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Imaging Cancer Using PET – the Effect of the Bifunctional Chelator on the Biodistribution of a ^{64}Cu -Labeled Antibody

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Abstract

Introduction—Use of copper radioisotopes in antibody radiolabeling is challenged by reported loss of the radionuclide from the bifunctional chelator used to label the protein. The objective of this study was to investigate the relationship between the thermodynamic stability of the ^{64}Cu -complexes of five commonly used bifunctional chelators (BFCs) and the biodistribution of an antibody labeled with ^{64}Cu using these chelators in tumor-bearing mice.

Methods—The chelators (*p*-NH₂-Bn-NOTA, BAT-6, *p*-NH₂-Bn-DOTA, DOTA, and SarAr) were conjugated to the anti-GD2 antibody ch14.18, and the conjugated antibody was labeled with ^{64}Cu and injected into mice bearing subcutaneous human melanoma tumors (M21) (*n* = 3-5 for each study). Biodistribution data were obtained from PET images acquired at 1, 24 and 48 hours post-injection, and at 48 hours post-injection a full *ex vivo* biodistribution study was carried out.

Results—The biodistribution, including tumor targeting, was similar for all the radioimmunoconjugates. At 48 h post-injection, the only statistically significant differences in radionuclide uptake (*p* < 0.05) were between blood, liver, spleen and kidney. For example, liver uptake of [^{64}Cu]ch14.18-*p*-NH₂-Bn-NOTA was 4.74 ± 0.77 per cent of the injected dose per gram of tissue (%ID/g), and for [^{64}Cu]ch14.18-SarAr was 8.06 ± 0.77 %ID/g. Differences in tumor targeting correlated with variations in tumor size rather than which BFC was used.

Conclusions—The results of this study indicate that differences in the thermodynamic stability of these chelator-Cu(II) complexes were not associated with significant differences in uptake of the tracer by the tumor. However, there were significant differences in tracer concentration in

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other tissues, including those involved in clearance of the radioimmunoconjugate (e.g., liver and spleen).

Keywords

Copper-64; SarAr; NOTA; protein radiolabeling; antibody biodistribution

1. Introduction

Radiolabeling proteins provides a method of characterizing their biodistribution. In the case of proteins designed or engineered to have certain pharmacological properties, such as antibodies with selective uptake in tumors, this can perform a dual role: in addition to providing a way of detecting the cancer when using a radionuclide for imaging, the antibody can also act as a vehicle for delivery of a radionuclide with cytotoxic emissions.

The detection and therapy of cancer using radiolabeled antibodies depends upon selective delivery of the radionuclide to the tumor, with minimal uptake in normal tissues. The degree to which a given radioimmunoconjugate (RIC) discriminates between normal and target tissues depends on the radioimmunoconjugate, which consists of the antibody, the bifunctional chelator that is attached to the antibody, and the radionuclide that is complexed by the chelator. The chemistry of conjugation must preserve the structural integrity of the antibody, with no extremes of temperature or pH. Rapid and stable chelation of the radiometal is also required. If the metal-chelate complex has low stability then the data will reflect the distribution of both the free radioisotope and the radiolabeled antibody. Therefore, the stability of the metal complex, and an understanding of the implications of stability for distribution of the radioimmunoconjugate within the body, is critical for successful antibody-based radiopharmaceutical development.

The radioisotope ^{64}Cu ($T_{1/2} = 12.7$ h) has been widely used to explore and develop the field of antibody targeting. Its half-life, in addition to being commensurate with the biological half-lives of antibodies or their engineered derivatives, also enables shipping from production sites to research centers. Copper is also attractive because there are a number of copper radioisotopes available, with a range of half-lives and emission profiles, resulting in a variety of options for both diagnostic imaging and radioimmunotherapy [1]. One disadvantage of the use of copper radionuclides is the high lability of Cu(II) which may result in loss of copper from the chelate and subsequent accumulation in non-target tissue such as the liver, probably in part due to transchelation of the copper to endogenous proteins involved in normal copper metabolism [2-4]. Because of this high lability, there has been extensive interest in the development of an optimal chelator with which to label proteins with copper radionuclides; one that combines high complex stability *in vivo* with radiolabeling procedures that will not damage the protein. The development of such a chelator would result in a more accurate representation of the distribution of the antibody.

