bifunctional chelates · Trastuzumab

Introduction

With the increasing focus on personalized medicine monoclonal antibodies (mAbs) are of particular interest in
the development of molecular imaging agents [1, 2]. The high target affinity of mAbs is advantageous for both diagnostic imaging and radiotherapy. Antibody-based imaging can be used to measure antigen expression in the entire body noninvasively, to stratify patient populations for specific treatments, and to monitor therapeutic response [3]. A targeted agent for radiotherapy [2, 4] can be obtained by incorporating a therapeutic radioisotope into an mAb; substitution of the therapeutic radioisotope with one suitable for imaging would produce an imaging agent that could be used for dosimetry calculations and dose planning [5].

While the specificity of mAbs make them attractive targeting vectors, their large size and sensitive functional groups pose issues for radiopharmaceutical development. The large size of mAbs (150 kDa) could be sterically hindering and may slow down the kinetics of radiolabeling. Increasing the reaction rate by using harsher radiolabeling conditions such as elevated temperature is not a viable option as these conditions may degrade mAbs. Achieving good target to background ratios for imaging and limiting non-target organ dose for therapy are also barriers in developing mAb-based radiopharmaceuticals because of mAbs’ long half-lives in the body and hepatic clearance. As well, mAbs can be slow to penetrate target tissue, such as tumors; therefore localization at the site of interest can take days. Thus, radioligands with longer half-lives are favored to allow imaging 24–72 h after administration of the mAb when optimal target to background ratios are obtained. Smaller antibody fragments or engineered constructs designed to clear rapidly and facilitate earlier imaging protocols have been examined, but frequently these moieties have lower target accumulation and higher kidney uptake [6, 7]. Consequently, despite the aforementioned drawbacks, antibodies are still considered a highly valuable class of targeting vectors in radiopharmaceutical development.

146Cu is a radioisotope that has been investigated for mAb-based radiopharmaceuticals [3, 8–16] due to its applicable physical properties. 146Cu decay generates β+ emissions (17.4%, E\text{max}=0.656 MeV) applicable to positron emission tomography (PET), as well as potentially therapeutic β− emissions (39%, E\text{max}=0.573 MeV). The half-life of 146Cu (T\text{1/2}=12.7 h) is long enough for imaging 24–48 h after administration to accommodate the mAb localization time. The concept of using the same radiopharmaceutical for both imaging and therapy also makes 146Cu an attractive radioisotope for drug development [9, 10].

Generally, a bifunctional chelate (BFC) is used to stably chelate the 146Cu and link it to the mAb. Current BFCs used are not ideal because of poor in vivo stability or the harsh conditions required to efficiently incorporate the radioisotope. One of the most widely used chelates for 146Cu 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) has been shown to have only moderate stability in vivo [3, 13, 17]. 146Cu-radiolabeled complexes with improved stability have been reported with 1,4,7-triazacyclonane-1,4,7-triacetic acid (NOTA) derivatives [18, 19] and cross-bridged 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (CB-TETA) [17, 20], but these chelates require harsher reaction conditions such as elevated temperature to incorporate the radioisotope. Only a few chelates or BFCs have been reported to have both high stability and efficient radiolabeling under mild conditions [15, 21, 22]. Our group previously reported on two novel BFCs, 1-oxa-4,7,10-triazacyclododecane-S-5-(4-nitrobenzyl)-4,7,10-triacetic acid (p-NO2-Bn-Oxo-DOTA) and 3,6,9,15-tetraazabicyle[9.3.1]pentadeca-1(15),11,13-triene-S-4-(4-nitrobenzyl)-3,6,9-triacetic acid (p-NO2-Bn-PCTA), with these desirable characteristics [21]. To further probe the utility of these BFCs, we examined their potential for use in antibody imaging with 146Cu.

