

Conjugation of Monoclonal Antibodies with TETA Using Activated Esters: Biological Comparison of ^{64}Cu -TETA-1A3 with ^{64}Cu -BAT-2IT-1A3

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A simple method for conjugation of monoclonal antibodies (mAbs) with the chelating agent 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA), has been developed using commercially available reagents. This method involved activation of a single carboxyl group of TETA with N-hydroxysulfosuccinimide and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide. The resulting activated ester of TETA was reacted with the anti-colorectal carcinoma mAb 1A3 at molar ratios ranging from 10:1 to 100:1 to give immunoconjugates modified with an average of 0.4 to 2.0 functional chelators per antibody molecule. The TETA-1A3 conjugate was labeled with ^{64}Cu at specific activities as high as 15.4 $\mu\text{Ci}/\mu\text{g}$, and the radiolabeled mAb exhibited high in vitro serum stability and minimal loss of immunoreactivity. The biodistribution of ^{64}Cu -labeled TETA-1A3 in hamsters bearing GW39 human colon carcinoma xenografts was compared to that of ^{64}Cu -BAT-2IT-1A3 (BAT = 6-(p-bromoacetamidobenzyl)-1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid; 2IT = 2-iminothiolane). Both conjugates showed high tumor uptake (6.60–9.05% injected dose/gram) from 24 to 48 h post-injection and generally similar blood clearance and non-target organ uptakes. Human absorbed dose estimates derived from the hamster biodistribution data showed the critical organs for both conjugates to be the large intestine and the red marrow. Our results suggest that the in vitro and in vivo performance characteristics of ^{64}Cu -TETA-1A3 compare favorably with those of ^{64}Cu -BAT-2IT-1A3 and that further evaluation of the diagnostic and therapeutic efficacy of ^{64}Cu -TETA-1A3 is warranted.

INTRODUCTION

Monoclonal antibodies (mAbs) have been labeled with ^{64}Cu ($T_{1/2} = 12.7$ h; β^+ 655 keV (17.4%); β^- 573 keV (39%)) and ^{67}Cu ($T_{1/2} = 61.9$ h; β^- 0.182 (1%), 0.392 (56%), 0.483 (23%), 0.576 (20%) keV; γ 0.091 (7.9%), 0.093 (31.8%), 0.185 keV (48.0%)) for diagnostic imaging and radioimmunotherapy (RIT) of cancer. Meares and

colleagues developed the bifunctional chelator BAT (6-(p-bromoacetamidobenzyl)-1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid, Figure 1),¹ using 2-iminothiolane (2IT)² as a linker for covalent attachment of radio-copper chelators to mAbs. DeNardo et al. used BAT to attach ^{67}Cu to mAb Lym-1 for imaging and RIT of non-Hodgkin's lymphoma.^{3,4} Our group has used BAT to complex ^{64}Cu to mAb 1A3, an anti-colorectal carcinoma monoclonal antibody,⁵ and this agent was shown to be effective in positron emission tomography (PET) imaging⁶ and to have potential for RIT.^{7,8}

Although BAT has been proven to be an effective chelator for attaching copper radionuclides to mAbs, a time-consuming multi-step syn-

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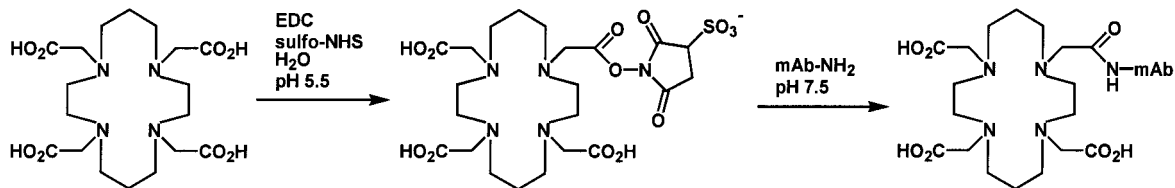


Figure 2. Formation of TETA-OSSu and Conjugation to Monoclonal Antibodies (mAbs).

it was used immediately, without purification, to prepare the TETA-conjugated antibody. The pH of the TETA-OSSu solution was adjusted to 7.5 by addition of 0.1 M Na₂HPO₄, pH 9.2. The TETA-OSSu concentration of the resulting solution was 3.73 mM. A solution of 1A3, 47.6 mg/mL in 0.1 M NaH₂PO₄, pH 7.5, was divided into four 3.57-mg (23.8 nmol) aliquots for conjugation with the TETA active ester at molar ratios of 10:1, 20:1, 50:1, and 100:1. The appropriate volumes of TETA-OSSu were added, respectively, to the four 75- μ L aliquots of mAb 1A3, and the reaction mixture was incubated on a tube rotator for 18–24 h at 4 °C. The reaction mixtures were concentrated to 50–100 μ L using Centricon-30 centrifugal filter devices, diluted with 2 mL of 10 mM NaH₂PO₄/150 mM NaCl, pH 7.5, and concentrated to 50–100 μ L again. The resulting solutions were diluted with 2 mL of 0.1 M NH₄Cit, pH 5.5, and concentrated to 50–100 μ L. This process was repeated five times. Recovery of TETA-1A3 was determined by absorbance at 280 nm ($\epsilon = 1.42$ (mg/mL)⁻¹), and the conjugates were analyzed for purity by FPLC.

