



The *In Vivo* Behavior of Copper-64-Labeled Azamacrocyclic Complexes

Teresa M. Jones-Wilson,¹ Kim A. Deal,¹ Carolyn J. Anderson,¹
Deborah W. McCarthy,¹ Zoltan Kovacs,² Ramunas J. Motekaitis,³ A. Dean Sherry,²
Arthur E. Martell³ and Michael J. Welch¹

¹MALLINCKRODT INSTITUTE OF RADIOLOGY, WASHINGTON UNIVERSITY SCHOOL OF MEDICINE, ST. LOUIS, MO 63110, USA;

²DEPARTMENT OF CHEMISTRY, UNIVERSITY OF TEXAS AT DALLAS, RICHARDSON, TX 75083, USA; AND ³DEPARTMENT OF CHEMISTRY, TEXAS A & M UNIVERSITY, COLLEGE STATION, TX 77843, USA

ABSTRACT. The use of copper radioisotopes in imaging and therapy applications has created a greater need for bifunctional chelates (BFCs) for complexing copper radioisotopes to biomolecules. It has been demonstrated that the charge and lipophilicity of the Cu-BFC complex has a significant effect on the *in vivo* behavior of the radiolabeled Cu-BFC-biomolecule conjugate. To evaluate the effects of charge, stability, and macrocyclic backbone size on the biological behavior of ⁶⁴Cu complexes, a series of macrocyclic ⁶⁴Cu complexes have been prepared, and the biodistributions of these agents were evaluated in normal Sprague-Dawley rats. Two macrocyclic backbones, dodecane and tetradecane, were evaluated; cyclen, DOTA, and DO2A were dodecane backbone derivatives, and cyclam, TETA, and et-cyclam were tetradecane backbone derivatives. The biodistributions of the ⁶⁴Cu-labeled complexes correlated with differences in the size of the macrocycle backbone and the formal charge of the complex. All compounds showed uptake and clearance through the liver and kidneys; however, the positively charged ⁶⁴Cu complexes showed significantly higher uptake in both of these organs than did the negatively charged or neutral complexes. ⁶⁴Cu-TETA, a negatively charged complex with the tetradecane backbone, had the most efficient clearance by 24 hours' postinjection. These data suggest that negatively charged complexes may have more favorable clearance properties when used as BFCs. NUCL MED BIOL 25;6:523–530, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. Copper-64, Biodistribution, Macrocyclic, Stability constant

INTRODUCTION

Copper-64 ($t_{1/2} = 12.7$ h; β^+ [19%]; β^- [40%]) has proven to be a versatile isotope with respect to its applications in both imaging (8, 19) and therapy (2, 7). Bifunctional chelates designed for attachment of various isotopes of copper(II) to antibodies and antibody fragments have employed primarily tetraazamacrocyclic backbones. Azamacrocyclic ligands provide excellent binding environments for Cu(II) ions, combining rapid metalation, small size and aqueous solubility with resistance to exchange of Cu(II) *in vivo* (17). Substituted azamacrocycles like 6-bromoacetamidobenzyl-1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (BAT) (5, 6, 17) and 4-[(1,4,8,11-tetraazacyclotetradec-1-yl)-methyl]benzoic acid (CPTA) (22) have shown blood stability *in vivo* (1, 20, 22) and have been used in clinical studies as bifunctional chelates conjugated to monoclonal antibodies (MAbs) (10, 19).

Rogers and co-workers showed that the charge of the bifunctional chelate used to complex ⁶⁴Cu to MAb fragments had a significant effect on the kidney clearance of the ⁶⁴Cu-BFC-MAB fragment conjugate (20); ⁶⁴Cu-CPTA-MAB 1A3-F(ab')₂ fragments showed much greater accumulation in the kidney and slower clearance than

⁶⁴Cu-BAT-2IT-1A3-F(ab')₂. Anderson and co-workers conjugated TETA (1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid) and CPTA to the somatostatin receptor ligand octreotide (3), and they observed completely different normal organ clearance characteristics between the two ⁶⁴Cu-BFC-octreotide conjugates. More than 90% of the injected dose of ⁶⁴Cu-CPTA-octreotide accumulated in the liver, with slow clearance over 24 h, whereas ⁶⁴Cu-TETA-octreotide cleared almost exclusively through the renal system, with ~80% being excreted in the urine by 24 h. The ⁶⁴Cu-CPTA complex is positively charged, whereas ⁶⁴Cu-BAT/⁶⁴Cu-TETA is negatively charged, and together these studies suggest that the negatively charged ⁶⁴Cu-BFC-conjugates have more favorable clearance characteristics.