The bifunctional chelator SarAr forms a very stable complex with Cu(II) [5], and SarAr has been used as a bifunctional chelator to label tumor targeting antibodies with ^{64}Cu [6], [7]. The extremely high thermodynamic stability of the ^{64}Cu -SarAr complex presents an opportunity to examine the influence of metal complex stability on antibody biodistribution by comparing the biodistribution of ^{64}Cu -labeled antibody conjugates with various bifunctional chelators (Figure 1) that form Cu(II) complexes with a range of thermodynamic stabilities (Table 1, references [8-12]). It is important to note that the thermodynamic stability constant, K , is measured in a simple chemical system at equilibrium, and that this is not necessarily reflective of the complex's stability in a complex biological environment, which is why we undertook this *in vivo* study.

The hypothesis tested in this study was that: *the differences in thermodynamic stability would result in different biodistributions of the ^{64}Cu -labeled antibody*. To test this hypothesis, the anti-GD2 antibody ch14.18 was conjugated with a series of bifunctional chelators which all bind copper readily at room temperature and thus are compatible with proteins - other ligands such as porphyrins or TE2A do not share this property. The antibody-chelate conjugates were then labeled with ^{64}Cu and injected into mice bearing GD2-expressing M21 melanoma tumors. The biodistribution of the radioimmunoconjugate was measured by small animal PET imaging as well as dissection and tissue assay.

2. Materials and Methods

2.1 General

Chemicals and reagents were obtained from Sigma-Aldrich (St Louis, MO) unless otherwise specified. Reagents 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS) were purchased from Pierce (Rockford IL). Metal-naïve pipette tips were purchased from Rainin Instrument (Oakland CA). Glass and plasticware were acid washed with 10 % HNO_3 and rinsed thoroughly with ultrapure water ($> 15 \text{ M}\Omega$ resistivity, Millipore, Billerica, MA) before use. Ultrapure water was also used in buffer preparation. Sodium acetate buffer (pH 5.0) was prepared by addition of glacial acetic acid to a 0.1 M solution of sodium acetate until the desired pH was reached. Metal contaminants in the buffer were decreased by either passing through a Chelex-100 resin column (Bio-Rad Laboratories, Hercules CA, USA), or by batch treatment with Chelex-100. Copper-64 was purchased from Washington University (St Louis, MO) and was supplied at 1.14 mCi/ μL (range = 0.48 – 2.1 mCi/ μL) in 0.04 M HCl. Radioactivity in the tissue samples was assayed using a Packard Cobra II automated gamma counter (Meriden, CT). MicroPET images were obtained using a Siemens Focus 120 camera (Siemens Medical Solutions USA Inc., Malvern PA).

2.2 Bifunctional chelators

SarAr was synthesized as described previously [12]. *S*-2-(4-aminobenzyl)-1,4,7,10-tetraazacyclododecane tetraacetic acid (*p*- NH_2 -Bn-DOTA) and *S*-2-(aminobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (*p*- NH_2 -Bn-NOTA) were purchased from Macrocyclics (Dallas, TX). BAT-6 (6-[*p*-(bromoacetamido)benzyl]-1, 4, 8, 11-tetraazacyclotetradecane-*N*, *N'*, *N''*, *N'''*-tetraacetic acid) was synthesized as previously reported [13]. 1,4,7,10-tetraazacyclododecane-*N*, *N'*, *N''*, *N'''*-tetraacetic acid (DOTA) was purchased from Sigma.

2.3 Antibody conjugation

The anti-GD2 ch14.18 antibody was provided by EMD Serono (Billerica, MA) [14]. The antibody was buffer exchanged and concentrated to approximately 10 mg/mL in an appropriate buffer using centrifugal filter units (30 kDa MWCO; Centricon, Millipore, Billerica MA).