Radiolabeling of TETA-1A3

The TETA-1A3 conjugate was labeled with ⁶⁴Cu, using modifications of previously published procedures.^{5,10} Representative labeling conditions are given here. To 7.53 mCi of ⁶⁴CuCl₂ in 12.1 μ L of 0.1 N HCl was added 343 μ L of 0.1 M NH₄Cit, pH 5.5, followed by 1.44 mg of TETA-1A3 (conjugated with 100 equivalents of TETA-OSSu) in 213 μ L of 0.1 M NH₄Cit, pH 5.5. The reaction mixture was incubated at 43 °C for 1 h, after which it was applied to a 3-mL G-25-50 spin column, which was equilibrated with 0.1 M NH₄Cit, pH 6.6. The column was eluted by centrifugation at 2500 rpm for 4 min at room temperature. The eluant, containing ⁶⁴Cu-TETA-1A3, and the column were measured in a dose calibrator to determine the percent incorporation of ⁶⁴Cu.

Conjugation of 1A3 with BAT/2IT and Radiolabeling of BAT-2IT-1A3

Monoclonal antibody 1A3 was conjugated with BAT/2IT as described previously,⁵ using a modification of a published procedure.² BAT-2IT-1A3 was labeled with ⁶⁴Cu as described previously.⁵

FPLC of ⁶⁴Cu-TETA-1A3

The radiochemical purity of ⁶⁴Cu-TETA-1A3 was determined by FPLC, using a Pharmacia Superose 12 HR 10/30 column and an isocratic mobile phase of 20 mM HEPES/150 mM NaCl, pH 7.3, at a flow rate of 0.4 mL/min. Fractions were collected at 1 min intervals and counted with a Beckman 8000 gamma counter (Fullerton, CA).

Determination of the Average Number of Chelators per Antibody

The average number of chelators per antibody molecule was determined using an isotopic dilution assay, as described previously.^{5,13} This assay is a modification of a procedure described by Meares and colleagues.¹⁴ The percent ⁶⁴Cu incorporation was plotted as a function of the quantity of copper added to TETA-1A3. The point of 50% reduction of mAb labeling, compared to labeling under no-carrier-added conditions, was determined, and this value represents approximately half the quantity of functional chelators attached to the antibody.

Immunoreactivity Determination

The immunoreactivity of ⁶⁴Cu-TETA-1A3 was determined using GW39 human colon carcinoma cells in suspension culture, under conditions of antigen excess, according to the method of Lindmo et al.¹⁵

Serum Stability Studies

An aliquot of 175 μ Ci of ⁶⁴Cu-TETA-1A3 in 70 μ L of 0.1 M NH₄Cit, pH 6.6, was added to 500

μL of rat serum. This mixture was incubated at 37°C for 48 h. At 0, 2, 4, 24, and 48 h, $25\text{-}\mu\text{L}$ aliquots of the serum were analyzed by FPLC.

Tumor Model and *in Vivo* Studies

All animal experiments were performed in compliance with guidelines specified by the Washington University Animal Studies Committee. Propagation of GW39 human colon cancer xenografts was achieved in 7- to 9-week-old male Golden Syrian hamsters. Hamsters were injected with tumor cell suspensions with $>90\%$ viability (25% (v/v), 0.5mL , $\sim 8.2 \times 10^6$ cells)¹⁶ in the right thigh musculature.

The ^{64}Cu -labeled TETA-1A3 ($40\ \mu\text{Ci}$ and $38\ \mu\text{g}$) and BAT-2IT-1A3 conjugates ($40\ \mu\text{Ci}$ and $40\ \mu\text{g}$) were administered by intracardiac injection two days after tumor implantation. Biodistribution studies were performed at 1, 6, 12, 24, and 48 h post-injection. Tissues collected included blood, skin, muscle, tumor, bladder, heart, lung, liver, spleen, kidney, stomach, small intestine, upper large intestine, lower large intestine, thyroid, and bone. Tumor weights at harvest ranged from 100–400 mg. Tissues were drained of blood, weighed, and counted in the gamma counter with a standard of the injected dose, such that decay-corrected values were calculated as $\%$ injected dose/gram ($\%ID/g$) and $\%ID/organ$.

Human Normal Organ Dosimetry

Human absorbed dose estimates to normal organs were derived from hamster biodistribution data according to methods described previously.^{7,17} The assumption was made that normal organ uptake and clearance of the radioimmunoconjugate in the hamster is the same as in humans. Time-activity curves (TACs) for 15 organs were generated using the percent injected activity per organ, decay-corrected to the time of injection. The residence time for each organ was determined by fitting the data to a sum of exponentials and integrating the area under the TAC analytically, assuming no excretion and physical decay after the last time point. Human dose estimates were calculated using standard MIRD techniques, using S-values (mean absorbed doses per unit cumulative activity) obtained from MIRDOSE3¹⁸ for the adult male reference model. The absorbed radiation doses to adrenals, bladder, bone, stomach, small intestine, upper large intestine, lower large intestine, heart wall, kidneys, liver, lungs, red marrow, muscle, and spleen were calculated as

the sums of the products of the residence times and the tabulated S-values for ^{64}Cu in a standard human geometry. Bone activity was assumed to be distributed equally between cortical bone and trabecular bone. Bone marrow activity was derived from blood pool activity according to the model of Siegel et al.,¹⁹ using a partition fraction of 0.3. Excretion through both the bladder and gastrointestinal tract was assumed to be minimal, based on previous dosimetry studies with ^{64}Cu -BAT-2IT-1A3 in humans.²⁰ The results accounted for 52% of the injected activity, with the rest being included as “missing” and assigned to the MIRD category “remainder-of-body,” thus assumed to be distributed uniformly in the body.

