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Labeling and *in Vivo* Evaluation of Novel Copper(II) Dioxotetraazamacrocyclic Complexes

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ABSTRACT. ^{64}Cu shows promise as both a positron emission tomography imaging and radiotherapeutic radionuclide due to its half-life ($T_{1/2}$ = 12.7 h), decay characteristics (β^+ [19%]; β^- [40%]), and the capability to produce it on a large-scale with high specific activity on a biomedical cyclotron. Macrocyclic chelators are generally used as bifunctional chelators to attach Cu(II) to antibodies and peptides due to their relatively high *in vitro* stability. To investigate neutral Cu(II) complexes, we performed labeling experiments with six tetraazamacrocyclic ligands with different chelate ring sizes. 1,4,8,11-Tetraazacyclotetradecane-3,9-dione (1), 1,4,8,11-tetraazacyclotetradecane-5,7-dione (2), 1,4,7,10-tetraazacyclotridecane-11,13-dione (3), 1,4,7,10-tetraazacyclotridecane-2,9-dione (4), 1,4,7,10-tetraazacyclododecane-2,9-dione (5), and 1,4,7,10-tetraazacyclotridecane-3,8-dione (6) were radiolabeled with ^{64}Cu. Only ^{64}Cu-labeled 1 readily formed a complex in high purity, and therefore was evaluated *in vivo*. The rapid blood, liver, and kidney clearance of ^{64}Cu-labeled 1 suggest that ligand 1 may be useful as a macrocyclic structure to design new bifunctional chelators for copper radionuclides in diagnostic or radiotherapeutic studies and is a potential alternative to currently used macrocyclic bifunctional chelators. NUCL MED BIOL 27;4:375–380, 2000. **© 2000 Elsevier Science Inc. All rights reserved.**

KEY WORDS: Copper-64, Biodistribution, Macrocycle

INTRODUCTION

^{64}Cu shows promise as both a positron emission tomography (PET) imaging and radiotherapeutic radionuclide due to its half-life ($T_{1/2}$ = 12.7 h), decay characteristics (β^+ [19%]; β^- [40%]), and the ability for its large-scale production with high specific activity on a biomedical cyclotron (2, 6, 14, 19). Macrocyclic chelators are generally used as bifunctional chelators to bind Cu(II) to antibodies and peptides due to their relatively high *in vitro* stability (5, 16). Rat and hamster biodistribution studies on monoclonal antibody (mAb) fragments and peptides labeled with ^{64}Cu indicated that the charge of the bifunctional chelate used to complex ^{64}Cu had a significant effect on the clearance properties of the conjugate fragments (3, 20). In another study on the biological behavior of ^{64}Cu complexes, six macrocyclic complexes with different formal charges were prepared and their biodistributions evaluated in normal Sprague-Dawley rats (11). The negatively charged compounds exhibited low liver uptake and rapid and efficient clearance through the kidneys, whereas the positively charged complexes showed higher retention in the kidney and liver out to 24 h postinjection (11). The neutral complex behaved similarly to the negatively charged Cu(II) complexes.

To investigate neutral Cu(II) complexes more thoroughly, we performed labeling experiments with six tetraazamacrocyclic ligands with different chelate ring sizes (7). 1,4,8,11-Tetraazacyclotetradecane-3,9-dione (**1**), 1,4,8,11-tetraazacyclotetradecane-5,7-dione (**2**), 1,4,7,10-tetraazacyclotridecane-11,13-dione (**3**), 1,4,7,10-tetraazacyclotridecane-2,9-dione (**4**), 1,4,7,10-tetraazacyclododecane-2,9-dione (**5**), and 1,4,7,10-tetraazacyclotridecane-3,8-dione (**6**) (Fig. 1) were radiolabeled with ^{64}Cu. Ligands **1** and **2** are both 14-membered rings differing only in the position of the dioxo groups; ligands **3**, **4**, and **6** are 13-membered rings also differing only in the placement of the dioxo groups; and ligand **5** is a 12-membered ring. ^{64}Cu-labeled **1** was also evaluated *in vivo*. The thermodynamic stability constants of the Cu(II) complexes of **1** through **6** have been reported (18), and these values are compared with the radiochemical and biological data presented here.