Trastuzumab is an antibody with a high affinity for the HER2/neu receptor which is overexpressed in 20–30% of breast cancers and is associated with poor prognosis [23]. The cold antibody is an approved drug (Herceptin, Genentech/Roche) and has been recognized as a potential targeting vector in nuclear medicine. Radiolabeling of trastuzumab, as well as related fragments and affibodies, has been investigated for both diagnostic and radiotherapeutic applications [3, 6, 7, 24–35]. Because trastuzumab is a validated targeting vector that has been widely studied, it is a good model system for comparing the properties of our novel bifunctional chelates to the commonly used DOTA chelate.

In this work we compared the trastuzumab conjugates of the two novel BFCs, p-SCN-Bn-PCTA and p-SCN-Bn-Oxo-DOTA, directly with a DOTA analog (p-SCN-Bn-DOTA) (Fig. 1). For each BFC the radiolabeling kinetics, yield, and the specific activity achievable were optimized. The immunoreactivity of the bioconjugates and the specific uptake of radioactivity in HER2/neu-expressing cell lines were measured to ensure the biological properties of the antibody were not compromised by either the conjugation chemistry or radiolabeling conditions. Finally, in vivo studies in tumor-bearing mouse models compared the target accumulation and target to background ratios of the three 146Cu-radiolabeled BFC-trastuzumab conjugates at 24 and 40 h.

Materials and methods

All solvents and reagents were used as received unless otherwise noted. The BFCs, S-2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-tetraacetic acid (p-SCN-
Bn-DOTA), 1-Oxa-4,7,10-triazacyclododecane-5-S-(4-isothiocyanatobenzyl)-4,7,10-triacetic acid (p-SCN-Bn-Oxo-DO3A), and 3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene-4-S-(4-isothiocyanatobenzyl)-3,6,9-triacetic acid (p-SCN-Bn-PCTA) were acquired from Macrocyclics Inc. (Dallas, TX, USA) and trastuzumab (Hoffman-La Roche, Mississauga, ON, Canada) was obtained from the British Columbia Cancer Agency Pharmacy. 64Cu and 111In were both obtained as dilute HCl solutions (MDS Nordion Inc., Ottawa, ON, Canada). Water was deionized using a Milli-Q Biocel A10 water purification system. Reaction vials were acid washed to remove impurities and trace metals.

The HPLC system used for analysis consisted of a Waters Alliance HT 2795 separation module equipped with a Raytest Gabi Star NaI(Tl) detector and a Waters 996 photodiode array (PDA) detector. Analysis of radiolabeled bioconjugates was done on a Phenomenex Biosep size exclusion column S2000 300×7.8 mm eluting with 0.1 M phosphate buffer pH 7 at 1 ml/min. Positive identifications of the 64Cu-radiolabeled bioconjugates were made by comparison of the retention time of the radiation detector peak and the UV detector peak associated with the bioconjugate.

Preparation of trastuzumab bioconjugates

The bioconjugates were prepared by incubating trastuzumab (42 μmol) with one of the bifunctional chelates, p-SCN-Bn-DOTA, p-SCN-Bn-Oxo-DO3A, or p-SCN-Bn-PCTA, (126 μmol or 3 equivalents) in 50 mM HEPES buffer at pH 8.5 for 20 h. Non-conjugated bifunctional chelates and other low molecular weight impurities were removed by size exclusion chromatography using PD-10 Desalting Columns (GE Healthcare, Waukesha, WI, USA) eluting with 10 mM sodium acetate buffer (pH 5.5).

The number of chelates per antibody was determined after purification. The antibody concentration was determined by a Coomassie protein assay. The chelate concentration was determined by radiolabeling with an excess of high specific activity 111In spiked with a nonradioactive In3+ standard. After 3 h the radiolabeling reaction was quenched with diethylenetriaminepentaacetic acid (DTPA) and HPLC analysis was used to determine the %In bound to the bioconjugate compared to In-DTPA. Number of chelates/mAb=%In bound to bioconjugate × concentration of In3+/concentration of mAb.