The SarAr, *p*- NH_2 -Bn-DOTA, and *p*- NH_2 -Bn-NOTA chelators were conjugated to the antibody using slight modifications of previously described methods [6]. For example: to SarAr (5 mg) in Me_2SO (56.25 μL) was added 3 volume equivalents of 0.1 M sodium acetate buffer (pH 5.0) to give a final SarAr concentration of 22 mg/mL. By the addition of 1 M NaOH, the pH was brought to 5.0. The chelator was added to the antibody at a molar ratio of 250:1, followed by buffer, and then finally 500 molar equivalents of EDC, which was made up immediately before use in ultrapure water at a concentration of 50 mg/mL. The volume of reagents added diluted the antibody to a final concentration of 5 mg/mL. The reaction was mixed gently by pipette, centrifuged to remove air bubbles, pH was confirmed

to be 5 using a micropH probe, and the reaction was placed in a 37 °C water bath. The final concentration of Me₂SO was always < 5% (typically ~ 1%). After 30 minutes, unbound chelate was separated from the immunoconjugate by size-exclusion HPLC using a BioSep SEC-S3000 column (Phenomenex, Torrance CA) with an isocratic aqueous phase consisting of 0.1 M sodium acetate, pH 5.0, and a flow rate of 1 mL/min. Retention time (RT) of the immunoconjugate was typically 8 min, with the unbound chelator eluting at approximately 11 min. The purified immunoconjugate fractions were pooled, concentrated, and stored in aliquots at -80 °C.

DOTA was conjugated to antibody using literature methods [15]. Briefly, DOTA-OSSu was prepared by adding 239 µL of a DOTA solution (18 mg in 900 µL H₂O, 20 mg/mL, brought to pH 5.5 by addition of approx. 72 µL of 1 M NaOH) to 117 µL of freshly prepared aqueous sulfo-NHS solution (20 mg/mL), all on ice. Then 8.25 µL of freshly prepared aqueous EDC (25 mg/mL) was added to the DOTA/sulfos-NHS mixture. After 30 minutes, 40 µL of Na₂HPO₄ (0.2 M, pH 9.2) was added to the reaction mixture, and the pH was confirmed to be 7.3. This solution was added to a solution of the antibody (41 µL, 24.3 mg/mL, DOTA-OSSu:Ab=20:1), mixed by pipette, and placed in a cold room (4°C) with constant agitation. After 24 h, the resulting immunoconjugate was purified by semi-preparative HPLC as described above, concentrated into NaOAc buffer (0.1 M, pH 5.0), and stored in aliquots at -80 °C.

BAT-6 was conjugated using previously reported conditions [16]. Briefly, to 1 mg of antibody (Ab concentration 24.6 mg/mL) in 41 µL of tetramethylammonium phosphate buffer (pH 8.0) was added 0.133 µM BAT-6 in ultrapure water and freshly prepared 2-iminothiolane (2IT, 0.01 mg in 13.2 µL) in 50 mM triethanolamine.HCl at pH 8.7, such that final concentrations were: Ab, 0.1 mM; BAT-6, 2 mM; 2IT, 1 mM. The pH was confirmed to be 7.8. The reaction was mixed by gentle pipetting, briefly centrifuged, and then placed in a 37 °C water bath for 30 minutes. Unbound chelator was removed by HPLC as before, and the immunoconjugate was concentrated into NH₄OAc buffer, 0.1 M pH 5.0, and stored in aliquots at -80 °C.