Hamster Tumor Dosimetry

The absorbed dose to a small GW39 tumor was determined from the hamster biodistribution data, using previously described methods.^{7,17} Each tumor was harvested, weighed, and counted to determine the $\%ID/organ$. Time-activity curves were generated by combining decay-corrected tumor uptake values for all animals at all time points. Using MIRDOSE3, the S-value for a small tumor was calculated, assuming the radioactivity to be uniformly distributed within a tumor sphere with a mean weight of 0.21 g. The S-value for ^{64}Cu in a 0.2-g tumor was 1.122 rad/ $\mu\text{Ci}\cdot\text{h}$. The assumption was made that tumors of similar size in different animals had similar uptakes, and the absorbed dose estimate represents the average of the doses absorbed by individual tumors.

Statistical Analysis

To compare differences between the ^{64}Cu -labeled 1A3 conjugates, a Student's *t*-test was performed. Differences at the 95% confidence level ($p < 0.05$) were considered significant.

RESULTS

Conjugation of mAb 1A3 with TETA-OSSu

Reaction of TETA-OSSu with monoclonal antibodies results in the formation of an amide bond between the chelating agent and the ϵ -amino group of lysine residues and/or the *N*-termini of the polypeptide chains. After activation of TETA with sulfo-NHS and EDC at a molar ratio of 10:10:1, respectively, the activated ester was not isolated. Rather, the theoretical yield of TETA-

OSSu, based on EDC as the limiting reactant, was used to define the reaction stoichiometry of bifunctional chelating agent to antibody. The mAb 1A3 was conjugated with 10, 20, 50, and 100 theoretical equivalents of TETA-OSSu, and the ratio of functional chelators per antibody molecule was determined by an isotopic dilution assay.⁵ A linear relationship was found between the TETA-OSSu:1A3 reaction stoichiometry and the chelator/mAb ratio, indicating that approximately 2% of the activated ester reacted with the antibody to form a functional chelator. At TETA-OSSu:mAb conjugation ratios of 10:1, 20:1, 50:1, and 100:1, respectively, the average chelator/mAb ratios were 0.40, 0.54, 0.95, and 2.00. It should be noted that the method used to determine the number of chelators/mAb is only a measurement of the average number of *functional* chelating agents; i.e., the chelators that are available for complexing Cu(II). Therefore, it is possible that there are more chelators present on the mAb than can be measured by the assay used.

Radiolabeling and *in Vitro* Evaluation of ⁶⁴Cu-TETA-1A3

TETA-1A3 was labeled with ⁶⁴Cu using modifications of previously published procedures,^{5,10} and the radiolabeled mAb was purified using a gel filtration spin column technique.⁵ The results of triplicate labeling experiments using 1A3 conjugated with 10, 20, 50, and 100 theoretical equivalents of TETA-OSSu are presented in Table 1. In these experiments, TETA-1A3 was reacted with ⁶⁴Cu citrate at ratios increasing from 5.00 $\mu\text{Ci}/\mu\text{g}$ to 30.0 $\mu\text{Ci}/\mu\text{g}$, until the labeling

efficiency dropped below 50%. The labeling efficiencies and specific activities obtained were directly proportional to the amount of TETA-OSSu used in the conjugation and to the average number of chelators conjugated to the mAb. When 1A3 was conjugated with 100 equivalents of the TETA activated ester, ⁶⁴Cu incorporation averaged $83.2 \pm 4.7\%$ ($n = 6$), and the specific activity of ⁶⁴Cu-TETA-1A3 reached a maximum of 15.4 $\mu\text{Ci}/\mu\text{g}$. Since the average specific activity of the cyclotron-produced ⁶⁴Cu has been measured at 12,800 Ci/mmol,¹¹ the maximum specific activity of ⁶⁴Cu-TETA-1A3 corresponded to 18% of the antibody molecules being labeled with ⁶⁴Cu. FPLC analysis of all ⁶⁴Cu-TETA-1A3 preparations indicated that the radiochemical purity of the labeled mAb was >99%, and no antibody aggregates were formed by the conjugation reaction.

The kinetic stability of ⁶⁴Cu-TETA-1A3 was assessed by incubating the radiolabeled mAb in rat serum at 37 °C for 48 h. FPLC analysis of this mixture showed that the conjugate, which had an initial radiochemical purity of 99.4%, lost 1.7% of its ⁶⁴Cu label over 48 h. Interestingly, the ⁶⁴Cu lost from TETA-1A3 was observed as a low molecular weight species and was not associated with any serum proteins.

The immunoreactivity of ⁶⁴Cu-TETA-1A3 was determined under conditions of antigen excess in 1A3 antigen-expressing GW39 human colon carcinoma cells. Under these conditions, the immunoreactivity of the conjugates ranged from 73.8% for the 100:1 TETA-OSSu:mAb conjugate to 87.3% for the 20:1 conjugate. Because the conjugate prepared with 10 equivalents of TETA-

TABLE 1
Copper-64 Labeling Efficiencies, Specific Activities, and Immunoreactivities for TETA-1A3 Conjugates ($n = 3$).

TETA-OSSu:mAb	⁶⁴ Cu/mAb ($\mu\text{Ci}/\mu\text{g}$)	Labeling (%)	Specific Activity ($\mu\text{Ci}/\mu\text{g}$)	Immunoreactivity (%)
10:1	5.00	39.4 ± 0.8	1.97	ND ^a
20:1	5.00	66.6 ± 0.2	3.33	87.3
50:1	10.0	74.0 ± 2.2	7.40	84.0
100:1	20.0	83.2 ± 4.7^b	15.4	73.8

^aND = not determined.