Radiolabeling and stability of bioconjugates

64Cu (100–400 MBq) was added to one of the bioconjugates, Oxo-DO3A-, PCTA-, or DOTA-trastuzumab, (10–1,000 μg) in 10 mM sodium acetate buffer pH 5.5 (1 ml). After incubation at room temperature for a period of 30 min to 2 h, the reaction was quenched with the addition of 50 μl of 1 M ethylenediaminetetraacetic acid (EDTA). The 64Cu-radiolabeled bioconjugates were purified from free 64Cu and other low molecular weight impurities by size exclusion chromatography using a PD-10 Desalting Column eluting with phosphate-buffered saline (PBS) or PBS with 0.1% bovine serum albumin (BSA) (PBSB). HPLC analysis was done before purification to determine radiochemical yield (RCY) and after purification to determine radiochemical purity of the final product.

A 400 μl (4 mCi) aliquot of each of the 64Cu-radiolabeled bioconjugates, Oxo-DO3A-, PCTA-, or DOTA-trastuzumab, was added to 500 μl of mouse serum and incubated at 37°C. HPLC analysis to determine any loss of 64Cu to serum proteins was done after 24 and 48 h.

Cell lines

LCC6Vector and LCC6HER2 cell lines were supplied by the BC Cancer Agency (Vancouver, BC, Canada) [36]. Cells were maintained in DMEM supplemented with 2 mM L-glutamine (StemCell Technologies, Vancouver, BC, Canada) and 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA). Frozen cells contained 500 μg/ml of the selective antibiotic G418 (Mediatech, Inc., Herndon, VA, USA). Cells were expanded in DMEM-10% FBS, with no G418, for at least one passage before in vitro studies were started. SK-BR-3 and MDA-MB-231 cell lines were purchased from American Type Culture Collection. SK-BR-3 cells were maintained in RPMI 1640 medium supplemented with 10% FBS.
FBS, under 5% CO₂ at 37°C. MDA-MB-231 cells were maintained in Leibovitz L15 medium supplemented with 10% FBS, under 100% air at 37°C. Prior to use, cells were detached from the surface of the tissue culture flask by treatment with 0.25% trypsin/EDTA.

Flow cytometry

Relative expression levels of HER2/neu for the four cell lines, LCC6HER2, LCC6Vector, SK-BR-3, and MDA-MB-231 were confirmed with monolayer cultures using the Becton Dickinson FACSCalibur Analyzer (Franklin Lakes, NJ, USA) and anti-HER2/neu-PE (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). All washes were performed with PBSB. Cells were harvested with trypsin/EDTA, counted with trypan blue, and prepared as aliquots of 1×10⁶ cells per tube. Cells were washed twice with PBSB (PBS with 0.1% BSA), 20.0 μl of anti-HER2/neu-PE was added, and the cells were incubated for 0.5 h at room temperature. The cells were then washed twice with PBSB, re-suspended with 400 μl PBSB, and filtered before the percent positive cells and fluorescent intensity were measured.

The affinity of the bioconjugates for the HER2/neu receptor was compared to trastuzumab using all three HER2/neu-expressing cell lines and the non-expressing LCC6Vector cell line as a control. Cells were washed twice in PBSB, then re-suspended with 100 μl buffer plus 20 μl of either trastuzumab or bioconjugate at various concentrations between 0.05 and 20.0 μg/ml, and then incubated on ice for 0.5 h. Cells were washed twice more and re-suspended in 100 μl PBSB buffer plus 1 μg/1×10⁶ cells of the secondary antibody [phycoerythrin (PE) conjugated F(ab')₂ fragment of affinity purified anti-human IgG F9(c) (goat)] (Rockland, Gilbertsville, PA, USA), specific for trastuzumab, and incubated again on ice in the dark for 0.5 h. Two final washes were performed; the cells were re-suspended in 400 μl cold PBSB, filtered, and analyzed by flow cytometry for a total of 10,000 events captured. Control studies were also done in a similar manner. A paired Student’s t test (p<0.05) was used to determine statistical differences between bioconjugates and trastuzumab binding with respect to the same cell line.