An understanding of the chemistry and clearance properties of a radiometal-labeled chelate is essential in the design and selection of a bifunctional chelate with desirable clearance characteristics. In this study, we compared the thermodynamic stability and *in vivo* behavior of six Cu(II) complexes, which differ in the macrocycle backbone and formal charge of the metal-ligand complex. TETA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), 1,4,8,11-tetraazacyclotetradecane (cyclam), 1,4-ethano-1,4,8,11-tetraazacyclotetradecane (et-cyclam), 1,4,7,10-tetraazacyclododecane (cyclen), and 1,4,7,10-tetraazacyclododecane-1,7-diacetic acid (DO2A) (Fig. 1) have been radiolabeled with ⁶⁴Cu(II) and evaluated *in vivo*. The Cu-complexes of these ligands differ in charge, with TETA and DOTA forming negatively-charged Cu(II) complexes, cyclam, cyclen, and et-cyclam forming positively charged complexes, and DO2A forming a neutral complex with Cu(II). TETA, cyclam, and et-cyclam are 14-membered macrocycle rings,

Address correspondence to: Carolyn J. Anderson, PhD, Division of Radiological Sciences, Washington University School of Medicine, 510 S. Kingshighway Blvd., Box 8225, St. Louis, MO 63110; e-mail: <andersoncj@mirlink.wustl.edu>.

The current address of Teresa M. Jones-Wilson is Ashland University, Department of Chemistry, 401 College Ave., Ashland, OH 44805.

Received 28 January 1998.

Accepted 6 March 1998.

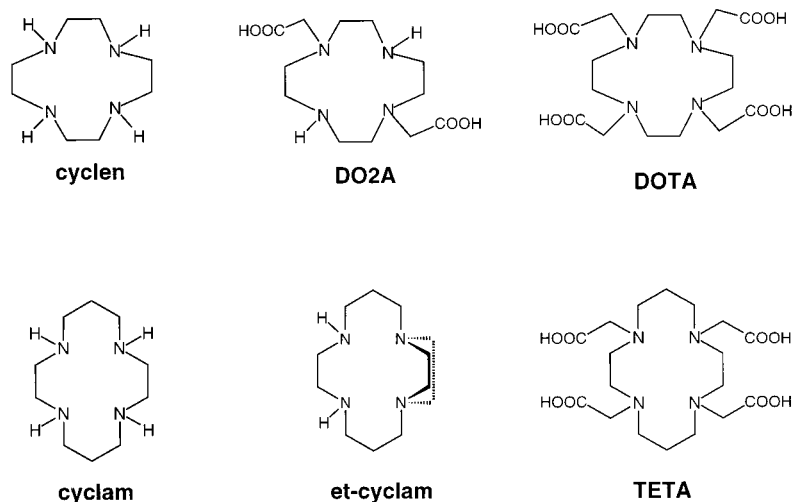


FIG. 1. Structures of cyclen, DO2A, DOTA, cyclam, et-cyclam, and TETA.

whereas DOTA, DO2A, and cyclen consist of 12-membered rings. Et-cyclam is a bridged macrocycle that was synthesized and evaluated to determine whether a more sterically rigid macrocycle may have more favorable *in vivo* behavior. The thermodynamic stability constants of the Cu(II) complexes of et-cyclam and DO2A are presented and compared with literature values for the other Cu(II) complexes studied.

MATERIALS AND METHODS

Reagents and Instrumentation

Ammonium acetate was purchased from Fluka; *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) was purchased from Sigma (St. Louis, MO). Cyclam, TETA, and cyclen were purchased from Aldrich Chemical Co. (Milwaukee, WI). DOTA was purchased from Parish Chemical Co. (Vineyard, UT). DO2A was prepared by the literature method (11). A preliminary preparation of et-cyclam has been briefly reported (12), and the complete synthesis is reported herein. All other chemicals and solvents were obtained from Aldrich Chemical and used without further purification. Aqueous solutions were prepared using filtered distilled deionized water through a Milli-Q filtration system. The $^{64}\text{CuCl}_2$ was obtained from two sources, the Missouri University Research Reactor (MURR) in Columbia, Missouri (26) or via in-house production on a biomedical cyclotron (16). No-carrier-added $^{111}\text{InCl}_3$ was obtained from Mallinckrodt, Inc.

Sample activity was measured using a Capintech CRC-12 Dose Calibrator. Radioactive thin-layer chromatograms (TLC) were analyzed using a Bioscan Imaging Scanner System 200, a position-sensitive radio TLC analyzer with Autochanger and gas flow detection. For animal and serum experiments, radioactivity was measured using a Beckman Gamma 8000 automatic well-type gamma scintillation counter. Electrophoresis was conducted using a TITAN gel chamber and TITAN III cellulose acetate plates with a BioRad model 2000/500 power supply.

