Radiolabeling and In Vivo Behavior of Copper-64-Labeled Cross-Bridged Cyclam Ligands

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Macrocyclic chelators and their metal complexes have widespread applications in the biomedical sciences, including radiopharmaceutical chemistry. The use of copper radionuclides in radiopharmaceuticals is increasing. Macrocyclic chelators have been found to have enhanced in vivo stability over acyclic chelators such as ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA). The currently used chelators of choice for labeling copper radionuclides to biological molecules are analogues of TETA (1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid); however, recent reports have demonstrated evidence of in vivo instability of the radio-Cu(II)−TETA complexes. A new class of structurally reinforced macrocycles, the "cross-bridged" cyclam derivatives, form highly stable complexes with Cu(II) that are resistant to dissociation in strong acid. Here, we evaluate a series of ^{64}Cu(II) cross-bridged macrocyclic complexes for biological stability and in vivo behavior. The ligands evaluated include the parent ligand, 1,4,8,11-tetraazabicyclo[6.6.2]hexadecane (**1**), and three 4,11-dipendant arm derivatives: 4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane (**2**); 4,11-bis(*N,N*-diethyl-amidomethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane (**3**); and 4,11-bis(amidoethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane (**4**). Copper-64 formed complexes with ligands **1**−**4** in high radiochemical yields. The ^{64}Cu−**2** complex was neutral, while ^{64}Cu complexes of **1**, **3**, and **4** were positively charged. All complexes showed no decomposition in rat serum out to 24 h. Biodistribution experiments in Sprague−Dawley rats indicated that ^{64}Cu−**1**, −**3**, and −**4** were taken up by the liver and kidney and cleared slowly over 24 h, whereas ^{64}Cu−**2** cleared rapidly from all tissues. The rapid clearance of the ^{64}Cu−**2** complex from the blood and liver, as well as liver metabolism experiments in rats, suggests that it is highly stable in vivo. A bifunctional chelator of **2** is a significant candidate for labeling copper radionuclides to biological molecules for diagnostic imaging and targeted radiotherapy.

Introduction

Macrocyclic polyamine ligands and their metal complexes[1−11] have applications in radiopharmaceutical, catalysis, and biomimetic chemistry.[12−22] Recently, the focus has also been on their functionalized, pendant-armed derivatives.[23−26] In addition, "structurally reinforced" polyamines featuring ethylene-bridging of adjacent nitrogens and their metal complexes have been investigated for some time.[27−31] The New Hampshire group has communicated the syntheses of several members of a novel class of ligands having nonadjacent nitrogens bridged by CH$_2$CH$_2$, the "cross-bridged" tetraamines.[32,33] These bicyclo[6.6.2], -[6.5.2], and -[5.5.2] tetraamines were designed to be capable of adopting conformations[32,33] having all four nitrogen lone pairs convergent upon a cleft (*in,in* at the bridgehead nitrogens),[34] for the complexation of small, hard metal ions as well as the encapsulation of protons.

A preliminary communication on copper(II) complexes of two of the cross-bridged macrocyclic ligands appeared

in 1996.[33] These first structural results showed that the [6.6.2] cross-bridged cyclams do indeed form cis-folded complexes. Subsequently, the New Hampshire group has prepared and characterized an extended series of related Cu(II) complexes of these ligands.[35] Since 1998, Busch and co-workers have also reported their work on 3d transition metal complexations[36−40] using the same cross-bridged ligands and a *C*-hexamethyl analogue.[40] Their experiments demonstrated the remarkable kinetic inertness of these complexes in solution that was expected based on the original ligand design.[32,33] For example, the copper(II) complex of the dimethyl cross-bridged cyclam was found to be over 8 orders of magnitude more kinetically stable ($>10^8$) to dissociation in strong acid than its closest nonbridged analogue.[36−39] This kinetic inertness is endowed in part by the ligand's relatively strain-free C_2 coordinating conformation, which has each of the 10-membered rings in the distorted diamond−lattice [2323] conformation.[32,33,35] Micheloni and Ciampolini, Springborg, and others have also reported a number of interesting cross-bridged tetraamines, having longer bridging groups,[41−47] as well as structures of several of their copper(II) complexes.[48−51] Although the kinetic stability of a few metal complexes of cross-bridged macrocycles in acidic media has been

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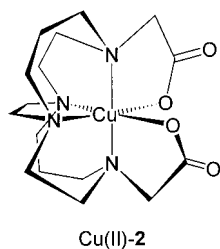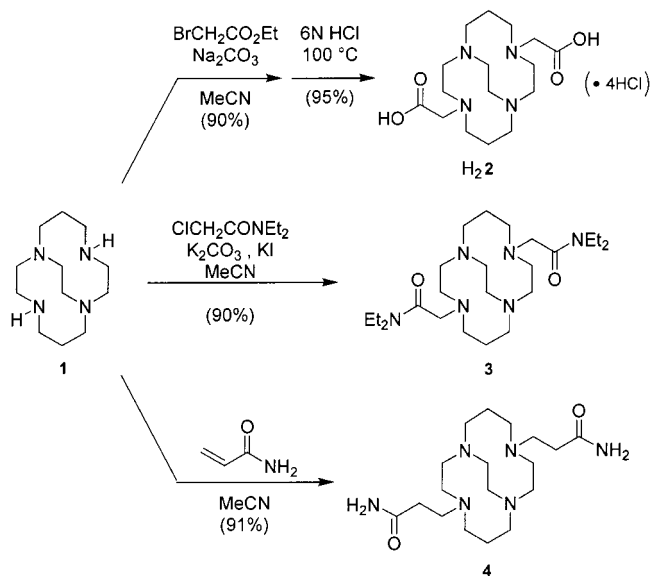‡ University of New Hampshire.
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Figure 1. Structure of the Cu(II)−**2** complex based on the crystal structure found by Wong et al.[35]

Scheme 1

reported,[36,39,40] to the best of our knowledge, there have been no studies on this class of complexes to confirm their stability under biological conditions, nor have thermodynamic stability constants been determined.