Cell binding

Cell binding studies with the ⁶⁴Cu-radiolabeled bioconjugates at a single concentration of bioconjugate (25 ng/ml based on trastuzumab concentration) were carried out in triplicate using LCC6HER2, LCC6Vector, SK-BR-3, or MDA-MB-231 cells (4 million cells per experiment). Cells were incubated in the presence of the 0.4 μCi ⁶⁴Cu-radiolabeled bioconjugates for 2 h at room temperature. In control or nonspecific uptake experiments the cells were first saturated by incubating with an excess of trastuzumab for 0.5 h. A reading of the total activity present was measured (pellet + supernatant), after which samples were centrifuged and washed twice in PBSB (0.1% BSA). The pellet was re-suspended in PBSB and the activity that remained with the cell pellet was measured. Student’s t test (p<0.05) was used to determine statistical differences in the cell binding of the radiolabeled bioconjugates.

PET imaging and biodistribution studies

HER2-negative (LCC6Vector) and -positive (LCC6HER2) tumors were grown subcutaneously on Rag2 (disrupted recombination activating gene) mice as reported previously [36]; briefly, 5×10⁶ cells (50 μl) were injected subcutaneously on the lower back of Rag2 mice. Once tumors reached ~100 mm³, mice were injected intravenously with the ⁶⁴Cu-radiolabeled trastuzumab conjugated to DOTA, PCTA, or Oxo-DO3A (5.5-7.4 MBq, 95±15 GBq/μmol) or with free ⁶⁴Cu in PBS. Groups (n=4) of mice (LCC6Vector and LCC6HER2) injected with one of the three radiolabeled bioconjugates (⁶⁴Cu-DOTA-, Oxo-DO3A-, or PCTA-trastuzumab) were imaged at 24 and 40 h post-injection. Mice bearing LCC6Vector and LCC6HER2 tumors injected with the same tracer were imaged in pairs (see Fig. 6). Imaging was carried out in the Siemens Inveon multimodality CT/PET small animal scanner [37]. PET data were carried out in list mode acquisition (30 min) and subsequently histogrammed in a single frame. Images were reconstructed in 3-D using ordered subset expectation maximization (OSEM)-MAP3D algorithms supplied by Siemens following CT-based attenuation scans to correct for the animal’s body mass.

Once imaged, mice were euthanized, and the blood, liver, kidney, muscle, and tumor tissue were harvested, weighed, and placed in a scintillation counter to determine the activity present per gram tissue. Three-dimensional regions of interest (ROIs) were placed on the whole tumor on reconstructed images to determine the per unit activity present in tissue.

Results

Chemistry

The bioconjugates were prepared by linking amine functional groups in trastuzumab with the bifunctional chelate via the formation of a thiourea. The average number of chelates per trastuzumab was determined to be 1.5±0.4, 1.3±0.2, and 1.7±0.5 for the PCTA-, Oxo-DO3A-, and DOTA-trastuzumab bioconjugates, respectively.
The bioconjugates were labeled with $^{64}$Cu at room temperature in a buffered aqueous solution at various concentrations and various reaction times. Radiolabeling efficiency and %RCY were mainly affected by the amount of bioconjugate used, with larger amounts of bioconjugate facilitating higher %RCY (Fig. 2). The bifunctional chelate component of the bioconjugate also influenced the reaction time and the %RCY. PCTA- and Oxo-DO3A-trastuzumab bioconjugates gave higher %RCY in shorter reaction times than the DOTA-trastuzumab bioconjugate. When using 0.5 mg of PCTA- or Oxo-DO3A-trastuzumab for labeling with $^{64}$Cu, >95% RCY was achieved in 15 min, while using 0.5 mg of DOTA-trastuzumab for $^{64}$Cu labeling gave only an 88% RCY even when the reaction time was increased to 2 h. The same trend of lower %RCY for the $^{64}$Cu labeling of DOTA-trastuzumab compared to PCTA- and Oxo-DO3A-trastuzumab was observed when using 0.1 mg of the respective bioconjugate (Fig. 2). As unlabeled and $^{64}$Cu-labeled bioconjugates were not separated, the specific activity achieved was a function of the amount of bioconjugate used in the reaction and the %RCY of that reaction. Because $^{64}$Cu radiolabeling reactions of DOTA-trastuzumab produced lower %RCY, the specific activity (SA) of $^{64}$Cu-DOTA-trastuzumab tended to be lower than the SAs of PCTA- and Oxo-DO3A-trastuzumab for reactions using the same amount of $^{64}$Cu and bioconjugate (Table 1). Purification using a size exclusion column to remove un-reacted $^{64}$Cu always yielded the radiolabeled bioconjugate with ≥99% radiochemical purity.