Carbon-13 and proton nuclear magnetic resonance spectra were obtained on a Gemini 300 MHz or Varian 500 MHz Fourier Transform NMR spectrometer. Proton chemical shifts are reported in ppm relative to internal tetramethylsilane (TMS). Carbon chemical shifts are reported relative to CDCl_3 . Positive ion fast atom bombardment (FAB) and electrospray mass spectra were obtained from Washington University Mass Spectroscopy Resources. Elemental analysis was performed by Galbraith Laborato-

ries. X-ray crystallographic data was obtained from the X-ray Crystallographic Facility, Department of Chemistry, Washington University.

Adult female Sprague-Dawley rats were purchased from Sasco (Omaha, NE). All animal experiments were performed in compliance with guidelines specified by the Washington University Animal Studies Committee.

Et-Cyclam

Et-cyclam was prepared in a one-step reaction from cyclam using a modified method of Wainwright (25). Cyclam (4.0 g, 20.0 mmol) was dissolved in 80 mL absolute ethanol under N_2 . Next, 1,2-dibromoethane (7.88g, 42 mmol) was added dropwise and the mixture heated at reflux for 68 h. The reaction solvent was removed by evaporation under reduced pressure yielding a sticky yellow solid. The reaction was monitored by TLC on silica gel with a 20:80 10% ammonium acetate/methanol mobile phase. Visualization was by iodine staining. The product and starting material migrated with R_f values of 0.3 and 0.0, respectively. The residue was dissolved in 20 mL H_2O , and 10 N NaOH was added dropwise to precipitate the free amine. The suspension was washed with CHCl_3 (2×40 mL) followed by six 20-mL portions of CHCl_3 . The combined organic layers were dried over MgSO_4 and filtered. Celite (5 g) was added, the solvent evaporated under reduced pressure, and the solids dried under vacuum (25 mm Hg). Et-cyclam was isolated by sublimation of the crude solids under vacuum (0.1 torr) at 37°C . Typical yield 1.13 g (0.5 mmol) 20% colorless, hygroscopic prisms. ^{13}C NMR (CDCl_3): δ 57.4 (C-4, C-5, C-11, C-12 (ethylene carbons)); 51.7; 50.2; 50.0 (C-1, C-8, C-3, C-6 (propylene carbons)); C-9, C-10 (ethylene carbons)); 25.90 (C-2, C-7 (ethylene carbons)). ^1H NMR (CDCl_3): δ 1.68 (s, 4H); 2.36 (m, 4H); 2.66 (t, 4H); 3.13 (m, 4H); 2.75 (s, 8H, C-7 H_2). FAB mass spectrometry of the product shows the parent ion peak at $(M + 1) = 227$. Anal. calc'd for $\text{C}_{12}\text{H}_{26}\text{N}_4$: C, 63.67; H, 11.58; N, 24.75. Found: C, 63.82; H, 11.53; N, 24.55.

X-Ray Diffraction Studies of Et-Cyclam

Sublimation of the crude reaction mixture produced colorless prisms of et-cyclam of sufficient quality for X-ray diffraction analysis. The sample was isolated and mounted under an Argon atmosphere. Data were collected at room temperature (296 K) on a Siemens P4 diffractometer using $\text{CuK}\alpha$ ($\lambda = 1.54178 \text{ \AA}$) radiation. The

TABLE 1. ⁶⁴Cu(II) Complex Formation and Chromatography Conditions

Ligand	Concn. (mM)	pH	Reaction time (min)	Radiochemical purity (%)	R _f
Cyclen	20	5.5	10	98	0.67 ^a , 0.12 ^b
Cyclam	20	5.5	10	97	0.67 ^a , 0.56 ^c
Et-cyclam	5	6.4	20	90	0.07 ^c , 0.37 ^d
DOTA	30	5.5	45	95	0.39 ^b
TETA	20	5.5	30	100	0.70 ^a , 0.56 ^b
DO2A	10	5.5	60	100	0.58 ^a , 0.17 ^b

^a Whatman No. 1 paper; 70:30 ethanol:0.1 M ammonium acetate, pH 5.45.

^b Silica; 1:1 methanol:10% ammonium acetate.

^c Reverse-phase C18; 92:8 acetonitrile:1 M ammonium acetate, pH 7.0.

^d Reverse-phase C18; 92:8 acetonitrile:1 M ammonium acetate, pH 2.0.

structure was based upon an empirical formula of C₁₂H₂₆N₄ and a formula weight of 226.4 g/mol. No empirical absorption correction was applied. Hydrogens were located using the Riding model with refined common isotropic U. All data reductions and structure refinements were carried out by using the Siemens SHELXTL PLUS structure-determination package.