A further enhancement on the already high kinetic stability of the cross-bridged ligand copper(II) complexes can be realized through attachment of ionizable pendant arms at the two secondary nitrogens.[35] These can serve to fully envelop the six-coordinate cation as well as neutralize its dicationic charge (Figure 1). The latter property is desirable for optimal radiolabeled Cu(II) complex biodistribution and clearance characteristics.[52,53] The New Hampshire group has prepared a number of these derivatives including those featuring carboxymethyl arms (Scheme 1, **2**−**4**) and has confirmed the expected full ligand coordination of the Cu(II) in the neutral complex Cu(II)−**2**.[35]

High stability toward metal loss is of considerable interest in systems designed for the in vivo delivery of copper radionuclides for nuclear medicine applications.[54−56] The group at Washington University has been particularly interested in ^{64}Cu, as it shows promise as both a radionuclide for positron emission tomography (PET) imaging and targeted radiotherapy due to its half-life ($t_{1/2}$ = 12.7 h), decay characteristics (β^+ (19%); β^- (40%)), and the ability for large-scale production with high specific activity on a biomedical cyclotron.[57−60] Macrocyclic chelators have historically been used as bifunctional chelators (BFCs) to bind ^{67}Cu(II) ($t_{1/2}$ = 62 h; β^- (100%)) to antibodies due to their relatively high in vivo stability as compared to acyclic chelators such as ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA).[61−64] Cole and colleagues showed that the relative in vivo stability of the radiometal-labeled BFC−mAbs (monoclonal antibodies) was paralleled by the in vivo stability of the metal-labeled BFCs themselves.[63] In addition, our group reported that the properties of radiometal-labeled 1,4,8,-11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA) and CPTA (cyclam) chelators were indicative of the in vivo behavior of their corresponding octreotide conjugates.[52,65] This correlation also extends to BFC−protein conjugates, which were predicted from the biological data of radiolabeled BFCs themselves.[55] Although TETA has been used to bind copper radionuclides for clinical imaging and therapy studies with both antibodies and peptides,[66−69] it was recently demonstrated that in rat liver in vivo ^{64}Cu dissociated from TETA-D-Phe^1-octreotide (TETA-OC) and bound to the protein superoxide dismutase (SOD).[70] Therefore, the cross-bridged macrocyclic chelators are being investigated under the expectation that they may provide enhanced in vivo stability. In this paper, the radiochemistry, in vitro and in vivo stability, and biodistribution data of ^{64}Cu complexes **1**−**4** based on cross-bridged cyclam and its pendant-armed derivatives are presented.

Results

Ligand Synthesis. Cross-bridged cyclam (1,4,8,11-tetraazabicyclo[6.6.2]hexadecane, **1**) was prepared as described previously[33] by hydrogenolysis of 4,11-dibenzyl-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane, which was synthesized by an alkylation/reductive ring expansion approach from a bisaminal adduct of cyclam and glyoxal. The other three pendant-arm cross-bridged ligands used in this study (**2**−**4**) were prepared from **1** as shown in Scheme 1. As reported in detail elsewhere,[35] alkylation of **1** with ethyl bromoacetate and subsequent acidic hydrolysis of the resultant isolated diester gave diacid H$_2$**2** as the tetrahydrochloride. Diamide **3** was prepared by the alkylation of **1** with 2-chloro-*N*,*N*-diethylacetamide and diamide **4** by conjugate addition of **1** to acrylamide. Synthesis and X-ray structures of cold Cu(II) complexes of ligands **1** and **2** (as the dicarboxylate) have been reported.[33,35]

Protonation and Stability Constants for Ligand 1. The overall log β_n (nH$^+$ + L = H$_n$L^{n+}, where L = **1**) protonation constants found were 12.42, 22.61, (20.23), and 24.00. From successive differences of these overall log β_n values (log K$_n^H$ = log β_n − log $\beta_{n−1}$), the stepwise log protonation constants for ligand **1** would therefore be 12.42, 10.20, −2.38, and 3.77. However, because the third stepwise protonation constant is negligible, it means that the third and fourth protonation steps occur simultaneously with a log value of 1.39. The log protonation constants with their defining equations are listed in Table 1.

Ligand **1** undergoes two high-pH stepwise protonations and one two-proton, single-step, low-pH protonation equilibrium. The ethylene cross-bridged **1** is characterized by a higher pH first protonation step and a lower second protonation step as compared to the parent compound cyclam. Factors that influence this no doubt include both intramolecular H-bonding and solvation

Table 1. Stability Constants and Molar Absorptivities for Cu(II) Complexes of Cyclam and Ligand **1** and Protonation Constants for the Uncomplexed Ligands[a]

ligand	K_{Cu-L}	Log $K_1{}^H$ $H^+ + L \rightarrow HL^+$	Log $K_2{}^H$ $H^+ + HL^+ \rightarrow H_2L^{2+}$	Log $K_3{}^H K_4{}^H$ $2H^+ + H_2L^{2+} \rightarrow H_4L^{4+}$	ϵ (588 nm)[d] (cm^{-1} M^{-1})	ϵ (510 nm)[e] (cm^{-1} M^{-1})
cyclam	27.2[b]	11.73	10.42	3.67[c]	4.4	92.9
ligand **1**	27.1	12.42	10.20	1.39	169.1	34.6