The stability of the $^{64}$Cu-radiolabeled bioconjugates was determined in the presence of mouse serum. After 24 h both $^{64}$Cu-radiolabeled PCTA-trastuzumab and Oxo-DO3A-trastuzumab were >95% intact, while $^{64}$Cu-radiolabeled DOTA-trastuzumab was only 54% intact. At 48 h $^{64}$Cu-radiolabeled PCTA-, Oxo-DO3A-, and DOTA-trastuzumab were 80, 78, and 26% intact, respectively.

In vitro validation

The relative level of HER2/neu expression in each of the cell lines was determined by flow cytometry. Both the mean fluorescence intensity and the percentage of positive cells confirmed a high level of receptors in the SK-BR-3 cell line ($2\times10^6$ receptors/cell) [26], a moderate level of receptor expression in the LCC6$^{HER2}$ (4.5×10^5 receptors/cell) [36] and MDA-MB-231 (4×10^4 receptor/cell) [25] cell lines, and low expression in the LCC6$^{Vector}$ cell line (1.5×10^4 receptors/cell) [36].

Comparison of the receptor binding of the bioconjugates and the native antibody was done in each of the cell lines. Negligible levels of fluorescence in the controls were observed. Although some variation was observed between each of the bioconjugates and trastuzumab in their binding to each of the cell lines (both % positive cells and mean fluorescence intensity), the differences were not statistically significant (Fig. 3).

Measurement of $^{64}$Cu binding to cells was done to confirm the receptor-specific targeting of the bioconjugates once labeled with $^{64}$Cu. The cell binding was highest in SK-BR-3 and negligible in the LCC6$^{Vector}$. The majority of the binding could be blocked by first incubating the cells with an excess of trastuzumab. The percent of total $^{64}$Cu bound fell to <2% for each of the bioconjugates.

In vivo results

Biodistribution studies were done on HER2/neu-positive (LCC6$^{HER2}$) and HER2/neu-negative (LCC6$^{Vector}$) tumor-bearing mice at 24 and 40 h after injection and the results are summarized in Figs. 4 and 5. While the biodistribution results were largely alike for all three $^{64}$Cu-radiolabeled bioconjugates, some statistically significant differences were noted. For all three, the majority of the activity was in the tumor, blood, and liver at both time points. Uptake in the HER2/neu-positive tumor was significantly higher than in the negative control tumor for each of the $^{64}$Cu-radiolabeled bioconjugates (p<0.01), while no difference in background tissue uptake was observed between the positive and negative tumor-bearing animals. Liver and kidney activity decreased from the 24-h to the 40-h measurements, while blood and muscle tissue remained
relatively constant. The most noteworthy difference was the significantly higher uptake in HER2/neu-positive tumor for $^{64}$Cu-radiolabeled PCTA- and Oxo-DO3A-trastuzumab compared to that for DOTA-trastuzumab ($p$=0.014 and $p$=0.006, respectively). Tumor to background ratios for PCTA- and Oxo-DO3A-trastuzumab were higher than for DOTA-trastuzumab at 24 h (Fig. 3), with the tumor to kidney ratio being >2 times higher ($p$=0.035). The biodistribution of free $^{64}$Cu at 24 h revealed 7.0±0.9%ID/g in the kidney and 20±12%ID/g in the liver, which were not found to be statistically different than the kidney and liver results for the $^{64}$Cu-radiolabeled bioconjugates at 24 h.