Stability Measurements

The stability constants of Cu-TETA (4), Cu-DOTA (4), Cu-cyclam (18), and Cu-cyclen (15) have been reported elsewhere, whereas the determination of the stability constants of Cu-et-cyclam and Cu-DO2A are presented here. The formation constants of Cu²⁺ with et-cyclam 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 protonation constants of et-cyclam were determined at 25, 60, 70, and 80°C by ordinary potentiometric titration. The protonation constants of et-cyclam were computed at 90°C by extrapolation of log(overall protonation constants) vs. 1/T where T is absolute temperature. The displacement reaction (1) was determined at 60, 70, 80, and 90°C, and log K_{disp} extrapolated to



25°C using plots of log K_{disp} vs. 1/T. The displacement constant was converted to the normal constant log K_{ML}.

Potentiometric determinations of protonation constants were obtained as described (14). Computations of protonation constants were done using program BEST (14), and pK_w was refined at temperatures above 25°C. Briefly, a 3.000-mL aliquot of a stock solution containing et-cyclam (5.597 × 10⁻³ M) was added to a 1.360-mL aliquot of a stock solution containing CuCl₂ (1.306 × 10⁻²). Both HCl (1.942 M) and 1.000 M KCl were added as necessary to obtain a nominal 0.1 M ionic strength, and the solution was diluted to 10.00 mL. This solution was 1.68 × 10⁻³ M in et-cyclam and 1.78 × 10⁻³ M in Cu²⁺. Five solutions were prepared for each temperature measured (60, 70, 80, and 90°C) each corresponding to calculated pH 1.110, 0.933, 0.757, 0.566, and 0.369. The peak at 560 nm was monitored, allowing sufficient time for equilibration.

The stability constant of DO2A with Cu(II) was determined by potentiometric titration. The pH measurements were performed with a Fisher Accumet 925 pH-meter, an Orion 8103 Ross

Combination electrode, and a Metrohm automatic buret in a jacketed vessel at 25 ± 0.1°C under a nitrogen atmosphere. The ionic strength was kept constant with 0.1 M KCl. Hydrogen ion concentrations were calculated from the measured pH values using a pK_w = 13.806 and a H⁺ activity coefficient of 0.82 determined in separate titrations. The Cu(II)-DO2A stability constants were obtained from the potentiometric data by using a Simplex nonlinear regression algorithm run on a PC (21). All measurements were in triplicate.

¹¹¹In-DTPA

Freshly prepared diethylenetriaminepentaacetic acid (DTPA) labeled with ¹¹¹In³⁺ was employed as an electrophoresis standard. A 3.3-mM DTPA solution in 0.1N NaOH was prepared. To a 300-μL aliquot of this solution, the desired quantity of ¹¹¹In-acetate in 600 μL 0.45 M sodium acetate, pH 5.45, was added. The pH of the solution was adjusted to 7.0 with 1N HCl. Complex formation was complete within 10 min as determined by radio-TLC on silica eluting with 1:1 methanol/10% sodium acetate.

⁶⁴Cu-Ligand Complexes

The ⁶⁴CuCl₂ from MURR was received in 18–19 mL 1 N HCl, whereas cyclotron-produced ⁶⁴Cu was prepared in 8–10 mL 0.5 M HCl. For use in radiopharmaceutical studies, both solutions were reduced to dryness with heating under a stream of nitrogen gas and the activity redissolved in 140 μL 0.1 M HCl. The ⁶⁴CuCl₂ was converted to ⁶⁴Cu-acetate by stirring with 1 mL 0.1 M ammonium acetate, pH 5.45, for 5 min.

Conditions for the preparation of the ⁶⁴Cu(II) complexes are summarized in Table 1. In general, the ligand was dissolved in water to obtain a solution of the desired concentration. The ⁶⁴Cu-labeled complex was prepared by addition of the desired quantity of ⁶⁴Cu-acetate to the ligand solution, and the pH was adjusted by addition of either 1N HCl or 1N NaOH. The mixture was stirred and allowed to stand at room temperature for the time specified in Table 1. Characterization of the complexes were accomplished by radio-TLC. Chromatographic conditions and R_f values are summarized in Table 1.