MATERIALS AND METHODS

Radiochemistry

Ligand **2** was purchased from Aldrich Chemical Co. (St. Louis, MO USA). Ligand **3** was prepared by Dr. Hidefumi Kato of Kurume Technical College (Kurume, Japan). Ligands **1** and **4** through **6** were prepared by methods reported previously (18). All materials were reagent grade unless otherwise specified. ^{64}Cu was prepared on the Washington University Medical School Cyclotron CS-15 cyclotron by the ^{64}Ni(p,n)^{64}Cu nuclear reaction as previously described (14). Water was distilled and then deionized (18 MΩ/cm^2) by passing through a Milli-Q® water filtration system (Millipore Corp., Bedford, MA USA). Ammonium acetate was purchased from Fluka Chemie AG (Buchs, Switzerland). Cu(II) chloride (99.999%) was purchased from Aldrich Chemical Co. (Milwaukee, WI USA) and diethylenetriaminepentaacetic acid (DTPA) from Sigma Chemical Co. (St. Louis, MO USA). Waters C18 silica gel thin layer chromatography (TLC) plates (KC18F, 60 Å, 200 μm)

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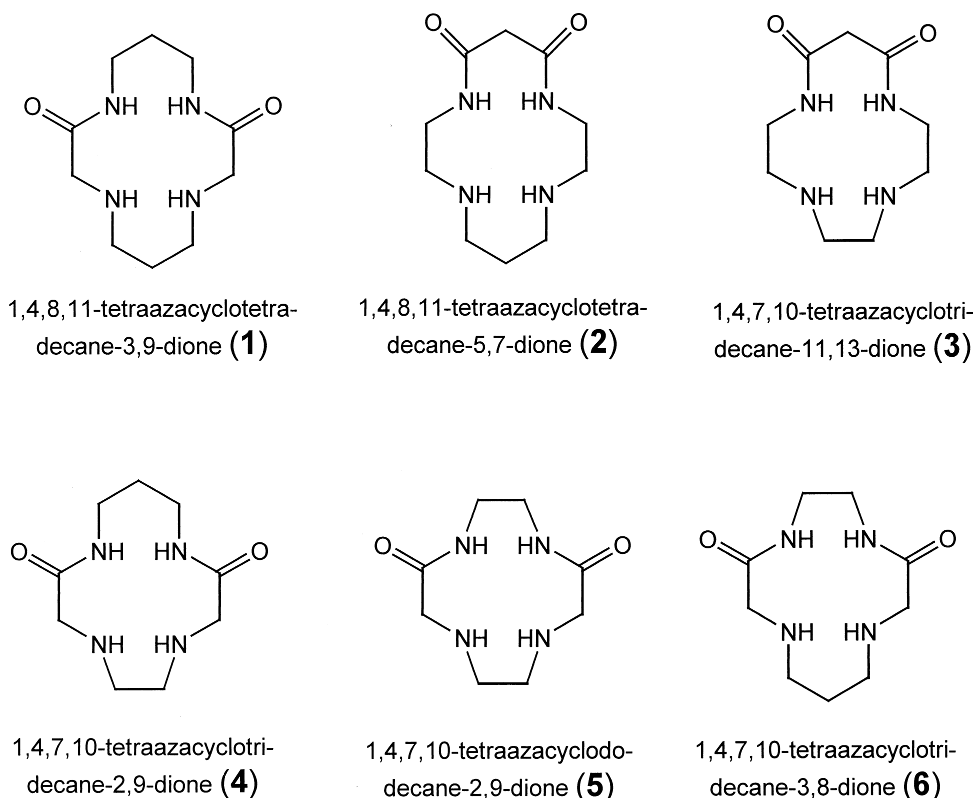

FIG. 1. Structures of the six dioxomacrocyclic ligands investigated in this study for labeling with ^{64}Cu. 1,4,8,11-tetraazacyclotetradecane-3,9-dione, 1; 1,4,8,11-tetraazacyclotetradecane-5,7-dione, 2; 1,4,7,10-tetraazacyclotridecane-11,13-dione, 3; 1,4,7,10-tetraazacyclotridecane-2,9-dione, 4; 1,4,7,10-tetraazacyclododecane-2,9-dione, 5; 1,4,7,10-tetraazacyclotridecane-3,8-dione, 6.

were purchased from Fisher Scientific (Pittsburgh, PA USA). The chemical purity of Cu(II)-**1** was determined by TLC using ultraviolet detection. Radio-TLC chromatograms were analyzed on either a Bioscan 200 imaging scanner (Bioscan, Inc., Washington, DC USA) or a Berthold Automatic TLC-Linear Analyzer (Berthold System, Inc., Pittsburgh, PA USA). Radioactivity was counted with a Beckman Gamma 8000 counter containing a NaI crystal (Beckman Instruments, Inc., Irvine, CA USA).