PET imaging was also done at 24 and 40 h post-injection, and ROI analysis of the reconstructed images supported the findings of the biodistribution measurements. HER2/neu-positive tumor, kidney, and liver were visible in each of the scans, while the control HER2/neu-negative tumor was more difficult to visualize (Fig. 6). Analysis of the average activity per unit volume of the ROIs around the positive and control tumors gave ratios of HER2/neu-positive to HER2/neu-negative tumor of 1.4, 2.3, and 1.7 at 24 h and 3.7, 2.8, and 3.4 at 40 h for $^{64}$Cu-radiolabeled DOTA-, Oxo-DO3A-, and PCTA-trastuzumab, respectively (Fig. 7).

Discussion

The $^{64}$Cu radiolabeling of the PCTA- and Oxo-DO3A-trastuzumab bioconjugates was superior to the radiolabeling of the DOTA-trastuzumab bioconjugate. Higher radiochemical yields were achievable with the PCTA- and Oxo-DO3A-trastuzumab in less than 30 min. These differences in radiolabeling efficiency for the bioconjugates were more pronounced than previous results with the basic chelate moieties [21], which showed relatively fast, high radiochemical yield labeling with $^{64}$Cu for PCTA and Oxo-DO3A as well as DOTA. The slower reaction kinetics when radiolabeling antibodies usually necessitates heating the reaction [10, 11], but efficient radiolabeling was possible under mild conditions, aqueous buffer, and room temperature. $^{64}$Cu-radiolabeled PCTA- and Oxo-DO3A-trastuzumab may not require purification, as radiochemical yields >95% were obtained for both the PCTA- and Oxo-DO3A-bioconjugates at specific activities that have been effectively used in antibody imaging [9, 10, 13, 16]. Radiochemical yields >95% and similar specific activities have also been reported for $^{64}$Cu radiolabeling of an antibody conjugate of the SarAr BFC (1-N-(4-aminobenzyl)-3,6,10,13,16,19-hexaazabicyclo[6.6.6]eicosane-1,8-diamine) [15]. This is a potential advantage over the DOTA-trastuzumab bioconjugate, which would require

<table>
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<tr>
<th>Amount of conjugate (mg)</th>
<th>Average specific activity (GBq/mg)</th>
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<tr>
<td>DOTA-trastuzumab</td>
<td>Oxo-DO3A-trastuzumab</td>
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<tr>
<td>0.5</td>
<td>0.24±0.13</td>
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<tr>
<td>0.1</td>
<td>0.34±0.05</td>
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<td>0.01</td>
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These specific activities achieved with >95% radiochemical purity without post-labeling purification.

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A

**Fig. 3** Flow cytometry analysis of trastuzumab and the bioconjugates Oxo-DO3A-, PCTA-, and DOTA-trastuzumab binding to four cell lines with different levels of HER2/neu expression (20 ng of trastuzumab or bioconjugate incubated with 1×10^6 cells and 10,000 events counted). a Percent positive cells. b Mean fluorescence intensity

B

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Fig. 4 Biodistribution of $^{64}$Cu-radiolabeled Oxo-DO3A- (red), PCTA- (blue), and DOTA-trastuzumab (yellow) at 24 h (a) and 40 h (b) post-injection in LCC6$^{HER2}$ xenograft (solid) or LCC6$^{Vector}$ (checkered) tumor-bearing mice.