Electrophoresis

Electrophoresis experiments were conducted using cellulose acetate plates and either 0.1 M HEPES, pH 7.34, or 0.1 M ammonium acetate, pH 5.45, buffer. For each sample, the radioactive complex was applied to the marked mid-point of the pre-equilibrated

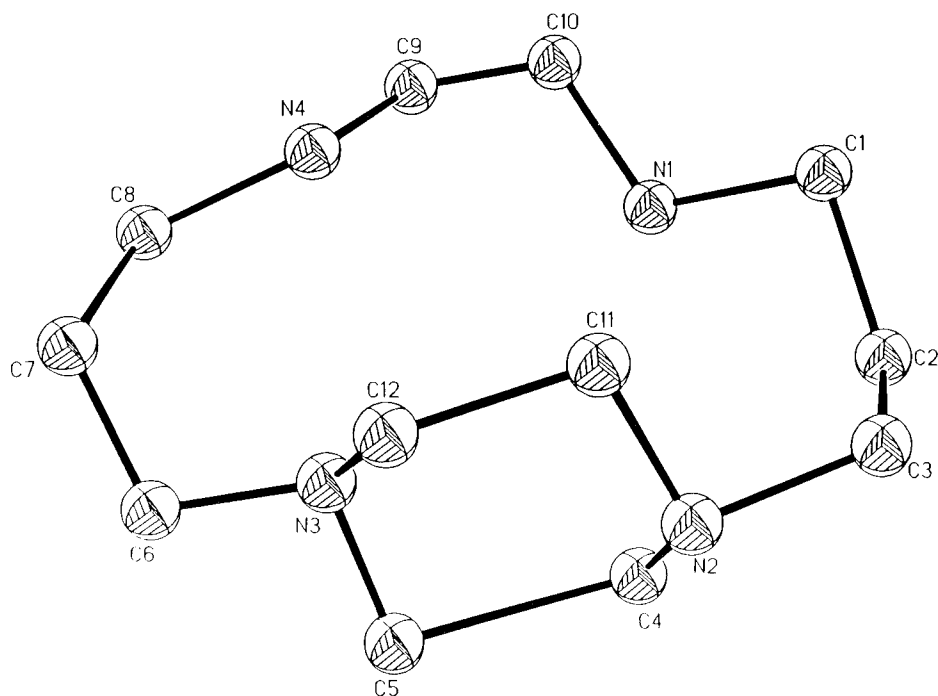


FIG. 2. ORTEP drawing of et-cyclam. Crystal dimensions = $0.24 \times 0.26 \times 0.32$, point group orthorhombic (Pna2), unit cell dimensions: $a = 16.7409(3)$ Å, $b = 9.663(2)$ Å and $c = 8.738(2)$ Å; unit cell volume $1413.5(2)$ Å³. Calculated density = 1.064 g/cm³. Adsorption coefficient = 5.06 cm⁻¹; $Z = 4$, $R = 0.0594$, $wR = 0.0828$; the goodness of fit = 1.57 .

cellulose plate. The strips were developed at 8 mA for 30 min. Migration of each radioactive complex was compared to a freshly prepared ¹¹¹In-DTPA²⁻ standard. Radioactivity was detected using the BioScan radio-TLC scanner.

Biodistributions

Animal biodistributions were performed for each ⁶⁴Cu-labeled macrocycle. The radioactive complex in 0.1 M ammonium acetate solution was diluted with saline. Mature, female Sprague-Dawley rats (160–180 g) were injected with the ⁶⁴Cu-labeled compound ($n = 4$ or 5). The injected volume of activity per animal did not exceed 0.2 mL for 7–15 μCi of activity. Animals were anesthetized and activity administered by bolus injection into the tail vein. Animals were allowed access to food and water *ad libitum*. At selected time points postinjection, animals were sacrificed. Organs of interest were removed, weighed, and counted. The percent injected dose per gram (%ID/g) and percent injected dose per organ (%ID/organ) were calculated by comparison to a weighed, counted standard solution.

Statistical Analysis

To determine whether the observed differences in biodistribution data between the ⁶⁴Cu-labeled azamacrocycles were significant, Student's *t* test was performed, and a 95% confidence level was assumed, with $p < 0.05$ being statistically different.

RESULTS

Chemistry

Preparation of et-cyclam resulted in a maximum of 20–25% isolated yield in a one-step reaction from cyclam using a modified procedure

of Wainwright (25). The use of celite resin in the purification via sublimation substantially increased the yield from less than 10% to 25%. The isolated material was extremely hygroscopic, and the compound was handled in a glove box. Sublimation of the crude et-cyclam produced X-ray quality crystals, and the ORTEP drawing is shown in Figure 2. Bond distances and bond angles with estimated standard deviations are presented in Table 2. The location of the ethylene bridge on the ethylene (not the propylene) side of the