Complex charge was determined by electrophoresis using a Helena Laboratories electrophoresis chamber (Beaumont, TX USA) with Sepraphore III cellulose polyacetate strips (Gelman Sciences Inc., Ann Arbor, MI USA) presoaked in either 0.1 M HEPES buffer, pH 7.4; 0.1 M ammonium acetate buffer, pH 5.5; or 0.1 M citrate buffer, pH 5.5. The strips were developed using a Bio-Rad model 1000/500 power supply (Richmond, CA USA) at a constant current of 8 mA and a voltage of 250 mV for 45 min. ^{111}In-DTPA, known to have an overall charge (2−) was used as a standard (11). The strips were analyzed using the Bioscan imaging scanner to determine the migration of radioactivity and overall charge of the complex.

Preparation of the ^{64}Cu Ligand Complexes

^{64}Cu chloride was converted to ^{64}Cu-acetate or -citrate by stirring with 0.1 M ammonium acetate, pH 5.5, or 0.1 M ammonium citrate, pH 5.5. ^{64}Cu-acetate or -citrate (0.1–0.5 mCi) was added to a ligand solution containing 0.5–5 mg of ligand in 0.3–1.0 mL of 0.1 or 0.4 M ammonium acetate, pH 5.5, or 0.1 M ammonium citrate, pH 5.5. Reaction times ranged from 1–20 h and temperatures ranged from room temperature to 55°C. The radiochemical purity was determined by TLC using C18 plates developed in MeOH:10% ammonium acetate (1:1). Table 1 summarizes reaction conditions with radiochemical yields for the ^{64}Cu ligand complexes.

*Preparation of ^{64}Cu-labeled Cu(II)-*1("Hot + Cold")

To 100 μL of a 5 mM ligand **1** solution in 0.4 M ammonium acetate, pH 5.5, 100 μL of a 5 mM solution of $CuCl_2$ in 0.4 M ammonium acetate, pH 5.5, was added. The ^{64}Cu-acetate solution (0.2 mCi) described above was added to this mixture. After a reaction time of 1 h at 37°C, the chemical and radiochemical purity was determined by TLC using C18 plates developed in MeOH:10% ammonium acetate (1:1).

Determination of Partition Coefficients

The partition coefficient (log P) of ^{64}Cu-**1** was determined by adding 4 μL of the labeled complex to a solution containing 500 μL of octanol and 500 μL of water (obtained from saturated octanol water solutions; N = 5). The resulting solutions were then shaken for 1 h at room temperature. From each of the five samples, an aliquot of 100 μL was removed from each phase and counted separately. The partition coefficient was calculated as a ratio of counts in the octanol fraction to counts in the water fraction. An average log P value was obtained from the five samples.

Serum Stability

In vitro serum stability experiments were conducted by placing 88–120 μL of ^{64}Cu-labeled ligand **1** in either 1 mL of freshly drawn rat serum or rat serum purchased from Sigma Chemical Co. The solutions were incubated at 37°C, and samples were removed at various time points and analyzed by the TLC methods described above.

TABLE 1. Labeling conditions for Complexing ^{64}Cu to Ligands 1 through 6

Ligand	Radiochemical purity (%)	R_f
1-h reaction time at 37°C		
1	96	0.83
2	20	0.66
3	2	0.81
4	21	0.79
5	0	—
6	0	—
20-h reaction time at 25°C		
1	99	0.83
2	65	0.82
3	7	0.83
4	26	0.83
6	4	0.83
2-h reaction time at 37°C		
1	98.5	0.82
2	44	0.67
3	4	0.82
4	41	0.79
5	0	—
6	0	—
0.5-h reaction time at 55°C		
1	96	0.86
2	57	0.78
4	20[a]	0.82

For all complexes, a 5 mM ligand solution (0.1 M ammonium acetate, pH = 5.5) was used. The complexes were analyzed by thin layer chromatography (C18 plates developed in 1:1 MeOH:10% ammonium acetate).
[a] 1.5-h reaction time.