^b $n=6$

OSSu did not label to high specific activity, its reactivity with GW39 cells was not determined. The results obtained with the other 3 conjugates (Table 1) indicate that while the immunoreactivity of all conjugates remained high, a slight decrease was observed for TETA-1A3 prepared with 100 equivalents of the activated ester. This slight decrease in immunoreactivity is likely due to non-specific conjugation of TETA to mAb 1A3, which possibly attaches a chelator in the region of the antigen binding site. We have observed similar results when other chelators were attached to mAb 1A3. For example, Rogers et al. reported a decrease in immunoreactivity to 75% from >90% when conjugating SCN-TETA to mAb 1A3, when only 0.24 chelators/mAb were added.²¹ It is likely that this particular mAb is susceptible to decreasing immunoreactivity when chelators are added.

***In Vivo* Distribution of ⁶⁴Cu-TETA-1A3 and ⁶⁴Cu-BAT-2IT-1A3**

The biodistribution of ⁶⁴Cu-TETA-1A3 in GW39 tumor-bearing Golden Syrian hamsters (Table 2) was compared to that of ⁶⁴Cu-BAT-2IT-1A3 (Table 3). As expected, both radiolabeled conjugates cleared relatively slowly from blood, which was the dominant normal tissue out to 48 h. Heart and spleen uptakes of the two conjugates were very similar, with values at 1 h of approximately 2% ID/g decreasing to about 1% ID/g by 48 h. However, lung uptake of ⁶⁴Cu-BAT-2IT-1A3 was approximately 3% ID/g at early time points, with accumulation decreasing to about 2% ID/g at 24 to 48 h. These values were 1.5- to 2-fold higher than the lung uptake observed for ⁶⁴Cu-TETA-1A3.

After blood, liver had the highest non-target or-

gan accumulation. The TETA conjugate showed initial hepatic accumulation of 3.74% ID/g at 1 h and modest clearance to 2.61% ID/g at 48 h, but this clearance was not statistically significant ($p > 0.05$). Following administration of ⁶⁴Cu-TETA-1A3, significant increases in radioactivity were seen in the gastrointestinal tract, particularly in the upper and lower large intestine. At 1 h, upper and lower large intestine uptakes of ⁶⁴Cu-TETA-1A3 were 0.251% ID/g and 0.208% ID/g, respectively, and the corresponding values increased to 0.964% ID/g and 1.17% ID/g at 48 h. In contrast, liver uptake of ⁶⁴Cu-BAT-2IT-1A3 was 3.08% ID/g at 1 h but cleared significantly ($p < 0.05$) to 1.69% ID/g by 48 h post-injection. At 48 h, uptakes of the BAT-2IT conjugate in the stomach and small intestine were significantly lower than those of ⁶⁴Cu-TETA-1A3. As with the TETA conjugate, the amounts of radioactivity from ⁶⁴Cu-BAT-2IT-1A3 increased in the upper and lower large intestine with time. At 1 h post-injection, upper and lower large intestine uptakes for the BAT-2IT conjugate were 0.183% ID/g and 0.168% ID/g, respectively, values similar to those seen with ⁶⁴Cu-TETA-1A3. However, by 48 h the uptakes for ⁶⁴Cu-BAT-2IT-1A3 in the upper and lower large intestine were only 0.478% ID/g and 0.439% ID/g, respectively, values 2- to 2.7-fold lower than the corresponding uptakes for ⁶⁴Cu-TETA-1A3.

After injection of ⁶⁴Cu-TETA-1A3, radioactivity in the kidney was initially 1.74% ID/g at 1 h and did not clear significantly out to 48 h, when renal uptake was 1.38% ID/g. At 1 h post-injection, kidney uptake of ⁶⁴Cu-BAT-2IT-1A3 (2.07% ID/g) was similar to that of the TETA conjugate, but by 48 h it had decreased to 1.05% ID/g, significantly lower ($p < 0.05$) than the corresponding value for ⁶⁴Cu-TETA-1A3. However,

TABLE 2. Biodistribution (%ID/g \pm s.d.) at 1, 6, 24, and 48 h of ⁶⁴Cu-TETA-1A3 in Golden Syrian Hamsters Bearing GW39 Human Colon Carcinoma Xenografts.

Organ	1 h (n = 4)	12 h (n = 5)	24 h (n = 5)	48 h (n = 5)
Blood	12.1 \pm 3.73	6.85 \pm 0.43	4.58 \pm 0.93	2.74 \pm 0.07
Muscle	0.11 \pm 0.03	0.26 \pm 0.02	0.33 \pm 0.03	0.30 \pm 0.03
Heart	2.25 \pm 0.34	1.68 \pm 0.11	1.23 \pm 0.13	0.96 \pm 0.07
Lung	2.74 \pm 0.60	2.32 \pm 0.27	2.31 \pm 0.90	1.08 \pm 0.06
Liver	3.74 \pm 0.33	3.25 \pm 0.31	2.99 \pm 0.15	2.61 \pm 0.16
Spleen	1.79 \pm 0.95	1.95 \pm 0.34	1.61 \pm 0.05	0.95 \pm 0.13
Kidney	1.74 \pm 1.08	2.22 \pm 1.08	1.63 \pm 0.18	1.38 \pm 0.07
Stomach	0.19 \pm 0.10	0.43 \pm 0.07	0.60 \pm 0.16	0.53 \pm 0.06
Small Intestine	0.94 \pm 0.56	1.23 \pm 0.09	1.20 \pm 0.12	0.88 \pm 0.11
Upper Large Intestine	0.25 \pm 0.16	1.03 \pm 0.16	1.04 \pm 0.21	0.96 \pm 0.05
Lower Large Intestine	0.21 \pm 0.11	0.89 \pm 0.17	1.21 \pm 0.28	1.17 \pm 0.12
Bone	0.91 \pm 0.18	0.80 \pm 0.27	0.75 \pm 0.10	0.55 \pm 0.03
Tumor	0.83 \pm 0.81	7.12 \pm 3.74	9.05 \pm 1.85	8.25 \pm 1.56