Fig. 5 Tumor to tissue ratios in LCC6$^{HER2}$ tumor-bearing mice at 24 h (solid) and 40 h (striped) post-injection for $^{64}$Cu-radiolabeled Oxo-DO3A- (red), PCTA- (blue), and DOTA-trastuzumab (yellow).
post $^{64}$Cu radiolabeling purification, as an average radiochemical yield of only 88% was obtainable under the same conditions, even with extended reaction times. Similar results have been reported for the $^{64}$Cu radiolabeling of other DOTA-antibody conjugates, although typically at 37 or 40°C rather than at room temperature [9, 11, 13]. The highly efficient radiolabeling of PCTA- and Oxo-DO3A-antibody bioconjugates under mild conditions suggests that these BFCs may be applicable to simple kit formulation for antibody labeling without need for post-labeling purification.

Despite the low number of chelates per antibody (average of 1.3–1.7), high specific activities, $>$3 GBq/mg, were achievable for all of the bioconjugates with $>$99% radiochemical purity after size exclusion purification; to the best of our knowledge these specific activities are higher than previously reported for antibody labeling with $^{64}$Cu.

Cell studies with both the unlabeled bioconjugates and the $^{64}$Cu-radiolabeled bioconjugates were done to validate the HER2/neu affinity and specificity of the agents. The preparation of the bioconjugates was optimized to minimize the number of BFCs attached to a single trastuzumab entity in an effort to limit the impact of the chelate on the biological properties of trastuzumab. Cell studies confirmed that the immunoreactivity of the antibody was not compromised and that each of the $^{64}$Cu-radiolabeled bioconjugates could selectively bind HER2/neu-expressing cells. Since the BFCs are similar in structure and are linked to trastuzumab in the same manner, it is not surprising that no significant differences between the bioconjugates were noted with respect to target affinity or specificity in vitro.

Unexpected differences in the biodistribution and imaging study results suggested that PCTA- and Oxo-DO3A-antibody bioconjugates may have advantages over DOTA-antibody bioconjugates in addition to the improved $^{64}$Cu radiolabeling noted. Because of their large size, antibodies typically dominate the pharmacokinetic properties of radiolabeled antibody agents. Hence, it was not expected for the different BFCs to have a large impact on the biodistribution of the $^{64}$Cu-radiolabeled bioconjugates. Other studies have shown small but significant differences in kidney and liver uptake and clearance for $^{64}$Cu-radiolabeled antibodies with different BFCs [14, 15]. In this work there was no statistical difference between the $^{64}$Cu-radiolabeled bioconjugates with respect to the %ID/g found in each of the background tissues measured (liver, kidney, muscle) or the blood and the results were in line with the other reports of $^{64}$Cu-radiolabeled antibody biodistributions at similar time points [9, 12, 13]. The unexpected outcome was the significantly higher HER2/neu-positive tumor uptake at 24 h for $^{64}$Cu-radiolabeled PCTA- and Oxo-DO3A-trastuzumab compared to that for $^{64}$Cu-DOTA-trastuzumab. Both the $^{64}$Cu-radiolabeled PCTA- and Oxo-DO3A-trastuzumab accumulated in the HER2/neu-positive tumor at approximately twice the level that $^{64}$Cu-DOTA-trastuzumab accumulated ($p=0.006$ and $p=0.014$, respectively). The higher HER2/neu-positive accumulation for $^{64}$Cu-radiolabeled PCTA- and Oxo-DO3A-trastuzumab was also confirmed by analysis of the PET images, as the ratio of the average ROIs for the
HER2/neu-positive to the HER2/neu-negative tumor were higher for $^{64}$Cu-radiolabeled PCTA- and Oxo-DO3A-trastuzumab compared to those for $^{64}$Cu-DOTA-trastuzumab at 24 h post-injection. The higher HER2/neu-positive tumor accumulation and equivalent background tissue uptake also resulted in higher tumor to background ratios for $^{64}$Cu-radiolabeled PCTA- and Oxo-DO3A-trastuzumab compared to $^{64}$Cu-DOTA-trastuzumab, most notably the tumor to kidney ratios ($p$<0.04). By the 40-h time point the initial high activity in the HER2/neu-positive tumor for the $^{64}$Cu-radiolabeled PCTA- and Oxo-DO3A-trastuzumab had diminished to the same level as for $^{64}$Cu-DOTA-trastuzumab, and no significant differences were observed in the biodistribution or PET analysis of the three $^{64}$Cu-radiolabeled bioconjugates. The unforeseen increased target accumulation at 24 h suggests $^{64}$Cu-radiolabeled antibody conjugates incorporating either PCTA or Oxo-DO3A may allow imaging at an earlier time point than the analogous DOTA-containing agent.