Biodistribution Studies

All animal studies were performed in compliance with guidelines set by the Washington University Animal Studies Committee. Mature female Sprague-Dawley rats (N = 4 per time point) weighing 150–200 g were anesthetized with Metofane (2,2-dichloro-1,1-difluoro-1-methoxyethanol) and injected with 5–10 μCi of activity in a volume of 100 μL via the tail vein. The rats were anesthetized prior to sacrifice (by decapitation) at each time point. The lung, liver, spleen, kidney, bladder, muscle, fat, heart, brain, and bone were removed from each animal, placed on absorbent paper, and weighed. Blanks and standards were prepared and counted along with the samples to calculate the percent injected dose per gram of tissue (%ID/gram) and percent injected dose per organ (%ID/organ).

RESULTS

Radiochemistry

Ligand **1** could successfully be labeled in >95% radiochemical purity with ^{64}Cu using all reaction conditions described above. Ligands **2** and **4** labeled with a maximum radiochemical yield of 65% and 40%, respectively. The dioxotetraazamacrocycles **3**, **5**, and **6** showed either no complex formation or only a very small amount of labeling with ^{64}Cu. Radiochemical analysis of each ^{64}Cu complex was accomplished by TLC (Table 1). For the ^{64}Cu complexes of ligands **2** through **6**, two species were present by radio-TLC: one at the origin and one that migrated with the R_f values reported in Table 1. The species at the origin was assumed to be ^{64}Cu-citrate or ^{64}Cu-acetate, because those standards also remained at the origin. In general the radiochemical yields with ^{64}Cu-citrate were lower than were those with ^{64}Cu-acetate. An experiment with the ^{64}Cu(II)/Cu(II)-**1** ("hot + cold") showed that the R_f value for the Cu(II) complex and the ^{64}Cu complex under the described conditions were identical.

^{64}Cu-labeled **1** was analyzed by electrophoresis to determine the charge of the complex. The migration of the ^{64}Cu complex was compared with the migration of an ^{111}In-DTPA standard. Although ^{111}In-DTPA migrated with a R_f of 0.44 in the direction of the anode, the ^{64}Cu-labeled **1** remained on the marked midpoint in both buffer systems (0.1 M ammonium acetate, pH 5.5, or 0.1 M HEPES, pH 7.4), indicating a complex of neutral charge. The octanol-water partition coefficient or log P of ^{64}Cu-labeled **1** was determined to be −1.02 ± 0.13. The *in vitro* stability of ^{64}Cu-labeled **1** showed the complex remained approximately 100% intact out to 2 h, indicating that it was stable in rat serum. Unfortunately, purification of ^{64}Cu ligands **2** and **4** from unlabeled ^{64}Cu-acetate was not possible in our hands, and therefore, biological stability of these agents could not be evaluated.

Biodistribution Experiments

The biodistribution results for ^{64}Cu-labeled **1** are shown in Figure 2. The ^{64}Cu-**1** complex showed initially high blood uptake (7 ± 1 %ID/organ at 15 min), which decreased rapidly out to 2 h (0.28 ± 0.061 %ID/organ) with no further clearance out to 24 h (0.23 ± 0.05 %ID/organ). The rapid blood clearance of ^{64}Cu-**1** indicates that the complex did not dissociate in the blood and likely is stable *in vivo*, consistent with the results of the *in vitro* serum stability data. Relatively low liver uptake was observed initially (2.1 ± 0.1 %ID/organ) and decreased over time (0.7 ± 0.1 %ID/organ at 24 h). No significant brain or heart uptake was observed (0.075 ± 0.02 and 0.17 ± 0.05 %ID/organ at 15 min, respectively). The uptake in the clearance organs shows ^{64}Cu-labeled **1** cleared fairly rapidly through the kidneys and into the bladder with a small amount clearing via the liver into the intestines. By 2 h > 90% of the activity was excreted.

DISCUSSION

In this study we evaluated six tetraazamacrocyclic ligands that differed in chelate ring size and placement of the dioxo groups. The aim of this study was to determine the effects of ring size and the dioxo groups on *in vivo* stability and biodistribution, with the overall goal being to design more optimal bifunctional chelates for labeling copper radioisotopes to biomolecules.