TABLE 3
Biodistribution (%ID/g \pm s.d.) at 1, 6, 24, and 48 h of ^{64}Cu -BAT-2IT-1A3 in Golden Syrian Hamsters Bearing GW39 Human Colon Carcinoma Xenografts.

Organ	1 h (n = 5)	12 h (n = 5)	24 h (n = 5)	48 h (n = 5)
Blood	8.78 \pm 1.34	4.98 \pm 0.75	4.17 \pm 0.83	2.54 \pm 1.29
Muscle	0.23 \pm 0.09	0.27 \pm 0.07	0.34 \pm 0.08	0.48 \pm 0.12
Heart	2.22 \pm 0.41	1.79 \pm 0.23	1.38 \pm 0.15	1.25 \pm 0.17
Lung	3.20 \pm 0.51	3.08 \pm 1.07	2.19 \pm 0.92	1.85 \pm 0.25
Liver	3.08 \pm 0.87	2.20 \pm 0.15	1.86 \pm 0.36	1.69 \pm 0.13
Spleen	1.63 \pm 0.30	1.98 \pm 0.26	1.68 \pm 0.36	1.69 \pm 0.25
Kidney	2.07 \pm 0.62	1.22 \pm 0.28	1.02 \pm 0.19	1.05 \pm 0.14
Stomach	0.24 \pm 0.08	0.27 \pm 0.04	0.29 \pm 0.04	0.24 \pm 0.04
Small Intestine	1.01 \pm 0.22	0.79 \pm 0.06	0.65 \pm 0.12	0.61 \pm 0.05
Upper Large Intestine	0.18 \pm 0.04	0.47 \pm 0.09	0.44 \pm 0.08	0.48 \pm 0.10
Lower Large Intestine	0.17 \pm 0.05	0.48 \pm 0.10	0.52 \pm 0.09	0.44 \pm 0.10
Bone	0.69 \pm 0.08	0.88 \pm 0.37	0.71 \pm 0.18	0.72 \pm 0.13
Tumor	1.13 \pm 0.44	6.00 \pm 1.01	6.70 \pm 1.77	6.60 \pm 1.35

kidney clearance of ^{64}Cu -BAT-2IT-1A3 from 1 to 48 h was not statistically significant at the 95% confidence level.

Tumor uptake of ^{64}Cu -TETA-1A3 reached a maximum of 9.05% ID/g at 24 h. The activity was retained in the tumor out to 48 h, at which time tumor uptake remained at 8.25% ID/g. Tumor-to-blood ratios of the TETA conjugate were 1.04 at 12 h, 1.97 at 24 h, and a maximum of 3.01 at 48 h. Maximum tumor uptake of ^{64}Cu -BAT-2IT-1A3 was 6.70% ID/g at 24 h, but this value was not significantly different from that observed with ^{64}Cu -TETA-1A3 at the same time point. The ^{64}Cu -labeled BAT-2IT conjugate was also retained in the tumor at 48 h, when the uptake was 6.60% ID/g. Tumor-to-blood ratios of ^{64}Cu -BAT-2IT-1A3 were 1.20 at 12 h, 1.61 at 24 h, and 2.60 at 48 h.

The tumor-to-normal tissue ratios of ^{64}Cu -TETA-1A3 were compared to those obtained with ^{64}Cu -BAT-2IT-1A3. Tumor-to-blood, tumor-to-liver, tumor-to-kidney, and tumor-to-bone ratios of the two conjugates at 24 h, the time of maximum tumor uptake, are shown in Figure 3. No significant differences ($p = 0.05$) in tumor-to-normal tissue ratios were observed for ^{64}Cu -TETA-1A3 versus ^{64}Cu -BAT-2IT-1A3.

Human Normal Organ and Hamster Tumor Dosimetry of ^{64}Cu -TETA-1A3 and ^{64}Cu -BAT-2IT-1A3

Human absorbed dose estimates to the normal organs for ^{64}Cu -TETA-1A3 and ^{64}Cu -BAT-2IT-1A3 are summarized in Table 4. The dosimetry of the two conjugates was similar for most normal organs, although moderate differences were observed in non-target organs such as the adren-

als, muscle, lungs, and bone surfaces. The largest discrepancies observed in non-target organs were to the heart wall, spleen, and the upper and lower large intestine. The heart wall dose imparted by ^{64}Cu -TETA-1A3 was 1.9 times higher than that of the BAT-2IT-1A3 conjugate, but the spleen dose was 1.8 times higher for ^{64}Cu -BAT-2IT-1A3. The slower liver clearance of the TETA conjugate resulted in a slightly higher dose to that organ, and doses to the upper and lower large intestine from ^{64}Cu -TETA-1A3 were 1.8 times higher than those from ^{64}Cu -BAT-2IT-1A3. Kidney and bladder wall absorbed doses were nearly identical for the two conjugates, despite the fact that retention of ^{64}Cu -BAT-2IT-1A3 in kidney was significantly less than that of the TETA conjugate. For both conjugates, critical organs were