[a] The stability constant of Cu(II)−**1** as determined spectrophotometrically by competition with Cu(II) cyclam. The molar absorptivities are listed for the Cu(II) ligand complexes. [b] Motekaitis et al.[16] [c] The actual resolved log stepwise protonation constants for the third and fourth protonation steps found are[71] $H^+ + H_2L^{2+} = H_3L^{3+}$ (1.44) and $H^+ + H_3L^{3+} = H_4L^{4+}$ (2.23). [d] λ_{max} = 588 nm, CuL where L = **1**. [e] λ_{max} = 510 nm, CuL where L = cyclam.
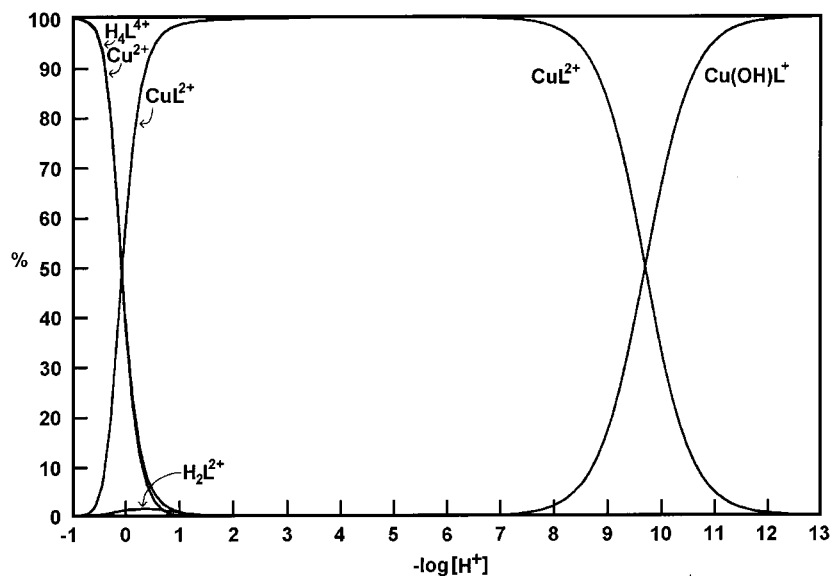

Figure 2. Species distribution at equilibrium at 25 °C and 0.100 KCl as a function of pH for a solution containing 0.003 61 M Cu^{2+} and 0.0362 M ligand **1**. The curves were computed from the thermodynamic equilibrium constants listed in Table 1 and show that from pH <1 to 8 the dominant species is $Cu-(\mathbf{1})^{2+}$ while at higher pH values the stable species is $Cu(OH)-(\mathbf{1})^+$. For comparison, cyclam (not cross-bridged) is of similar stability with Cu(II) but is kinetically less inert, forms a planar complex, and hence does not form the $Cu(OH)L^+$ species.

effects. The log protonation values of cyclam measured for this study under the same conditions are 11.73, 10.42, 1.44, and 2.23. While with cyclam it was possible to measure all four steps, the ethylene cross-bridge in **1** prevents the occurrence of the single protonation associated with the third step. Note that even cyclam itself shows a tendency toward a two-step third and fourth protonation reaction.[71] In **1**, the low pH protonation equilibrium is a single two-proton step.

The stability constant obtained for Cu(II)−**1** is log K_{ML} = 27.1. The uncertainty is about ±0.1, which reflects both the uncertainty in the reference ligand cyclam and the approximations introduced in the temperature extrapolation of the results. Given two ligands L and L′ and a Cu^{2+} ion in 1:1:1 ratio, the copper will distribute itself according to the displacement equilibrium K_x (charges omitted for simplicity):

$$CuL + L' \rightleftharpoons CuL' + L \quad K_x = \frac{[CuL'][L]}{[CuL][L']} \quad K_x = \frac{K_{CuL'}}{K_{CuL}} \tag{1}$$
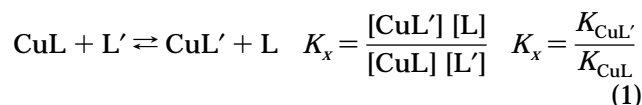
where

$$[L] = T_L/(1 + K_1{}^H[H] + K_1{}^H K_2{}^H[H]^2) \quad [L'] = T_L/(1 + K'_1{}^H[H] + K'_1{}^H K'_2{}^H[H]^2) \tag{2}$$

and also

$$K_{CuL} = \frac{[CuL]}{[Cu][L]} \quad K_{CuL'} = \frac{[CuL']}{[Cu][L']} \tag{3}$$

The free total ligand terms T_L and $T_{L'}$ were computed from stoichiometry. Equation 1 shows two ways of computing K_x. Both [CuL] and [CuL′] were directly computed from spectrophotometric measurements. The protonation constants $K_1{}^H$, $K_2{}^H$, $K'_1{}^H$, and $K'_2{}^H$ and the extinction coefficients for both CuL and CuL′ were determined in separate experiments (Table 1).

The extrapolated value of log K_x to 25 °C was found to be −0.05. Substituting the known log K_{CuL} = 27.2 for cyclam into eq 1 gives log $K_{CuL'}$ = 27.1 for Cu(II)−**1**. The speciation curves are shown in Figure 2.

Radiochemistry. The reaction conditions, thin-layer chromatography (TLC) conditions, and ^{64}Cu-labeling yields are presented in Table 2. Ligands **3** and **4** were labeled in high radiochemical purity at both carrier-added (CA) and no-carrier-added (NCA) conditions after incubation at 55 °C in EtOH for 1.5 and 0.5 h, respectively. In basic EtOH, ligand **1** was labeled by CA and NCA ^{64}CuCl$_2$ in yields of ca. 99% after a 1 h incubation at 75 °C.

The reaction between NCA ^{64}CuCl$_2$ and ligand H$_2$**2** showed two species (at a ratio of 3/1) by radio-TLC in either ammonium citrate buffer or EtOH even under very basic conditions, as described by Wong et al.[35] The R_f values of the two species of ^{64}Cu−**2** were 0.7−0.8 and 0.8−0.9. At CA levels with molar ratios of Cu(II):ligand

Table 2. Formation of ^{64}Cu(II)-labeled **1**–**4** under CA and NCA Conditions[a]

^{64}Cu-labeled ligand	reaction conditions	radiochemical purity (%)	R_f	Log P
1	100 μL of 10 mM ligand in EtOH; 5 μL 1N NaOH (in 100% EtOH); 75 °C, 1 h	99	0.42	-2.11 ± 0.22
2 (CA)	100 μL of 10 mM ligand in EtOH or 0.1M ammonium citrate, pH 6.5; Cu(II)/ligand **2**: from 1:100 to 1:1; 75 °C, 4 h	>95	0.78	-2.42 ± 0.04
2 (NCA)	100 μL of 10 mM ligand in EtOH; 5 μL 1N NaOH (in 100% EtOH); 75 °C, 1 h	76 23	0.76 0.88	
3	10 mM ligand; EtOH; 55 °C, 1.5 h	99	0.44	-1.95 ± 0.26
4	5 mM ligand; EtOH 55 °C, 0.5 h	87	0.72	-2.30 ± 0.10

[a] In all reactions, <1 μL of ^{64}CuCl$_2$ was added to the ligand solution. The TLC conditions were C18 plates as the stationary phase and 1:1 MeOH:10% ammonium acetate as the eluant, except for ligand **2**, where the eluant was 9:1 MeOH:0.1 M ammonium citrate.