2.4 Isotope dilution assay

Ratio of protein molecules to available chelating molecules was found using the method of Meares *et al.* [17], except that ⁶⁴Cu was used rather than ⁵⁷Co. Typically, a solution of immunoconjugate of known concentration (approx. 0.3 mg/mL) was prepared. A small amount of ⁶⁴Cu was added to a solution of cold copper (e.g. 10 mM) prepared from a commercially available standard (Sigma Aldrich, St Louis MO) that was approximately twice the estimated concentration of available chelators on the antibody. This solution was vortexed, centrifuged, and left for at least 1 h. To 10 µL of NaOAc buffer in a microcentrifuge tube was added 10 µL of the cold copper/⁶⁴Cu mixture followed by 10 µL of the antibody. The reaction was then mixed by pipetting and incubated at room temperature for at least 1 h. Then 1 µL of the labeled protein was removed and added to 9 µL phosphate buffer (0.1 M, pH 8) containing 100 mM EDTA. After 5 minutes, a 1 µL aliquot was spotted onto an ITLC strip (Instant Thin Layer Chromatography Si Gel impregnated glass fiber sheets; Pall Life Sciences, Ann Arbor MI), which was allowed to air dry and then developed using phosphate buffer (0.1 M, pH 8, 100 mM EDTA) as the mobile phase. The strip was cut into four sections and radioactivity in each was detected using a gamma counter. Using these conditions, labeled antibody remains at the baseline of the strip with free copper moving with the solvent front. TLCs were also run of antibody incubated with no cold copper, 10 mM cold copper (to block all binding sites) and concentrations of copper lower than and higher than the target concentration (e.g. 5 mM). The number of available binding sites was calculated by the reported method with the modification that account was taken for labeling achieved at 0 and 10 mM non-radioactive copper.

2.5 Radiolabeling

For radiolabeling with ^{64}Cu , typically, approximately 3 volume equivalents of pH 5, 0.1 M acetate buffer was added to 2 mCi ^{64}Cu in 5-10 μL HCl (0.04 N). A solution of the antibody, also in acetate buffer (250 μg , 75 μL), was then added to the ^{64}Cu solution. After 30 minutes incubation at 25 $^{\circ}\text{C}$, a TLC was run as before to assess radiolabeling. If the TLC assay showed that the radiochemical purity was <95%, the product was purified using centrifugal filter units (as before) and the post-filtration purity was confirmed by TLC. The radioimmunoconjugate was diluted with saline and sterile filtered (0.2 μm) before injection.

2.6 Cell culture and xenograft tumors

All animal procedures were carried out under a protocol approved by the Children's Hospital Boston Institutional Animal Care and Use Committee. Cell culture reagents were obtained from Mediatech (Herndon, VA) unless otherwise stated.

M21 melanoma cells (described in Mueller *et al* [18]) were generously provided by P.M. Sondel of the University of Wisconsin, Madison, WI. These cells are known to have a high expression of the disialoganglioside GD2 antigen [18] and therefore provide a suitable target for the anti-GD2 ch14.18 antibody-based radioimmunoconjugates under examination. The cells were grown in RPMI 1640 supplemented with antibiotics (100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin), L-glutamine (2 mM), and 10% FCS (Hyclone, Perbio Cell Culture Division, Logan UT) as a semicontinuous adhesion culture, with passaging by trypsinization every 3 - 4 d. Cells were used within 2 weeks of initiation of culture, and were grown *in vitro* for no longer than 12 weeks. For tumor implantation, cells were trypsinized (0.05% Trypsin, 0.53 mM EDTA in HBSS), pelleted by centrifugation, resuspended in medium, counted, recentrifuged and resuspended in phosphate-buffered saline (PBS) at 4 $^{\circ}\text{C}$ at a concentration of 4×10^7 cells/mL. The cells were then mixed 1:1 with Matrigel (BD Biosciences, Woburn MA). Athymic (nu/nu) female mice (Charles River, Wilmington MA) 2-3 months of age and weighing 20-25 g were anesthetized with isoflurane (2-4 % in a constant flow of oxygen) and injected subcutaneously with 0.1 mL of cell suspension on the left flank (2×10^6 cells each).