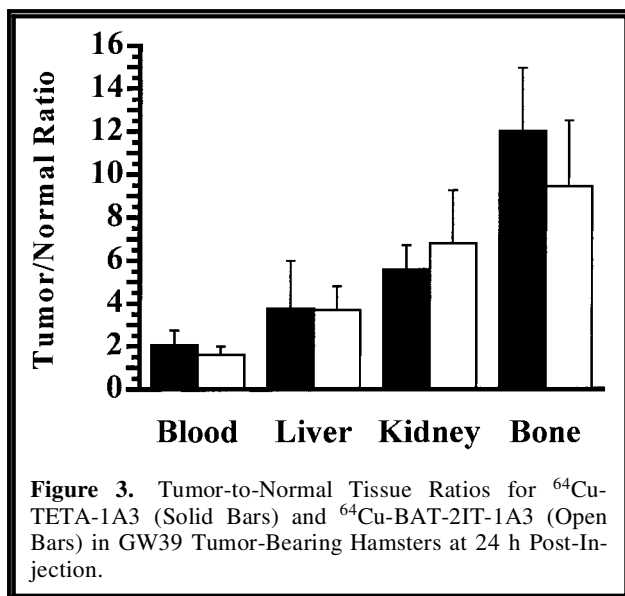


Figure 3. Tumor-to-Normal Tissue Ratios for ^{64}Cu -TETA-1A3 (Solid Bars) and ^{64}Cu -BAT-2IT-1A3 (Open Bars) in GW39 Tumor-Bearing Hamsters at 24 h Post-Injection.

TABLE 4
Human Absorbed Dose Estimates for ^{64}Cu -TETA-1A3 and ^{64}Cu -BAT-2IT-1A3 Obtained in GW39 Tumor-Bearing Hamsters. Values are Given in rad/mCi (mGy/MBq).

Organ	^{64}Cu -TETA-1A3	^{64}Cu -BAT-2IT-1A3
Adrenals	0.107 (0.029)	0.091 (0.025)
Bladder Wall	0.060 (0.016)	0.052 (0.014)
Bone Surfaces	0.160 (0.043)	0.194 (0.052)
Stomach Wall	0.160 (0.043)	0.118 (0.032)
Small Intestine	0.136 (0.037)	0.105 (0.028)
Upper Large Intestine	0.532 (0.144)	0.300 (0.081)
Lower Large Intestine	0.506 (0.137)	0.278 (0.075)
Heart Wall	0.906 (0.245)	0.471 (0.127)
Kidneys	0.332 (0.090)	0.300 (0.081)
Liver	0.634 (0.171)	0.456 (0.123)
Lungs	0.129 (0.035)	0.128 (0.035)
Red Marrow	0.251 (0.068)	0.320 (0.086)
Muscle	0.072 (0.019)	0.066 (0.018)
Spleen	0.086 (0.023)	0.157 (0.042)

the bone marrow and the large intestine. While the upper and lower large intestine doses were higher for ^{64}Cu -TETA-1A3, the red marrow dose was slightly higher for the BAT-2IT-1A3 conjugate.

The tumor absorbed doses in hamsters with small, 2-day-old tumors are presented in Table 5. The dose from ^{64}Cu -BAT-2IT-1A3 to a 0.4-g tumor was 227 rad/mCi. The absorbed dose delivered by ^{64}Cu -TETA-1A3 to a 0.2-g tumor was slightly higher at 284 rad/mCi, but this value was not significantly different from that of ^{64}Cu -BAT-2IT-1A3.

DISCUSSION

The macrocyclic chelator TETA selectively forms Cu(II) complexes with high thermodynamic stability and higher kinetic stability than non-macrocyclic chelators such as DTPA,^{1,22,23} making it useful for labeling biomolecules with copper radioisotopes. Meares and coworkers synthesized a bifunctional derivative of TETA, BAT, for labeling monoclonal antibodies with ^{67}Cu

($T_{1/2} = 62 \text{ h}$).¹ After conjugation with BAT and the 2IT,² the mAb Lym-1 was labeled with ^{67}Cu and evaluated for imaging and radioimmunotherapy of non-Hodgkin's lymphoma.^{3,4} Our group has used BAT and 2IT to label mAb 1A3 with ^{64}Cu for PET imaging and RIT of colorectal carcinoma.⁵⁻⁷ While conjugation with BAT allows for very efficient labeling of antibodies with copper radioisotopes, the bifunctional chelator is the product of a lengthy synthesis, a factor that may limit the use of this method for the development of radiocopper-labeled mAbs. We report here an alternative method for conjugation of TETA to mAbs using a simple, water-soluble procedure. This method utilizes only commercially available reagents, making it applicable to widespread use in labeling antibodies with all copper radioisotopes, including the longer-lived ^{67}Cu , which is well-suited for radioimmunotherapy.

The preparation of NHS esters of DOTA and TETA in organic solvents (DMSO) for conjugation of mAbs has previously been presented by Srivastava et al.²⁴ In this report, the NHS esters of DOTA and TETA were prepared in DMSO

TABLE 5
Absorbed Doses Delivered to GW39 Hamster Tumors by ^{64}Cu -TETA-1A3 and ^{64}Cu -BAT-2IT-1A3. Values are Given in rad/mCi (mGy/MBq).