Motekaitis and colleagues (18) recently reported the solution chemistry of the Cu(II) complexes of ligands **1** through **6**. Each of the six macrocycles consist of two amino groups and two endocyclic amide groups. The amide groups can coordinate to Cu(II) only upon loss of the nitrogen-bound proton. Although Cu(II) complexes of tetraamine ligands such as cyclam generally form complexes of the type ML^{2+}, the protons on the two amide amines in these ligands are more acidic than those on cyclam, and neutral $MH_{-2}L$ complexes are formed instead. The expression of formation constants for systems that lose protons while forming the predominant complex in solution can be problematic. The terms H_{-1} and H_{-2} indicate that one and two protons, respectively, were displaced from the amides during the formation of the metal complex. In Table 2, the log $\beta_{CuH_{-2}L}$ is therefore listed as a measure of thermodynamic stability. Another important marker for thermodynamic stability

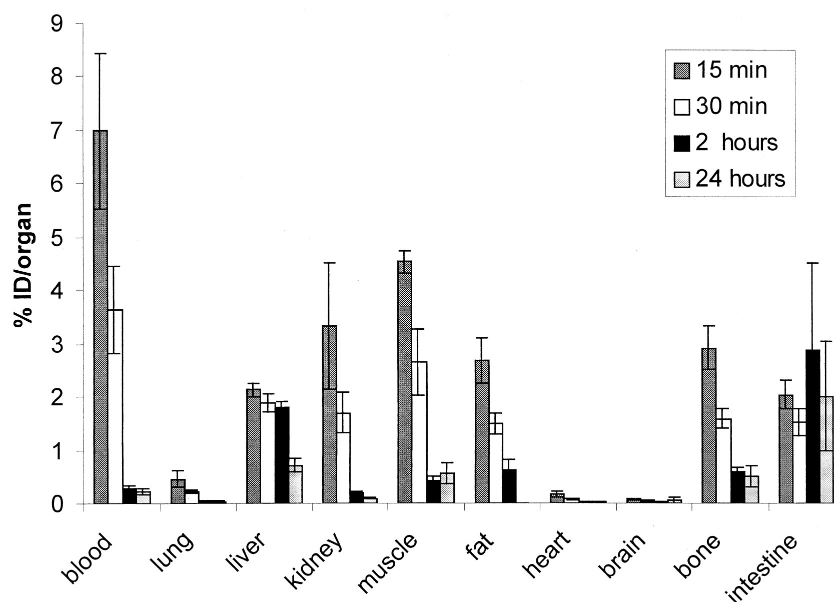

FIG. 2. Biodistribution of ^{64}Cu-1,4,8,11-tetraazacyclotetradecane-3,9-dione (1) in normal Sprague-Dawley rats. The data is presented as percent injected dose per organ (N = 4 for each time point).

that is relevant to radiopharmaceutical chemistry is the pM value. pM values are the free metal ion concentrations in equilibrium with the ligand at a specified pH in the presence of excess ligand. Thus, the ligand acts as a metal ion buffer and pM is analogous to pH. In this case, pM is defined as $-\log[Cu^{2+}]$ at 25°C and $\mu = 0.100$ (KCl) with 100% excess ligand at pH 7.4. Table 2 also lists the pM values for the Cu(II) complexes of ligands **1** through **6**.

The log $\beta_{CuH_{-2}L}$ and the pM values for the Cu(II) complex of **1** demonstrate that it is more thermodynamically stable than the Cu(II) complexes of ligands **2** through **6**. This is consistent with the fact that the ^{64}Cu-**1** complex labeled in >95% yield. ^{64}Cu-labeled ligands **2** and **4** labeled 60% and 45%, respectively, and the pM values of these complexes were 13.2 and 12.0, respectively. Ligands **3**, **5**, and **6** did not complex with ^{64}Cu to any measurable extent, and these Cu(II) complexes showed the lowest pM values. It appears that the labeling efficiencies of the ^{64}Cu ligands directly correlate with the thermodynamic stability. Our results indicate that the ring size and the arrangement of the two dioxo groups in ligand **1** are the most effective for complex formation with ^{64}Cu. These experiments also suggest that the smaller macrocyclic ring size causes a decrease in the stability of the Cu(II) complexes. In the case of ligands **1** and **2**, the position of the dioxo groups appeared to influence the extent of ^{64}Cu complex formation.