H$_2$**2** from 1:100 to 1:1, a single species of ^{64}Cu-labeled ligand **2** was obtained in high yields (R_f = 0.7–0.8, >95%) in either ammonium citrate buffer or EtOH after incubation at 75 °C for 4 h. This suggests that the second species at the NCA level probably resulted from the large excess of ligand H$_2$**2**. All ^{64}Cu complexes of ligands **1**–**4** that were formed in EtOH or basic EtOH remained stable when transferred to various solutions, e.g., 0.1 M ammonium citrate, 0.1 M (or 0.4 M) ammonium acetate, and water.

The partition coefficients (log P values, Table 2) demonstrated that the ^{64}Cu–**2** complex is less lipophilic than the other three complexes. Because of the neutral charge of this complex, this is somewhat surprising. This decreased lipophilicity is also reflected in the solvent system employed for the TLC analysis of the ^{64}Cu–**2** complex that requires a higher methanol concentration in the TLC eluant to decrease the R_f values.

Preliminary capillary electrophoresis experiments suggest a neutral complex for Cu(II)–**2** and a positive charge for Cu(II)–**1**, consistent with anticipated charge neutralization in the former. (CE experiments were performed by S. A. Tomellini and A. E. Thomsen, University of New Hampshire, on a Hewitt-Packard 3D CE instrument with an applied voltage of 25 kV across a fused silica capillary.)

Serum Stability. The in vitro serum stability of ^{64}Cu complexes of ligands **1**–**4** was evaluated out to 24 h postadministration of the ^{64}Cu complexes. Chromatography results indicated that all four ^{64}Cu complexes of ligands **1**–**4** showed no decomposition in rat serum out to 24 h. In these experiments, the concentration of ligand was in the range of 0.83–1.66 mM, while the protein concentration in rat serum for copper was in large excess. The ^{64}Cu–**2** complex also remained 100% intact at ligand concentrations in rat serum of 0.19 and 0.02 mM. These data suggested high in vitro stability of the ^{64}Cu complexes and indicated that they were worthy of in vivo investigation.

Biodistribution. The biodistribution of all four complexes at both NCA and CA levels was performed in normal Sprague–Dawley rats. In all cases, the NCA and CA data were statistically indistinguishable. The biodistributions of the ^{64}Cu complexes of ligands **1**–**4** were highly dependent on the ligand. The blood, liver, and kidney clearance of these agents are presented in Figure 3. The blood clearance was rapid for all four complexes; however, the percent injected dose (%ID) in
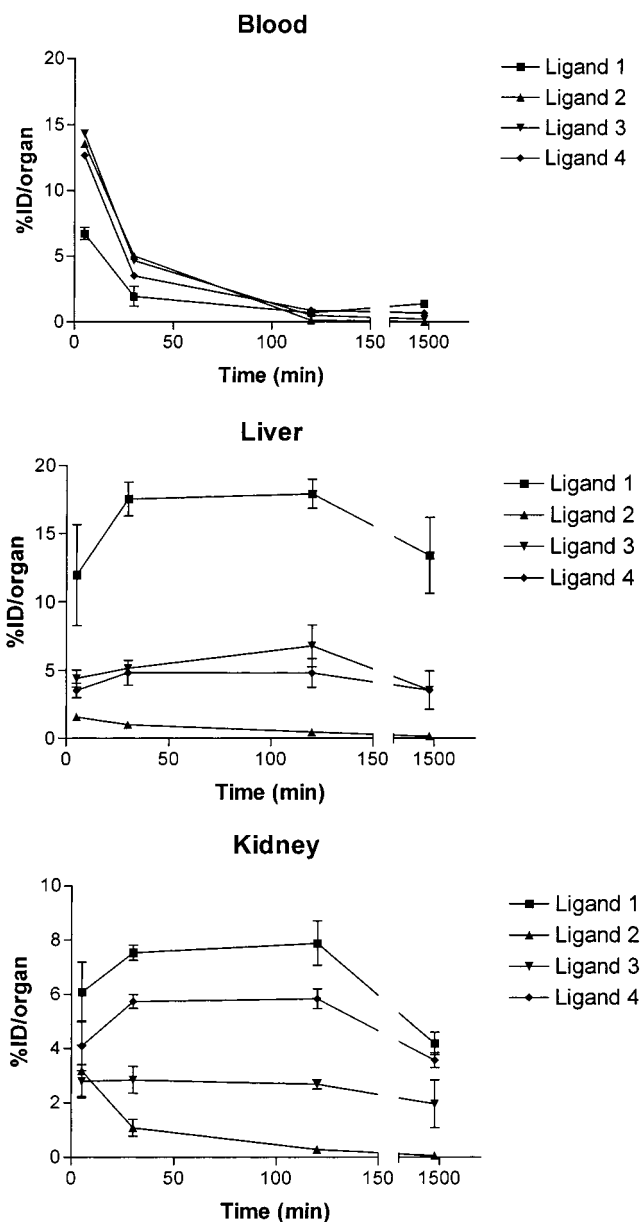

Figure 3. Biodistribution of ^{64}Cu-labeled **1**–**4** in normal, female, Sprague–Dawley rats. The data are presented as %ID/organ $\pm$ sd ($n = 4$).