2.7 Biodistribution studies

Approximately 3 weeks after inoculation the tumors were palpable and of suitable size for imaging studies. Radiolabeled antibody was injected i.v. into the tail vein (50 μg of antibody, mean activity 5.7 MBq, ^{64}Cu (mean 155 μCi , range 79-243 μCi), diluted to 0.1 mL with saline). Mice were then anesthetized using isoflurane as before and PET data were collected for 30 min. using a Siemens Focus 120 scanner at 1, 24 and 48 h post-injection. At 48 h post-injection, CT images were obtained using a Siemens MicroCAT II scanner. After CT imaging, the mice were euthanized by CO_2 inhalation, and a full biodistribution analysis was carried out. Tissues were collected, weighed and radioactivity was counted. PET and CT images were registered manually using AMIDE software (A Medical Imaging Data Examiner, <http://amide.sourceforge.net/index.html>). Data from volumes of interest (VOIs) within relevant tissues were used to calculate biodistribution throughout the imaging study.

Statistical analysis was carried out using SPSS V 14.0 for Windows. The 48 h *ex vivo* biodistribution data was analyzed for statistical significance by ANOVA, with *post hoc* analysis using the Bonferroni correction. Differences were considered significant at the 5 % level ($p < 0.05$).

3. Results and Discussion

3.1 Radiolabeling – Isotope Dilution Assay

The number of chelators on each protein was found to be: *p*-NH₂-Bn-NOTA 0.81 ± 0.05; BAT-6 2.17 ± 0.23; DOTA 0.82 ± 0.05; *p*-NH₂-Bn-DOTA 0.85 ± 0.19; SarAr 0.81 ± 0.02 (means of three separate determinations ± SD). At these chelator-to-protein (C:P) ratios, the proteins were typically labeled to specific activities of 185 – 370 MBq (5 – 10 μCi) per μg protein.

The biodistribution of a radiometal labeled protein in normal tissues can also be altered by the C:P ratio, so it was necessary to achieve similar chelator-to-protein (C:P) ratios for the RICs. For example, Grunberg *et al.* [19] reported that increasing the number of DOTA chelators on a F(ab')₂ molecule from 3 to 5 produced significant differences in radionuclide (⁹⁰Y) uptake in liver, kidneys and tumor. In addition, the C:P ratio was kept low (1 – 2 chelators per protein molecule) in order to retain antigen binding and therefore uptake in the target site, which can be reduced at higher chelate ratios due to loss of immunoreactivity [20], [21]. Conversely, C:P ratios significantly lower than 1 would have made it difficult to inject adequate activity for imaging without at the same time injecting an excessive amount of protein.

3.2 Biodistribution studies

Imaging studies were carried out 3 weeks after the tumors were inoculated (n = 21, mean size = 0.120 ± 0.131 (SD) g, range 0.011 – 0.471 g). Mean injected activity was 5.7 ± 2.0 MBq (155 ± 56 μCi) (range 2.9 to 9.0 MBq) per mouse. Figure 2 shows typical small animal PET images obtained for the antibody conjugated with *p*-NH₂-Bn-NOTA, *p*-NH₂-Bn-DOTA and SarAr at 1, 24 and 48 h registered with the CT images. The 1 h images mainly show blood pool distribution, at 24 h the radionuclide is distributed throughout the body, and by 48 h clearance from normal tissues and tumor targeting is evident, with predominant normal tissue uptake being in the liver. The PET images showed higher uptake of ⁶⁴Cu in the liver and kidneys with [⁶⁴Cu]ch14.18-SarAr compared with [⁶⁴Cu]ch14.18-*p*-NH₂-Bn-NOTA. This observation is confirmed by the quantification of imaging data presented in Figure 3 and by the 48 h *ex vivo* biodistribution data presented in Figure 4. These data are presented in order of ascending copper complex thermodynamic stability as given in Table 1.