It is unclear why $^{64}$Cu-radiolabeled PCTA- and Oxo-DO3A-trastuzumab demonstrated higher target accumulation at 24 h post-injection. Stability differences would be one potential answer, as DOTA has been reported to lose $^{64}$Cu to proteins such as superoxide dismutase with the $^{64}$Cu then retained in the liver [17], and the instability of a $^{64}$Cu-DOTA antibody conjugate when incubated in serum has been reported [13] and was also observed here. We have previously described the high stability of $^{64}$Cu-labeled PCTA and Oxo-DO3A BFCs; both had <4% loss of $^{64}$Cu from the BFC when incubated in serum or at low pH for 48 h [21]. It was surprising that no statistical difference in liver uptake and clearance was observed between the $^{64}$Cu bioconjugates, considering the lower stability of $^{64}$Cu-radiolabeled DOTA-trastuzumab. But while the average liver uptake for free $^{64}$Cu at 24 h (20±12%ID/g) was higher than the liver uptake of the $^{64}$Cu-radiolabeled bioconjugates, it was not statistically significant due to the variability (large standard deviations) in the liver %ID/g values. The similar preference for liver clearance/accumulation of both free $^{64}$Cu and radiolabeled antibodies appears to hinder the use of liver uptake as an indication of stability in these circumstances. The bioconjugates all gave similar results in the cell studies, suggesting that receptor affinity or target uptake are not likely the cause of the higher tumor accumulation for $^{64}$Cu-radiolabeled PCTA- and Oxo-DO3A-trastuzumab; neither was the specific activity as all of the $^{64}$Cu-radiolabeled bioconjugates were prepared for the animal studies in the same manner with similar specific activities. The structural differences of the BFCs themselves and differences in the location of the conjugation could also have influenced the 24-h biodistribution results, but their impact would be expected to be small considering their relative size compared to trastuzumab. A second set of imaging and biodistribution studies at the 24-h time point were done to confirm these findings and yielded the same results.

Conclusions

The novel bifunctional chelates $p$-SCN-Bn-PCTA and $p$-SCN-Bn-Oxo-DO3A were compared to $p$-SCN-Bn-DOTA for antibody labeling and imaging using trastuzumab. Both were found to have advantageous properties. Under mild conditions, the trastuzumab bioconjugates of PCTA and Oxo-DO3A were more rapidly radiolabeled with $^{64}$Cu (>95% radiochemical yield in 30 min) compared to DOTA-trastuzumab. A kit type formulation whereby $^{64}$Cu is simply added to the bioconjugate at room temperature without need for post-radiolabeling purification may be feasible for antibody bioconjugates incorporating PCTA or Oxo-DO3A. $^{64}$Cu-radiolabeled PCTA- and Oxo-DO3A-trastuzumab also showed high stability in mouse serum compared to $^{64}$Cu-DOTA-trastuzumab. Finally, $^{64}$Cu-radiolabeled PCTA- and Oxo-DO3A-conjugated antibodies may allow for earlier imaging protocols with better tumor to background ratios. Confirmation of these results with other antibodies would be worthwhile.

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Conflicts of interest

Cara L. Ferreira and Corinne Bensimon are employed by MDS Nordion. Paul Jurek and Garry Kiefer are employed by Macrocyclics Inc. The other authors declare that they have no conflicts of interest.

References