the blood at 24 h was substantially different for each ^{64}Cu complex. For example, ^{64}Cu–**2** cleared the blood considerably better than the other three complexes, as

shown by the %ID/blood at 24 h for the four complexes (^{64}Cu−**1**, 1.40 ± 0.065; ^{64}Cu−**2**, 0.032 ± 0.014; ^{64}Cu−**3**, 0.23 ± 0.11; ^{64}Cu−**4**, 0.67 ± 0.11). The liver and kidney uptake of ^{64}Cu−**1** were the highest of the four complexes, with ^{64}Cu-labeled **3** and **4** being intermediate. The %ID/organ of ^{64}Cu−**2** in the liver and kidney was almost negligible at 24 h (0.14 ± 0.03 and 0.064 ± 0.012, respectively). The rapid clearance of ^{64}Cu−**2** from the blood, liver, and kidneys suggests that the intact complex was clearing, since dissociated ^{64}Cu binds to proteins and remains trapped in tissues, hindering clearance.[70]

Metabolism. Metabolism studies of ^{64}Cu−**2** as compared to ^{64}Cu−TETA were carried out in normal rats using reported methods.[70,72] Rat liver homogenates from samples excised at 20 h postinjection were analyzed by size exclusion chromatography to determine whether the ^{64}Cu transchelated to proteins. The percent authentic intact (%AI) of ^{64}Cu−TETA and ^{64}Cu−**2** was calculated from the integration of the radio high-performance liquid chromatography (HPLC) chromatograms as previously described.[70] The calculated %AI of ^{64}Cu−**2** in the liver at 20 h postinjection was 50.3 ± 16.8%AI ($n = 3$), which was significantly higher than the value for ^{64}Cu−TETA (4.6 ± 2.1%AI, $n = 4$) ($p < 0.005$).

Discussion

In this study, a series of ^{64}Cu-labeled bridged cyclam derivatives were synthesized and evaluated for potential use as BFCs for complexing copper radionuclides to biological molecules. The goal of this work was to determine if the bridged macrocycles offer advantages with respect to thermodynamic and/or in vivo stability of the Cu(II) complexes as compared to the parent macrocycle, cyclam. The concentration of competing proteins in vivo is 10^3−10^5 higher in concentration than the BFCs; therefore, the in vivo stability of radiometal−BFC−protein or peptide conjugates largely depends on the kinetic stability of the radiometal−chelate rather than the thermodynamic stability, although thermodynamic stability is also important.[63] Jones-Wilson et al. reported that thermodynamic stability was not necessarily indicative of optimal in vivo behavior; therefore, we feel that although stability constants can be informative, they can also be misleading.[52] The Cu(II) complexes of cross-bridged ligands were reported to have high stability in concentrated acid solutions, and that is desirable for in vivo applications. Therefore, the cross-bridged macrocyclic chelators were investigated under the expectation that they may provide enhanced in vivo stability, specifically that they will be resistant to the transchelation of ^{64}Cu from BFCs to proteins.

The group at Washington University previously reported the synthesis of an adjacent-bridged cyclam ligand, 1,5,8,12-tetraazabicyclo[10.2.2]hexadecane, **5**, where the ethylene bridge spans the ethylene side of cyclam.[27,52,73] Structural data on related adjacent-bridged cyclam Cu(II) complexes revealed the ligand coordinating in a square-planar manner.[31] The log stability constant of the Cu(II)−**5** complex was experimentally determined to be 26.1; however, the biodistribution of the ^{64}Cu−**5** complex showed a large amount of activity in the liver that did not clear by 24 h postinjection.[52] In this study, we investigated a series

of cross-bridged cyclam ligands. The advantage of this series of ligands is the attachment of ionizable pendant groups on the secondary nitrogens, which can enhance the stability as well as change the charge of the Cu(II) complex. In particular, the attachment of carboxylate arms neutralizes the charge of the Cu(II) complex, which was previously shown to improve the in vivo clearance of ^{64}Cu-labeled azamacrocyclic complexes as compared to positively charged complexes.[52]

A noteworthy observation is the formation of two species for ^{64}Cu complexation with H$_2$**2** at the tracer level but only one species at a lower ligand:Cu(II) ratio (1:1 to 100:1). One hypothesis for the formation of these two species is that when the ligand:Cu(II) ratio is as high as 10^5:1 at the NCA level, it is possible to form a 2:1 ligand:Cu(II) species of the type [Cu−L$_2$]$^{2-}$. This rationale was supported by the observation that in the presence of carrier Cu(II) the second species was no longer detectable. A series of dilutions of ligand H$_2$**2** with concentrations from 1.0 to 0.001 mM in EtOH were evaluated to determine if the radiochemical purity of the desired species could be increased at the NCA level by simply varying the ligand:Cu(II) ratios (10^4:1 to 10:1). As the ligand concentration decreased, the second species disappeared (<2% at 1.0 mM and not observable at lower concentrations), while the desired species reached a yield of 90.6%. A radiochemical purity of 85% is attainable for the labeling of ligand H$_2$**2** when its concentration was decreased to 0.001 mM. These results also support the hypothesis of the formation of a 2:1 ligand:Cu(II) species at the NCA level in addition to the desired 1:1 complex.

An alternative hypothesis to the presence of two species by TLC of ^{64}Cu−**2** is that the ligand contained an undetectable trace amount of an impurity that had a higher affinity for Cu(II). At CA conditions, the relative amount of the ^{64}Cu-labeled impurity would decrease since the trace impurity ligand was readily saturated with cold Cu(II).