Overall, the patterns of distribution of radionuclide for the five different RICs are remarkably similar. Radionuclide uptake was high in the liver and kidney for all RICs, as would be expected for organs involved in clearance of an antibody. The ⁶⁴Cu concentration was low in the brain due to exclusion of the large antibody molecules by the blood-brain-barrier, and was also low in the muscle. Higher uptake in the lung was most likely due to the prolonged residence of whole antibody in the blood. Radioactivity in tumor shows the most variation. Temporal variation was due to gradual accumulation of the tracer in the tumor from the blood. Interestingly, while lower normal tissue uptake of ⁶⁴Cu was found for the NOTA conjugated protein than for the other RICs, this was not accompanied by a reduction of uptake in the tumor (see Figure 5): there were no significant differences in tumor uptake between the five RICs. In fact, the only variation in tumor uptake between the chelates is due to variations in tumor size. This is a major determinant of RIC uptake, with larger tumors having lower ⁶⁴Cu uptake per gram of tissue, with a significant increase in uptake occurring in tumors below around 0.1 – 0.2 g. This effect is demonstrated graphically in Figure 5, in which tumor size is plotted against radionuclide uptake in the tumor at 48 h (*ex vivo* biodistribution data). This effect is in agreement with previous studies [22–24], and suggests that radiolabeled antibodies may be especially useful in the detection and treatment of small cancer lesions.

Analysis of the 48 h *ex vivo* biodistribution data revealed statistically significant differences between the RICs in blood ($p = 0.012$), liver ($p = 0.029$), spleen ($p < 0.001$) and kidney ($p = 0.002$). In liver, the concentration of SarAr (8.06 ± 0.77 %ID/g) was higher than that of NOTA (4.74 ± 0.77 %ID/g) ($p = 0.017$). The spleen uptake of [^{64}Cu]ch14.18-SarAr (8.94 ± 1.57 %ID/g) was higher than that of the other chelators ($p < 0.05$) as was the kidney uptake (10.45 ± 2.47 %ID/g, $p \leq 0.021$). Since these organs (liver, spleen, kidney) are involved in the metabolism and excretion of antibodies, it is assumed that the differences are due to chemical differences between the RICs and resulting effects on metabolism and biodistribution. It is interesting to note that the biodistribution of the RICs based on the two forms of DOTA (i.e. DOTA attached directly to the antibody via one of the carboxylate arms versus *p*-NH₂-Bn-DOTA, which could be attached to the antibody via the amino group) were not different from each other.

The aim in undertaking this study was to investigate the effect of these BFCs, particularly of their different thermodynamic stability, on the biodistribution of the ch14.18 antibody. However, it is difficult to distinguish the effects of different copper complex stability from differences in other properties of these molecules. For example, the local charge conferred on the antibody by the radiolabeled copper complexes is different for different BFCs. The copper complexes of the DOTAs and BAT-6 confer a net negative charge, *p*-NH₂-Bn-NOTA does not change the local charge, and the SarAr complex is positively charged. It is important to note that these charges are based on the BFCs that are labeled with Cu(II) and conjugated to the antibody, as is appropriate for consideration of these radioimmunoconjugates. For example, *p*-NH₂-Bn-NOTA has a -3 charge due to the deprotonated carboxyls (pKa's in the range 1.5 – 5.0), a + 2 charge for the Cu(II), and confers a net +1 charge regionally on the antibody because a glutamic acid group that would otherwise have been deprotonated at pH 7.4 (pKa = 4.07) is part of an amide bond, resulting in an overall change in charge of 0.

The result is significant differences in the regional charges on the protein. Furthermore, the chelators are probably attached at different sites on the antibody. SarAr, *p*-NH₂-Bn-DOTA, and *p*-NH₂-Bn-NOTA all react with carboxylate residues on the protein (e.g., glu, asp) while BAT-6 and DOTA react with amine residues (e.g., lys), although it should be noted that the carboxyl groups of the amine-containing BFCs are also available for reaction with amine groups on the protein. It is reasonable to expect that these differences will also alter the biodistribution of the RICs.