Conjugate	Tumor Dose
^{64}Cu -TETA-1A3	284 (76.8)
^{64}Cu -BAT-2IT-1A3	227 (61.4)

and were stored at 0°C prior to use. Disadvantages of this method include having to add a DMSO solution of the active ester to the mAb, which could cause protein denaturation and subsequent lowering of immunoreactivity. The method for preparing activated esters of DOTA described by Lewis et al.^{9,10} uses only aqueous buffer solutions and precludes the need to use organic solvents altogether.

In addition to forming highly stable TETA complexes, Cu(II) also has high affinity for the chelator DOTA, as demonstrated by the serum stability studies of a ⁶⁷Cu-labeled DOTA-conjugated mAb.²³ In fact, Kukis et al. reported enhanced serum stability of the ⁶⁷Cu-labeled DOTA-conjugated mAb (BAD-2IT-mAb) over the ⁶⁷Cu-labeled BAT-2IT-mAb conjugate.²³ We compared ⁶⁴Cu-labeled TETA and DOTA conjugates of the somatostatin analog, tyrosine³-octreotide (Y3-OC) and found higher liver and kidney accumulation for ⁶⁴Cu-DOTA-Y3-OC than for ⁶⁴Cu-TETA-Y3-OC.²⁵ Although no metabolism studies have been reported, we hypothesize that this higher accumulation of ⁶⁴Cu in the non-target organs is due to dissociation of ⁶⁴Cu from the DOTA chelator. For this reason, we chose to evaluate the TETA conjugates of mAb 1A3 rather than the DOTA conjugates.

In the Lewis method of conjugating DOTA to mAbs using EDC and sulfo-NHS,^{9,10} the limiting reagent is EDC, and this stoichiometry favors activation of a single carboxyl group of polyazamacrocyclic polycarboxylate ligands such as DOTA or TETA. The use of 10 equivalents of TETA in the reaction discourages multiple activation of the tetracarboxylic acid, and the use of 10 equivalents of sulfo-NHS allows for efficient conversion of the TETA-EDC intermediate to the activated ester. These conditions should minimize formation of potential reaction by-products, such as the TETA-EDC *N*-acylurea or the *bis*-active ester of TETA, which would likely exhibit reduced chelate stability compared to the monoamide derivative of TETA.

We applied the method of Lewis et al. to the conjugation of mAb 1A3 with TETA and compared the ⁶⁴Cu labeling chemistry, *in vitro* behavior, and targeting properties of ⁶⁴Cu-TETA-1A3 in tumor-bearing hamsters with ⁶⁴Cu-BAT-2IT-1A3. As the amount of TETA active ester used in the conjugation reaction was increased, the antibody became modified with increasing numbers of functional chelators, and the resulting TETA-1A3 conjugates could be labeled

to progressively higher specific activities with ⁶⁴Cu. When 1A3 was reacted with 100 equivalents of TETA-OSSu, the resulting conjugate was modified with an average of 2 functional chelators per antibody molecule and could be labeled with ⁶⁴Cu at specific activities in excess of 15 mCi/mg, a value sufficiently high for RIT applications. However, heating of the mAb conjugate to 43 °C was necessary to achieve maximum specific activity labeling. This finding was in contrast to BAT-2IT-1A3, which could be labeled to high specific activity with ⁶⁴Cu at room temperature. The difference in ⁶⁴Cu labeling efficiency between the TETA and BAT-2IT conjugates may reflect a difference in the charge or protonation state of the two chelators. Recently Keire and Kobayashi²⁶ demonstrated that the yttrium complexation rates of a butylamide derivative of DOTA were approximately one-half those of DOTA between pH 4.6 and 6.5. In this case, the rate-limiting step is proton loss from the mono-protonated yttrium chelate to form the fully coordinated complex, which is accompanied by a significant rearrangement of the macrocyclic ring.²⁷ Thus, it was suggested that the replacement of one of the carboxylic acid groups of DOTA with an amide introduced a weaker proton transfer mediator and decelerated the metal loading reaction.²⁶ It is possible that a similar situation is encountered when one of the carboxylate arms of TETA is converted to an amide, resulting in a decrease in the rate of deprotonation of the copper chelate and requiring elevated temperature to accelerate the labeling reaction.

Conversion of one of the carboxylic acids of TETA to an amide group appeared to have a minimal effect on the kinetic stability of ⁶⁴Cu-TETA-1A3 *in vitro*. After incubation of the ⁶⁴Cu-labeled mAb in rat serum at 37 °C for 48 h, only 1.7% of the radiolabel was lost, a result comparable to that observed for ⁶⁷Cu-BAT-2IT-Lym-1.²³ TETA conjugation also had a minimal effect on the immunoreactivity of 1A3. After modification with 20 or 50 equivalents of the TETA activated ester, the antigen binding activity of ⁶⁴Cu-TETA-1A3 was nearly identical to that of ¹²⁵I-1A3,⁵ while modification with 100 equivalents of TETA-OSSu resulted in only a slight decrease in immunoreactivity.

Biodistribution studies in hamsters bearing GW39 human colon carcinoma xenografts demonstrated that ⁶⁴Cu-TETA-1A3 and ⁶⁴Cu-BAT-2IT-1A3 had similar pharmacokinetic properties. Both conjugates exhibited maximum tumor up-

take at 1 day post-injection. In contrast, previous studies in the GW39 hamster model²⁸ showed that tumor uptake of ¹¹¹In-labeled 1A3 reached a maximum at 3 days post-injection. The more rapid tumor targeting characteristics of the ⁶⁴Cu-labeled conjugates would enable imaging at earlier times post-injection than with the ¹¹¹In-labeled mAb. Furthermore, because of the 12.7-h half-life of ⁶⁴Cu, relatively rapid tumor targeting has important implications for radioimmunotherapy. Both the TETA and BAT-2IT conjugates attained tumor-to-blood ratios in excess of 1:1 by 12 h post-injection, with values reaching 2:1 to 3:1 by 24–48 h.