The rat biodistributions of ^{64}Cu-labeled **1**−**4** are consistent with what has previously been reported for ^{64}Cu-labeled azamacrocyclic complexes. Jones-Wilson et al. reported that positively charged ^{64}Cu-labeled cyclam and Et-cyclam had a higher accumulation in the liver of normal rats, while the negatively charged ^{64}Cu−TETA cleared significantly through the liver and kidneys by 24 h postinjection. The neutral complex, ^{64}Cu-labeled 1,4,8,11-tetraazacyclotetradecane-3,9-dione (3,9-DOC), behaved similarly to ^{64}Cu−TETA but had a slightly better liver clearance.[53] The biodistribution data of ^{64}Cu-labeled **1** showed that the blood activity actually increased from 2 to 24 h postinjection, while the liver uptake decreased over this time period. This may be consistent with hepatic radiocopper incorporation into ceruloplasmin and release into blood. Of the four complexes studied here, ^{64}Cu-labeled **2** had the best blood, liver, and kidney clearance (Figure 3). In fact, ^{64}Cu-labeled **2** showed more optimal clearance at 24 h postinjection than either ^{64}Cu−TETA or ^{64}Cu−3,9-DOC (Table 3).

To verify our hypothesis that more rapid clearance from the liver was indicative of higher in vivo stability, metabolism studies were carried out in normal rats to determine the extent of ^{64}Cu transchelation to proteins

Table 3. Comparison of Rat Biodistribution Data for ^{64}Cu–TETA, ^{64}Cu–3,9-DOC, and ^{64}Cu–**2** at 24 h Postinjection[a]

ligand	blood	liver	kidney
TETA[b]	0.21 ± 0.05	0.49 ± 0.11	0.21 ± 0.03
3,9-DOC[c]	0.23 ± 0.05	0.70 ± 0.10	0.097 ± 0.03
ligand **2**	0.032 ± 0.014	0.14 ± 0.03	0.064 ± 0.012

[a] Data are presented as %ID/organ $\pm$ sd. [b] Ref 52. [c] Ref 53.

observed after the injection of ^{64}Cu–**2** and ^{64}Cu–TETA. These metabolism studies of ^{64}Cu–**2** and ^{64}Cu–TETA demonstrated that ^{64}Cu–**2** was significantly more resistant to transchelation than ^{64}Cu–TETA (50.3 ± 16.8%AI at 20 h postinjection vs 4.6 ± 2.1%AI at 20 h postinjection, respectively). The major ^{64}Cu-labeled protein present for both agents had a molecular mass of 32 kDa, consistent with ^{64}Cu–SOD, which is in agreement with what was previously reported.[70]

The relatively high in vivo stability of ^{64}Cu–**2** as compared to ^{64}Cu–TETA is consistent with the findings of Hubin et al.,[40] which demonstrated that the lower limit of the half-life of Cu(II) dissociation from the ligand is more than six years and the two-carbon cross-bridge increased the stability in acid solution of the corresponding cyclam complexes by 6–8 orders of magnitude.[36,40] We previously showed that ^{64}Cu dissociated from ^{64}Cu–TETA–octreotide in rat liver.[70] We have also demonstrated that in a tumor-bearing rat model blood levels of ^{64}Cu–TETA–octreotide initially cleared the blood rapidly but after 24 h the accumulation of ^{64}Cu in the blood actually increased,[59] suggesting instability of the ^{64}Cu–TETA complex in vivo. In human PET-imaging trials, approximately 5%ID/blood of ^{64}Cu–TETA–octreotide remained at 24 h postinjection.[69] In human radioimmunotherapy trials with ^{67}Cu-labeled mAbs, where the BFC was a TETA derivative, it was found that ^{67}Cu dissociated from the chelator, and ^{67}Cu-labeled ceruloplasmin was found in the blood.[56] Because of the increasing use of $^{64}Cu/^{67}Cu$-labeled biological molecules as agents for cancer imaging and therapy, there is a distinct need for a more stable BFC for copper radionuclides. The synthesis of a BFC of **2** may provide a means of labeling copper radionuclides to biological molecules, whereby the radiolabeled Cu(II) forms a stable complex in vivo. Such a biologically stable ^{64}Cu–BFC–biomolecule will likely have a more favorable clearance from blood, liver, and kidneys and would significantly enhance imaging and possibly therapy characteristics of the radiopharmaceutical.

Conclusions

The radiochemistry and biological evaluation of four ^{64}Cu-labeled cross-bridged cyclam ligands (**1**–**4**) are reported. All ^{64}Cu complexes were labeled in high radiochemical purity and were found to be stable in serum out to 24 h. In normal rats, the positively charged ^{64}Cu complexes of ligands **1**–**3** exhibited rapid uptake in the liver and kidneys with slow clearance, whereas the neutral complex, ^{64}Cu–**2**, cleared rapidly from all tissues. The ^{64}Cu complex of **2** showed a significantly higher resistance to transchelation than ^{64}Cu–TETA in rat liver metabolism studies. In addition, ^{64}Cu–**2** demonstrated the most rapid clearance through blood, liver, and kidney in normal rats of ^{64}Cu-labeled macrocyclic chelators investigated by our group, suggesting that a

BFC of **2** has significant potential for labeling copper radionuclides to biological molecules for diagnostic imaging and targeted radiotherapy.

Experimental Section

General Methods and Materials. Unless indicated otherwise, ^1H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were recorded at 360.13 and 90.56 MHz, respectively, and referenced against internal tetramethylsilane. IR spectra were recorded as KBr pellets on a Nicolet MX-1 FT spectrophotometer. Low-resolution MS and elemental analyses were obtained from the University of New Hampshire University Instrumentation Center. Melting points are uncorrected. Reactions were run under nitrogen atmosphere with magnetic stirring. Bulk solvent removal was by rotary evaporation under reduced pressure, and trace solvent removal from solids was by vacuum pump. Unless specified otherwise, anhydrous Na_2SO_4 was used to dehydrate organic solutions. Solvents were either used as purchased or dried according to standard literature procedures.[74] Water was distilled and then deionized (18 MΩ/cm²) by passing through a Milli-Q water filtration system (Millipore Corp., Bedford, MA). 2-Chloro-N,N-diethylacetamide and acrylamide were obtained from the Aldrich Chemical Co. 1,4,8,11-Tetraazacyclotetradecane (cyclam) was obtained from the Strem Chemical Company. Sodium acetate was purchased from Fluka Chemie AG (Buchs, Switzerland).