Jones-Wilson *et al.* [25] reported that the distribution of unconjugated radiocopper complexes of azamacrocyclic and polycarboxyltetraazamacrocycles was greatly influenced by the formal charge of the complex. While all complexes had uptake in the liver and kidneys, those with a positive charge had significantly higher uptake in these organs, and a relationship between stability of the complex and biodistribution can be seen in the data. Rogers *et al.* [26] compared 4 copper chelators (BAT-6, SCN-TETA, CPTA and PCBA) attached to an antibody (1A3) and its F(ab')₂ fragment. They found that biodistribution of the RICs varied, with uptake in liver and kidney being significantly different. These differences were largely attributed to the differences in charge and lipophilicity of the chelators, although the stability of the copper complexes varied significantly. It was found that radioactivity in the liver was associated with the antibody for a relatively long period of time, with a metabolite of 35 kDa MW appearing by 5 days post injection. In comparison, the F(ab')₂ fragments were rapidly broken down in the kidney by 1 day post injection into small molecular weight metabolites which co-chromatographed with a radiocopper-chelate- ϵ -lysine standard. This study differs from ours because, while both use TETA derivatives, Rogers *et al.* compared them with complexes of lower stability, while our work considers the effect of higher metal complex stabilities on biodistribution. In Kukis *et al.* [27], the *in*

vitro serum stability of the radiocopper complexes of four chelators conjugated to an antibody including a NOTA derivative (BAN), two TETA derivatives (BAT-2 and BAT-6) and a DOTA derivative (BAD) were compared. It was found that BAT-2 complex was the least stable in this group, with 4.4% of the radionuclide lost to serum proteins per day compared to around 1 % for the others. That is, the stability of the NOTA-derived BFC Cu(II) complex was comparable to the DOTA and TETA BFCs in these conditions. In the present study we have found that use of the thermodynamically less stable copper chelator NOTA resulted in lower radionuclide retention in clearance organs.

It is often a concern when using radiocopper-labeled antibodies that loss of copper from the RIC will result in high liver uptake, confounding, for example, detection of hepatic lesions as well as increasing the radiation dose to a non-target tissue (as suggested in [4]). Part of the inspiration for this study was the emergence of the BFC SarAr, which forms a copper complex with extremely high thermodynamic stability, as a potential solution for this problem. It was assumed that if loss of copper from BFCs was leading to sequestration in the liver, then use of a very stable copper chelator would reduce the amount of ^{64}Cu that accumulated in the liver. We have observed, however, that labeling the ch14.18 antibody with the BFC that forms the least thermodynamically stable copper complex (^{64}Cu -NOTA) produced the lowest concentration of ^{64}Cu in the liver.

This point leads us to a consideration of what properties of a ligand make it a good bifunctional chelator for nuclear medicine imaging. The thermodynamic stability constants quoted in Table 1 are often used as an indication of *in vivo* stability. However, the kinetic stability of the metal complex, the rate at which the metal is lost from the ligand, the effect of *in vivo* chelators, and pH are perhaps more important when considering the stability of the complex in a biological environment. The observation that kinetic stability is of paramount importance in radiometal labeling a biological molecule has been made before ([25], [27], [28]). The BFCs used in this study vary in their properties (e.g., thermodynamic stability, charge, lipophilicity). For example, Cu-SarAr appears to be extremely stable but, depending on its degree of protonation, has a high positive charge *in vivo*. This charge (apparently) affects the biodistribution of the RIC and, based on previous experience with other BFCs, is presumably responsible for the higher kidney uptake seen in this study.

The results of this antibody-based study agree with those of Prasanphanich *et al.* in which they used a peptide as the pharmacophore. Their blocking study data suggested that kidney residence of NOTA/NO2A-peptide was non-specific [29]. A further study comparing DOTA and NOTA as the chelating moieties found that in tissues where uptake was specific Cu-NOTA-peptide uptake was not decreased, but in tissues where uptake was non-specific Cu-NOTA-peptide uptake was lower than Cu-DOTA-peptide [30]. In other words, non-specific uptake of the Cu-NOTA agent was lower than for the DOTA version, suggesting that NOTA would be a better chelating moiety for peptides, as we have seen in this study based on an antibody.