Normal organ biodistributions were generally similar for ⁶⁴Cu-TETA-1A3 and ⁶⁴Cu-BAT-2IT-1A3. The most significant differences in non-target organ uptakes were observed in the clearance organs. Both conjugates showed considerable accumulation of radioactivity in liver and kidney. However, in contrast to the TETA conjugate, ⁶⁴Cu-BAT-2IT-1A3 cleared significantly from the liver and had significantly lower retention in the kidney. The TETA conjugate also exhibited significantly greater uptake in the gastrointestinal tract, particularly in the large intestine. These differences in clearance between the two conjugates may reflect differences in metabolism of the two copper chelates, which might result from differences in charge or kinetic stability. Jones-Wilson et al. evaluated the effects of charge on the biological behavior of ⁶⁴Cu-labeled macrocyclic complexes.²⁹ These studies demonstrated that positively charged ⁶⁴Cu chelates had significantly higher liver and kidney uptakes than did negatively charged or neutral complexes. Compared to ⁶⁴Cu-BAT-2IT-1A3, in which the chelate is expected to have a -2 charge at physiologic pH, conversion of one carboxylate of the macrocycle to an amide group, as in ⁶⁴Cu-TETA-1A3, reduces the charge of the chelate to -1. This reduction in negative charge may lead to increased retention of ⁶⁴Cu in the liver and kidney. Alternatively, the ⁶⁴Cu-BAT complex may be more stable *in vivo* than the amide-conjugated TETA chelate. Bass and colleagues³⁰ determined that ⁶⁴Cu dissociates from amide-conjugated TETA-octreotide and is transchelated by superoxide dismutase (SOD) in rat liver. *In vivo* dissociation of copper radionuclides from biomolecule-chelate conjugates may be the result of thermodynamic or kinetic instability. Jones-Wilson et al.²⁹ found that some of the most thermodynamically stable copper chelates appeared to be the least stable *in*

vivo, suggesting that kinetic stability may be the more important determinant of the metabolic fate of copper radiopharmaceuticals. In the present study, the serum stability of ⁶⁴Cu-TETA-1A3 was found to be very similar to that previously reported for ⁶⁷Cu-labeled BAT-2IT conjugates,²³ yet it is possible that more ⁶⁴Cu dissociates from the TETA chelator *in vivo*, giving rise to greater liver and kidney retention of radioactivity.

Despite the differences in clearance between ⁶⁴Cu-TETA-1A3 and ⁶⁴Cu-BAT-2IT-1A3, no significant differences in tumor-to-liver, tumor-to-kidney, or any other tumor-to-normal tissue ratios were observed for the two conjugates at 24 h post-injection, the time of maximum tumor uptake. However, increased uptakes of ⁶⁴Cu-TETA-1A3 in clearance organs had variable effects on human absorbed dose estimates. In the kidney, the significantly greater retention of ⁶⁴Cu from the TETA conjugate caused only a marginal increase in absorbed dose. In contrast, the greater uptakes and slower clearance of ⁶⁴Cu-TETA-1A3 from the liver and gastrointestinal tract resulted in a 39% increase in liver dose and nearly 2-fold increases in doses to the upper and lower large intestine. A similar situation was encountered in the heart wall, where greater uptake of the TETA conjugate at early time points produced a nearly 2-fold increase in absorbed dose compared to the BAT-2IT conjugate. For PET imaging applications, a 10-mCi dose of ⁶⁴Cu-TETA-1A3 would give reasonable doses of 5 rad to the upper and lower large intestine. For RIT applications, the large intestine is expected to be a critical organ, and absorbed dose differences between the two conjugates may become an important consideration. The bone marrow is also expected to be a critical organ in RIT with ⁶⁴Cu-labeled 1A3. The red marrow absorbed dose estimate for ⁶⁴Cu-BAT-2IT-1A3 was 27% higher than that of the TETA conjugate.

Previous RIT studies⁷ demonstrated that 2 mCi of ⁶⁴Cu-BAT-2IT-1A3 produced complete regressions of small (0.2–0.4 g) GW39 tumors in the hamster model, with no signs of overt toxicity. In this study, the estimated absorbed dose to the GW39 tumor was 227 rad/mCi (61.4 mGy/MBq), which corresponds to 454 rad (4.54 Gy) for a 2-mCi injected dose. The tumor dose estimate for ⁶⁴Cu-TETA-1A3 was slightly higher at 284 rad/mCi (76.8 mGy/MBq), or 568 rad (5.68 Gy) for a 2-mCi injected dose, but this difference was not statistically significant. It is important to note that these are absorbed dose esti-

mates for the hamster tumor xenograft and not estimated doses to human tumors.

In conclusion, we have developed a simple procedure for conjugation of monoclonal antibodies with the macrocyclic chelating agent TETA, using commercially available reagents. This procedure represents an alternative to the use of BAT, which is the product of a lengthy multi-step synthesis, for labeling mAbs with copper radioisotopes. The radiolabeling efficiency, serum stability, immunoreactivity, biodistribution, and dosimetry properties of ^{64}Cu -TETA-1A3 compared favorably to those of ^{64}Cu -BAT-2IT-1A3. The results presented in this paper provide a rationale for further evaluation of the diagnostic and therapeutic efficacy of ^{64}Cu -TETA-1A3 in the GW39 hamster model, in preparation for clinical trials.

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