Copper-64 was prepared on the Washington University Medical School Cyclotron CS-15 cyclotron by the $^{64}Ni(p,n)^{64}Cu$ nuclear reaction at a specific activity range of 50–200 mCi/μg as previously described.[60] Waters C18 silica gel TLC plates (KC18F, 60 Å, 200 μm) were purchased from Fisher Scientific (Pittsburgh, PA). Radio-TLC was accomplished using a Bioscan 200 imaging scanner (Bioscan, Inc., Washington, DC). Radioactivity was counted with a Beckman Gamma 8000 counter containing a NaI crystal (Beckman Instruments, Inc., Irvine, CA).

Ligand Synthesis. The experimental details of the preparations of parent cross-bridged cyclam **1**[33] and bis(carboxymethyl) pendant-arm ligand H$_2$**2** (as the tetrahydrochloride) have been reported elsewhere.[35]

4,11-Bis-(*N,N*-diethylacetamido)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane Monohydrate (3). 1,4,8,11-Tetraazabicyclo[6.6.2]hexadecane (**1**) (184 mg, 0.812 mmol) was dissolved in CH_3CN (2 mL). K_2CO_3 (0.45 g, 3.3 mmol), KI (0.55, 3.3 mmol), and 2-chloro-N,N-diethylacetamide (483 mg, 3.23 mmol) in CH_3CN (2 mL) were added successively, and the resulting mixture was stirred at 60 °C for 24 h. The reaction mixture was then concentrated, the residue was dissolved in 3 M aqueous HCl (20 mL), and the solution was extracted with toluene (6 × 25 mL). The aqueous phase was cooled in an ice/H_2O bath, adjusted to pH 14 with KOH (pellets), and extracted with toluene (6 × 25 mL). The toluene extracts were dried and concentrated to give 342 mg (90%) of **3** as a waxy solid monohydrate; mp 81.5–82.5 °C. ^1H NMR (CD$_3$CN, ref central line of CD$_2H$CN set at 1.94): δ 1.03 (~t (X$_3$ of ABX$_3$), 6H, NCH$_2$CH_3, $J = 7.1$ Hz), 1.12 (~t (X$_3$ of ABX$_3$), 6H, NCH$_2$CH_3, $J = 7.1$ Hz), 1.27–1.41 (m, 2H, NCH$_2$CH_2N), 1.42–1.53 (m, 2H, NCH$_2$CH_2CH$_2$N), 2.15–2.25 (br s, H_2O), 2.25–2.60 (m, 4H), 2.29–2.39 (XX′ of AA′XX′, 2H, bridge NCH_2CH_2N) 2.71 (td, 4H, $J = 10.5, 3.6$ Hz), 2.93 (d, 2H, $J = 14.2$ Hz, NCH$_A$$H_X$C=O), 3.08–3.42 (m, 4H), 3.07–3.17 (AA′ of AA′XX′, 2H, bridge NCH_2CH_2N), 3.34 (d, 2H, $J = 14.2$ Hz, NCH_AH$_X$C=O), 3.47–3.59 (m (A of ABX$_3$), 2H, NCH_2CH$_3$), 3.87 (ddd, 2H, $J = 15.4, 12.0, 4.3$ Hz). ^{13}C NMR (CD$_3$CN, ref CD$_3$CN set at 1.39): δ 13.38 (CH$_3$), 14.67 (CH$_3$), 28.74, 40.24, 41.90, 52.67, 55.06, 57.72, 57.78, 58.15, 59.44, 170.83 (C=O). IR (KBr): 1645 cm^{-1}. MS (EI) m/z: 452.6 (M$^+$). Anal. (C$_{24}$H$_{48}$N$_6$O$_2$·H$_2$O) C, H, N.

4,11-Bis-(2-carbamoylethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane (4). Acrylamide (0.108 g, 1.52 mmol) was added to a solution of **1** (0.171 g, 0.753 mmol) in CH_3CN (15 mL), and the resulting mixture was stirred at room temperature for 3 weeks. The reaction mixture was then concentrated, the residue was dissolved in water (10 mL), the pH of the

solution was adjusted to 14 by the addition of KOH pellets with cooling (ice/H_2O bath), and the aqueous solution was extracted with $CHCl_3$ (4 × 15 mL). Combined $CHCl_3$ extracts were dried and concentrated to afford an oil, which was triturated with Et_2O to give 0.252 g (91%) of **4** as a hygroscopic white waxy solid; mp 120−122 °C. ^1H NMR ($CDCl_3$): δ 1.42−1.47 (m, 2H, $NCH_2CH_2CH_2N$), 1.57−1.70 (m, 2H, NCH_2CH_2-CH_2N), 1.68 (br s, H_2O), 2.26−3.0 (m, 18H), 4.10 (dt, 2H, $J =$ 13.4, 6.9 Hz), 5.50 (br s, 2H, amide NH), 7.67 (br s, 2H, amide NH). ^{13}C NMR ($CDCl_3$, ref central line of $CDCl_3$ set at 77.23): δ 26.64, 33.75, 52.34, 52.50, 53.43, 54.69, 55.50, 58.77, 175.56 (C=O). IR (KBr): 3356, 3184, 1669 cm^{-1} (C=O). MS (EI) m/z: 368.5 (M$^+$). Despite careful handling, NMR always showed the presence of H_2O and the elemental analysis was consistent with nonstoichiometrically hydrated **4**. Anal. Calcd for ($C_{18}H_{36}N_6O_2$·(H_2O)$_{1.23}$): C, 55.34; H, 9.92; N, 21.51. Found: C, 55.78; H, 9.60; N, 21.07.

Protonation and Stability Constants. Determinations of protonation constants were obtained under argon at 25.0 °C by potentiometry as described.[75] The 10.00 mL initial volume of aqueous test solution contained 0.1122 mmol of **1** with 0.100 M KCl for ionic strength control and was titrated with 0.0970 M standard KOH (CO_2-free) Dilut-It up to pH 11.7. Computations of protonation constants were done using the program BEST.[75] The standard deviation (sd) in pH between all 51 calculated and observed titration points was 0.002. Similarly, the protonation constants for cyclam (Aldrich) were redetermined under the experimental conditions for ligand **1** (25.0 °C and μ = 0.100 (KCl)).