TABLE 2. Comparison of ^{64}Cu-labeled Ligands 1 through 6 Complex Formation with Stability Constants of the Cu(II) complexes

Ligand	Radiochemical yield (%)[a]	log $\beta_{CuH_{-2}L}$[b]	log β_{CuL}[b]	pM[b]
1	98.5	1.86	8.33	16.1
2	44	0.56	8.99	13.2
3	4	−2.43	7.73	10.7
4	41	−2.56	6.71	12.0
5	0	−6.65	6.56	8.4
6	0	−3.47	6.08	9.7

[a] After 2-h reaction time at 37°C and thin layer chromatography on C18 1:1 MeOH:10% ammonium acetate.
[b] Motekaitis *et al.* (18).

To explain the differences in the complexation behavior, preliminary molecular modeling studies have been carried out on the Cu(II) complexes of ligands **1** and **2**. The Cu(II) complexes of both ligands **1** and **2** were examined by density functional calculations (B3LYP/lacvp**). Consistent with the observed experimental data, the Cu(II) complex with ligand **1** was found to be more stable by 3.488 kcal/mol compared with the complex with ligand **2**. The reasons for the difference in stabilities are currently under investigation (D. Reichert and M. Welch, manuscript in preparation).

The biodistribution of ^{64}Cu-**1** is shown in Figure 2. Comparisons can be made between the *in vivo* behavior of ^{64}Cu-**1** and other ^{64}Cu-labeled macrocyclic complexes. Ligand **1** forms a neutral complex with ^{64}Cu(II), whereas the 14-membered ring macrocycles cyclam and 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA) form 2+ and 2− complexes, respectively. Jones-Wilson and colleagues (11) showed that positively-charged ^{64}Cu macrocyclic complexes had slower blood clearance and higher accumulation in liver and kidney compared with negatively charged complexes. In Figure 3 the blood, liver, and kidney clearances are shown for ^{64}Cu-**1**, cyclam, and TETA. The biodistribution of ^{64}Cu-**1** was more similar to the negatively charged complexes. For example, ^{64}Cu-**1** was rapidly cleared through the kidneys (Fig. 3), with 0.097 ± 0.03 %ID remaining 24 h postinjection; this is less than ^{64}Cu-labeled TETA (0.21 ± 0.03 %ID/organ) (11), a chelator widely used as a bifunctional chelator for attaching ^{64}Cu to antibodies and peptides (1, 3). The liver uptake of ^{64}Cu-**1** was also comparable to (Fig. 3), although somewhat greater than, ^{64}Cu-TETA (11).

A proposed element of *in vivo* stability, and also of kinetic stability, is the reduction potential of Cu(II) complexes. The redox behavior of certain classes of Cu(II) complexes play a role in their biological action. For example, the redox behavior of Cu(II) thiosemicarbazones affects their uptake in normal versus hypoxic tissue in the heart (8, 9) and in tumors (12), a finding that has been exploited in the design of PET tracers for hypoxia. If the Cu(I) chelate complex is not stable, then it is likely that reduction of Cu(II) to Cu(I) causes the Cu(I) to dissociate from the chelate and bind to proteins. A similar mechanism of Cu(II) dissociation has been proposed for the trapping of Cu(II)PTSM [pyruvaldehyde-