TABLE 2. Selected Bond Distances and Bond Angles for et-Cyclam

Bond lengths (Å)		Bond angles (deg)	
N(2)—C(11)	1.436(7)	C(11)—N(2)—C(3)	114.0(5)
N(2)—C(4)	1.462(8)	C(3)—N(2)—C(4)	118.5(5)
C(12)—N(3)	1.460(8)	C(11)—C(12)—N(3)	110.5(5)
N(3)—C(5)	1.452(9)	C(12)—N(3)—C(5)	114.9(4)
C(7)—C(8)	1.534(10)	N(3)—C(6)—C(7)	121.0(5)
N(4)—C(9)	1.425(7)	C(7)—C(8)—N(4)	113.0(5)
C(10)—N(1)	1.477(7)	N(4)—C(9)—C(10)	110.1(4)
C(1)—C(2)	1.498(10)	C(10)—N(1)—C(1)	114.6(5)
C(4)—C(5)	1.478(11)	C(1)—C(2)—C(3)	114.1(7)
N(2)—C(3)	1.497(10)	N(2)—C(4)—C(5)	109.1(6)
C(11)—C(12)	1.486(9)	C(11)—N(2)—C(4)	107.4(5)
N(3)—C(6)	1.403(12)	N(2)—C(11)—C(12)	109.9(5)
C(6)—C(7)	1.509(12)	C(12)—N(3)—C(6)	112.3(6)
C(8)—N(4)	1.473(7)	C(6)—N(3)—C(5)	113.1(7)
C(9)—C(10)	1.486(7)	C(6)—C(7)—C(8)	121.1(7)
N(1)—C(1)	1.437(7)	C(8)—N(4)—C(9)	115.8(4)
C(2)—C(3)	1.478(15)	C(9)—C(10)—N(1)	109.4(4)
		N(1)—C(1)—C(2)	110.7(6)
		N(2)—C(3)—C(2)	113.0(5)
		N(3)—C(5)—C(4)	111.6(6)

TABLE 3. Thermodynamic Stability Constants for Cu(II) with Azamacrocycles: Conditions for Cu-Et-cyclam and Cu-DO2A: $\mu = 0.1$ M KCl; 25°C

Macrocycle	Log K	Reference
Cyclen	24.8	(15)
Cyclam	28.09	(18)
Et-cyclam	26.1	this work
DOTA	22.7	(4)
TETA	21.9	(4)
DO2A	18.9	this work

cyclam backbone was confirmed. Et-cyclam is in a typical boat conformation, with the propylene sides of the molecule bent out of the plane. The ethylene bridge points in toward the macrocyclic cavity and slightly out of the plane. The C—C bond distances are uniform and in the normal range for C—C single bonds (Table 2). The C—N bonds are also in the normal range, but are less uniform than the C—C bonds. The ethylene bridge bonds are slightly compressed compared to the propylene N—C bonds (Table 2). This bond compression is not surprising considering the steric restraints imposed by the ethylene bridge.

Thermodynamic stability constants for the Cu(II) complexes of the ligands are presented in Table 3. The log stability constant (log K) for Cu(II)-et-cyclam is 26.1 and the log protonation constants are 12.54, 9.42, and 0.73 at 25°C. The log K for DO2A with copper(II) is 18.87, as determined by potentiometric titration. The protonation constants of DO2A in 0.1 M KCl (10.91, 9.45, 4.19, 3.18) have been reported elsewhere (11). The log stability constants for the monoprotinated species, Cu-HDO2A, and the diprotinated species, CuH2DO2A, are 3.82 and 2.72, respectively. These two species are significantly less stable, but they are unlikely to be biologically relevant. All the reported stability constants, with the exception of DO2A, are greater than log K = 20. These ligands form thermodynamically stable complexes with Cu(II); however, the kinetic stability may favor DO2A, DOTA, and TETA owing to the carboxylate groups that favor 6-coordinate complexes over 4-coordinate complexes.

All six ligands were labeled with ^{64}Cu -acetate in yields >90% at ambient temperature; average radiochemical purities are presented in Table 1. Radiochemical purity analysis of each ^{64}Cu complex except DOTA was accomplished by a combination of two different TLC systems. The ^{64}Cu -acetate remained at the origin, $R_f = 0.0$, under all TLC conditions.

Metal-ligand complex charge was determined by electrophoresis; the migration of each ^{64}Cu complex was compared to the migration of an ^{111}In -DTPA $^{2-}$ standard. At pH 5.45 (0.1 M ammonium acetate, pH 5.45), ^{111}In -DTPA $^{2-}$ standard migrated with an R_f of +0.9 in the direction of the anode; ^{64}Cu -cyclam, -cyclen, and -et-cyclam migrated with R_f values ranging from -0.8 to -0.9, toward the cathode, indicating a positive overall charge at pH 5.45. At physiological pH (0.1 M HEPES pH 7.34), two radioactive peaks were observed for ^{64}Cu -et-cyclam with R_f values of -0.42 and -0.90, in the direction of the cathode, indicating the presence of more than one positively charged species. In both buffer systems, ^{64}Cu -TETA and -DOTA migrated in the direction of the anode, with R_f values of +0.8, indicating their negative charge. The ^{64}Cu -DO2A migrated at pH 5.45 and pH 7.34 with an R_f of +0.04, indicating that the complex was neutral. The slight migration of the