The kinetic stability of a copper complex is a function of the rigidity of the ligand structure [31], and also of the change of its degree of protonation as a response to changes in pH [32]. Once injected, the targeting agent will find itself in different cellular organelles, such as vesicles which have a pH lower than 7.4. Pippin *et al.* [33] showed that Cu-NOTA is far more stable than either Cu-TETA ($\sim 60\times$) or Cu-DOTA ($\sim 287\times$) at pH 4.6, which might explain the differences in biodistribution seen in the current work. The Cu-DOTA- and Cu-TETA-based complexes are dissociating and the Cu is being trapped intracellularly in normal tissues, whereas the Cu-NOTA-Ab is either targeting the tumor or being excreted, resulting in lower normal tissue uptake of the radionuclide and superior images.

4. Conclusion

The results of this study indicate that, despite large differences in the thermodynamic stability of the Cu(II) complexes of these chelators, these differences were not associated with significant differences in uptake of the tracer by the tumor. There were, however, significant differences in tracer concentration in other tissues, including liver and spleen.

In order to be useful in the clinical setting, the ease and reproducibility with which stable, reactive, radioimmunoconjugates can be prepared will necessarily guide further development of these imaging agents. To this end, this study has shown that the inherent thermodynamic stability of the Cu(II) BFC complexes, while important, may not be as critical a factor in predicting biodistribution. Work is currently being undertaken in this laboratory to evaluate the relative kinetic stabilities of Cu chelates in the presence of competing ligands and at a range of physiologically-relevant pH to provide stability data that will inform copper chelate design.

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Figure 1. Chemical structures of the bifunctional chelators used in this study

Top row (l-r): *p*-NH₂-Bn-NOTA, BAT-6, DOTA; bottom row *p*-NH₂-Bn-DOTA and SarAr.

Figure 2. Representative small animal PET images of radionuclide localization

Coronal (top) and transverse (bottom) images of antibody distribution radiolabeled with ^{64}Cu using selected bifunctional chelators. The images show that the radionuclide gradually distributed throughout the body, and then cleared from normal tissues and accumulated in the tumor throughout the study. PET images are in color, CT in grayscale. For orientation, head, tail, left and right are indicated on the images. Yellow arrow indicates liver, white arrow indicates tumor (48 h images). Mice were injected with 5.7 MBq ^{64}Cu (range 2.9 – 9.0 MBq), anesthetized with isoflurane (2-4 % in a constant flow of oxygen), and the PET data were collected over 30 min.

Figure 3. Small animal PET imaging - biodistribution data

The PET images were registered with the CT images for anatomic reference and then volumes of interest were drawn. Data from the major tissues were converted into percent of injected dose per gram of tissue (%ID/g) at 1, 24 and 48 hours post injection (white, gray and black, respectively; mean $\pm$ SD, n = 3-5). (*N.B. Some 1 hour imaging data for p-NH₂-NOTA-conjugated antibody was unavailable due to technical issues, leading to n=1/4.*)

Figure 4. Results from the *ex vivo* biodistribution study

At 48 hours post injection tissues were assayed for radioactivity, which is expressed as percent of injected dose per gram (mean $\pm$ SD, n = 3-5).

Figure 5. The relationship between tumor size and accumulation of radiolabeled antibody
Tumor uptake (%ID/g) of antibody varied between studies, but this was due to variations in tumor size rather than a reflection of radioimmunoconjugate performance. The individual data show an increase in accumulation at tumor sizes below ~ 0.1 g, as has been previously reported.

Table 1
Thermodynamic stability constants of the bifunctional chelators used in this study

Data are from references 8,9 (NOTA); 10,11 (TETA and DOTA); and 12 (SarAr).

Chelates	Stability constant (log <i>K</i> at 25 °C)	References
NOTA	21.6, 19.8	8,9
TETA	21.9, 21.6	10,11
DOTA	22.7, 22.21	10,11
SarAr	Negligible <i>in vitro</i> dissociation	12