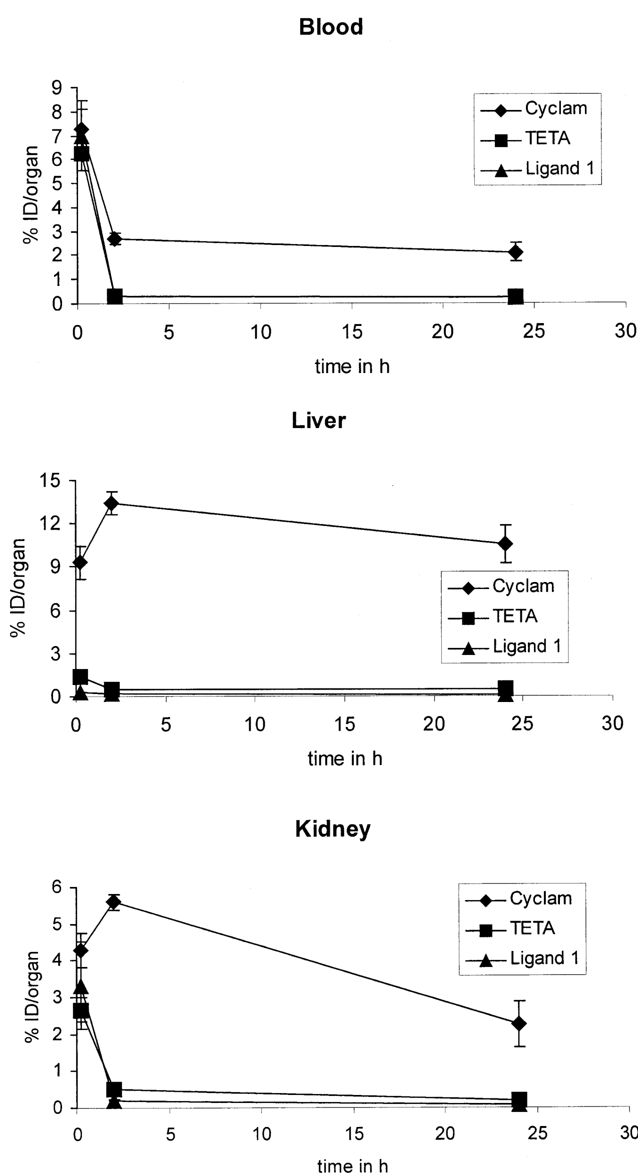

FIG. 3. Comparison of blood, liver, and kidney clearance for ^{64}Cu-labeled cyclam, 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA), and ligand 1,4,8,11-tetraazacyclotetradecane-3,9-dione (1) in Sprague-Dawley rats. Data presented as %ID/organ (N = 4 for each time point).

bis(N^4-methyl-thiosemicarbazone)] in heart, brain, or tumor tissues (10, 13). In this mechanism, the Cu(II)-PTSM is reduced to Cu(I), the Cu(I)-PTSM dissociates, and the Cu(I) is re-oxidized to Cu(II) with subsequent binding to intracellular proteins.

Miyoshi and colleagues (15) determined the reduction potentials ($E_{1/2}$) for a series of Cu(II/I) azamacrocyclic complexes and showed that Cu(II) cyclam is 150 mV more difficult to reduce than Cu(II)-15aneN5 (using a saturated calomel reference electrode). This correlates with data from our group showing that the ^{67}Cu-15aneN5 (1,4,7,10,13-pentaazacyclopentadecane [PCBA])-conjugated mAb 1A3 conjugate dissociated to a greater extent in the liver than in the ^{67}Cu-cyclam (or CPTA) conjugate (20). In addition, we also showed that ^{64}Cu dissociated from TETA-octreotide in rat liver, forming ^{64}Cu-superoxide dismutase (SOD) (4). Miyoshi *et al.* (15) also reported that the $E_{1/2}$ for Cu(II/I)-**2** was 119 mV more difficult to reduce than Cu(II/I) cyclam. Although ^{64}Cu-**1** and ^{64}Cu-TETA have similar pM values (16.1 compared with 16.2, respectively; pM Cu(II)-cyclam is 21.4 [17]), the data from Miyoshi *et al.* suggest that the dioxocyclam ligands may form Cu(II) complexes that are more stable to reduction *in vivo*. Further investigation of this is underway.

The favorable biodistribution and potentially greater kinetic stability of ^{64}Cu-**1** compared with ^{64}Cu-TETA suggests that ligand **1** may be worthwhile investigating as a bifunctional chelator for attaching ^{64}Cu to proteins and peptides.

CONCLUSIONS

In summary, six new dioxomacrocyclic chelates were evaluated for labeling ^{64}Cu in the hopes of forming neutral, lipophilic complexes. Of the six ligands synthesized, only one of these readily formed a complex with ^{64}Cu. The rapid blood, liver, and kidney clearance of ^{64}Cu-labeled **1** suggest that ligand **1** (1,4,8,11-tetraazacyclotetradecane-3,9-dione) may be useful as a macrocyclic structure to design new bifunctional chelates for copper radionuclides in radiodiagnostic or radiotherapeutic studies and may be a potential alternative to TETA.

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