TABLE 4. Biodistribution of ^{64}Cu -Azamacrocycles with the 14-Member Macrocycle Backbone

Time (h)	0.25	2	24
Cyclam			
Blood	7.26 ± 0.83	2.69 ± 0.26	2.11 ± 0.39
Liver	9.30 ± 1.13	13.4 ± 0.83	10.5 ± 1.3
Kidney	8.57 ± 0.93	11.2 ± 0.43	4.5 ± 1.25
Et-cyclam			
Blood	3.19 ± 0.29	0.69 ± 0.24	1.40 ± 0.07
Liver	16.3 ± 2.0	25.2 ± 12.7	13.4 ± 2.8
Kidney	15.1 ± 1.9	21.7 ± 10.2	8.38 ± 0.83
TETA			
Blood	6.25 ± 0.45	0.32 ± 0.13	0.21 ± 0.05
Liver	1.42 ± 0.09	0.54 ± 0.19	0.49 ± 0.11
Kidney	2.66 ± 0.33	0.52 ± 0.08	0.21 ± 0.03

Data are expressed as the %ID/organ ± SD with 4 or 5 animals per data point.

complex toward the cathode was due to electro-osmotic transport and does not indicate the presence of charge (9).

BIODISTRIBUTION

The uptake and clearance of the ^{64}Cu -labeled azamacrocycles were determined through biodistribution studies. Blood, liver, and kidney data at 15 min, 2 h, and 24 h postinjection are shown in Tables 4 and 5. In comparing the 12-member (cyclen) and 14-member (cyclam) ring systems, ^{64}Cu -cyclen showed significantly decreased liver (all times) and kidney (2 h and 24 h) retention than ^{64}Cu -cyclam. Statistical differences were observed between ^{64}Cu -labeled DOTA and TETA in the liver (2 and 24 h) and kidney (2 and 24 h) where ^{64}Cu -TETA showed more rapid clearance.

Both copper(II)-labeled cyclam and et-cyclam have the same parent backbone and same formal charge. At all time points, the blood activity of ^{64}Cu -et-cyclam was significantly lower than ^{64}Cu -cyclam. At 15 min postinjection, ^{64}Cu -et-cyclam showed significantly higher accumulations in the liver and kidney than ^{64}Cu -cyclam, whereas at 2 h postinjection there were no significant differences. At 24 h, the amount of ^{64}Cu -et-cyclam remaining in kidney was significantly higher than ^{64}Cu -cyclam.

Comparisons were made between compounds of the same parent

TABLE 5. Biodistribution of ^{64}Cu -Azamacrocycles with the 12-Member Macrocycle Backbone

Time (h)	0.25	2	24
Cyclen			
Blood	6.60 ± 0.73	2.05 ± 0.43	1.73 ± 0.14
Liver	7.25 ± 0.64	4.83 ± 0.73	3.90 ± 0.40
Kidney	9.06 ± 0.99	7.38 ± 1.9	2.14 ± 0.89
DOTA			
Blood	6.74 ± 1.35	0.79 ± 0.26	0.58 ± 0.19
Liver	1.91 ± 0.74	1.14 ± 0.08	1.05 ± 0.16
Kidney	2.64 ± 0.48	1.26 ± 0.14	0.54 ± 0.08
DO2A			
Blood	6.18 ± 0.71	0.50 ± 0.08	0.46 ± 0.08
Liver	1.52 ± 0.10	0.90 ± 0.09	0.86 ± 0.14
Kidney	3.15 ± 0.15	0.89 ± 0.05	0.35 ± 0.10

Data are expressed as the %ID/organ ± SD with 4 or 5 animals per data point.

backbone but differing formal charge of the Cu(II) species. The ^{64}Cu -et-cyclam (+2) was compared to ^{64}Cu -TETA (-2); both complexes have the 14-member backbone. At all time points, ^{64}Cu -TETA had significantly lower accumulation in the liver and kidney than did ^{64}Cu -et-cyclam. The ^{64}Cu -TETA cleared more slowly initially (15 min), but at 24 h had significantly lower uptake in the blood than did ^{64}Cu -et-cyclam. Also, ^{64}Cu -TETA (-2) had significantly lower uptake in the liver and kidneys than ^{64}Cu -cyclam (+2) at all times.