The stability constant of Cu−cyclam has been reported elsewhere,[16] whereas the determination of the stability constants of Cu(II)−**1** is presented here. The formation constants of Cu^{2+} with ligand **1** could not be determined by direct titration because the stability constant is so large that the formation takes place fully below pH 2. Furthermore, the extreme kinetic inertness for copper−ligand equilibration below pH 1.0, where copper is in equilibrium with the ligand, precludes ordinary spectrophotometric determination. The spectrophotometric competition was carried out from absorbance readings at 588 nm (Cu(II)−**1**). In separate experiments, the molar extinction coefficient was determined to be 169.1 cm^{-1} M^{-1}. Cu(II)−cyclam absorbs at 510 nm, but it contributes 4.4 cm^{-1} M^{-1} at 588 nm.

The spectrophotometric stock solutions of Cu(II), cyclam, and **1** used were 0.004 90, 0.005 78, and 0.005 00 M, respectively, adjusted to pH 9.0. It was found necessary to employ iminodiacetic acid (IDA; ∼4 mg/mL) as a transfer catalyst. Aliquots of this solution were heated at 73.0 °C for 9 h until the spectrum stabilized. Heating for additional time did not result in further visible equilibration. Similarly, experiments at 83.0 °C were complete in 5 h and at 93.0 °C in ∼3 h. Without IDA, the exchange reaction does not take place.

The protonation constant of (OH)Cu(II)−**1** was determined at 25 °C by ordinary potentiometric titration. The exchange equilibrium constant K_x (**1**), where L and L′ are cyclam and **1**, respectively, was determined at 77, 83, and 93 °C. From plots of values of log K_x vs $1/T$, its value at 25.0 °C was found. The exchange constant K_x was then converted to the normal constant log K_{ML} for Cu−**1** using the protonation constants of both the ligands and the known log K_{ML} for Cu−cyclam.

Radiochemistry. A 5 or 10 mM solution of each of the ligands was prepared by dissolving the compounds in 0.4 M ammonium acetate, pH 5.5, 0.1 M ammonium citrate, pH 5.5, or ethanol (see Table 2 for specific reaction conditions for each ligand). A solution of $^{64}CuCl_2$ in 0.1 N HCl (0.5 μL, 0.1−0.5 mCi) was added to the ligand solutions. Samples were incubated at either 75 or 55 °C. After several time points, ^{64}Cu-labeled **1**, **3**, and **4** were analyzed by radio-TLC using C18 plates as the stationary phase and 1:1 MeOH:10% ammonium acetate as the eluant. The mobile phase used to analyze ^{64}Cu−**2** was 9:1 MeOH:0.1 M ammonium citrate buffer. Under all of these conditions, free ^{64}Cu−acetate remained at the origin.

For CA reactions, a 100 mM solution of $CuCl_2$ in Milli-Q water was added to 10 mM ligand solutions. The $^{64}CuCl_2$ solution described above was added to this mixture. After the solutions were incubated at 75 °C for a certain time period, the radiochemical yields were determined using C18 plates as the stationary phase and 1:1 MeOH:10% ammonium acetate as the eluant, except for ligand **2**, where the eluant was 9:1 MeOH:0.1 M ammonium citrate. The incubation times, temperatures, radiochemical yields, and R_f values are summarized in Table 2.

The partition coefficients (log P) of $^{64}Cu/Cu(II)$ complexes (CA) were determined by adding 4−8 μL of the CA 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 (Table 2).

Serum Stability. In vitro serum stability experiments were carried out by adding 100 μL of ^{64}Cu-labeled ligands **1**−**4** (5−10 mM) to 0.5 mL of rat serum (Sigma, St. Louis, MO). For ligand **2**, two lower concentrations in rat serum were additionally tested by adding 2 and 20 μL of the labeled complex (5 mM), respectively, to 0.5 mL of rat serum. The solutions were incubated at 37 °C, and samples were analyzed by radio-TLC at 10, 30, and 60 min and at 2, 4, and 24 h postadministration to rat serum.

Biodistribution Studies. Copper-64-labeled **1**−**4** solutions were diluted with saline. Mature, female, Sprague−Dawley rats (180−200 g) were injected with ^{64}Cu-labeled ligands **1**−**4** ($n = 4$). The injected volume of activity per rat did not exceed 0.2 mL for 22 μCi of activity. At selected time points postinjection, animals were sacrificed. Organs of interest were removed, weighed, and counted. The %ID per gram (%ID/g) and %ID per organ (%ID/organ) were calculated by comparison to a weighed, counted standard.

Metabolism Studies. The metabolism of ^{64}Cu−TETA and ^{64}Cu−**2** in rat liver in vivo was performed in male Lewis rats using reported methods.[70] Briefly, ^{64}Cu−**2** and ^{64}Cu−TETA (20 mCi each) were injected into rats via the tail vein. The rats were sacrificed at 20 h postinjection, and the livers were immediately excised and placed on ice. Tissue samples were homogenized in 65:35 ethanol/ammonium acetate buffer (0.1 M, pH 5.5) followed by 1 minute of sonication. The precipitated protein was separated by centrifugation at 23 500g for 30 min at 4 °C. The supernatant was isolated, counted for radioactivity, and analyzed by a size-exclusion HPLC that was calibrated using molecular weight standards. Greater than 95% of the radioactivity was recovered from the HPLC column. Liver controls were performed where either the ^{64}Cu−TETA or the ^{64}Cu−**2** injectate was added directly to liver tissue excised from rats. These samples were worked up in the same manner as the experimental rats as described above. The %AI was determined from the integration of the radio-HPLC chromatogram as previously described.[76] An unpaired t-test on the metabolism data was performed using Prism, version 3.00 (Graphpad, San Diego, CA).

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