Complexes having a 12-member backbone with different formal charges, ^{64}Cu -cyclen (+2), ^{64}Cu -DOTA (-2), and ^{64}Cu -DO2A (0), were compared. The ^{64}Cu -DOTA cleared the liver and kidneys faster than did ^{64}Cu -cyclen, having significantly lower accumulation at all times. Statistical differences were observed for labeled cyclen and DO2A in the blood (2 h and 24 h); liver (all times) and kidney (all times), with ^{64}Cu -DO2A showing significantly more clearance in these tissues. The ^{64}Cu -DO2A showed a similar biodistribution pattern to ^{64}Cu -DOTA; however, statistical differences were observed in the blood (2 h), liver (2 h), and kidney (2 and 24 h), with ^{64}Cu -DO2A having somewhat lower accumulation.

The blood clearance of ^{64}Cu -labeled azamacrocycles varied with the chelate. The ^{64}Cu -et-cyclam had the slowest clearance; approximately 55% of the dose at 15 min postinjection had cleared by 24 h. In addition, ^{64}Cu -TETA had the fastest clearance, with 95% of the 15-min dose cleared in 2 h. Also, ^{64}Cu -DO2A had similar blood clearance to ^{64}Cu -TETA, with 92% of the 15-min dose cleared in 2 h. ^{64}Cu -cyclam, cyclen, and DOTA all demonstrated between 70–75% of the 15-min dose clearing in 24 h.

The kidney clearance of ^{64}Cu -labeled azamacrocycles was similar to the blood-clearance profiles. ^{64}Cu -TETA and DO2A had more rapid clearance, with about 90% of the 15-min dose cleared in 24 h. ^{64}Cu -et-cyclam and cyclam had 45–59% clearance, while ^{64}Cu -DOTA had slower clearance (25%). The liver clearance of ^{64}Cu -labeled azamacrocycles ranged from less than 20% (cyclam) to 65% (TETA) of the 15-min dose in 24 h. The ^{64}Cu -cyclam showed an increase in the liver dose with time. Both ^{64}Cu -et-cyclam and ^{64}Cu -DOTA showed slower liver clearance, with 80% of the 15-min dose remaining at 24 h. The ^{64}Cu -TETA had the most rapid liver clearance, with 65% of the 15-min dose cleared by 24 h. Both ^{64}Cu -cyclen and DO2A had similar liver clearance, with 45–50% of the 15-min dose clearing by 24 h.

Other organs were collected to determine whether any tissue specificity existed. No significant uptake was observed outside of the clearance organs. In particular, no significant uptake in the myocardium was found for any of the radiometal complexes. The heart/blood ratio was less than 0.9 at all time points. None of the complexes appeared to cross the blood-brain barrier. The brain/blood ratio was less than 0.4 at all time points.

DISCUSSION

In this study we evaluated a series of ^{64}Cu -macrocyclic complexes comparing complexes that differed in backbone size and charge. The goals were to determine the effects of charge and backbone size on *in vivo* stability and biodistribution with the overall goal of designing more optimal bifunctional chelates for labeling copper radioisotopes to biomolecules.

Preparation of ligands that are pre-organized for metal ion binding can reduce the complexation time of radiometal labeling. A simple method for further pre-organizing a macrocycle is to reinforce the ring by the addition of a carbon bridge. The parent macrocycle of et-cyclam is cyclam, a 14-member macrocycle with

four nitrogen donors (Fig. 1). Re-enforcement of cyclam led to the addition of an ethylene bridge on the ethylene side of cyclam (Fig. 2). Radiolabeling et-cyclam with ^{64}Cu yielded the expected complex in high radiochemical purity. Reaction time was twice as long as for the parent complex (Table 1), ^{64}Cu -cyclam, suggesting the cavity is highly sterically hindered. The addition of the ethylene bridge slightly lowered the stability of the resulting Cu(II) complex. Although the stability constant for Cu-et-cyclam is high, the drop in log K from 28.1 for Cu-cyclam to 26.1 suggests that the reinforced macrocycle exhibits a high degree of ring strain and rigidity.

The macrocycles were readily labeled with ^{64}Cu under a variety of conditions (Table 1). The differences in stability constants did not affect the ability to label the macrocycles *in vitro*. The complexes were expected to differ in lipophilicity, and an attempt was made to measure the octanol-water partition coefficients. Unfortunately, octanol-water partition coefficients could not be determined for the ^{64}Cu -labeled complexes. No back-extraction of the radiolabeled macrocycles into the octanol layer was detected. Aqueous partitioning of a charged species would be expected considering the ability of water to stabilize charge. Green and co-workers found positive octanol-water partition coefficients for positively charged compounds (23, 24), indicating that electrostatic interactions are not the sole factor governing octanol-water partition. The hydrophilic nature of the azamacrocyclic ligands, in addition to the charge distributions, explains the complexes' exclusive preference for the aqueous layer.

The protonation constants were determined for the ligand et-cyclam. Because of strong coulombic interactions, only three protonation constants could be determined. The difference between the second and third log protonation constant is very great (from 9.42 to 0.73) because the third proton is also subjected to a great deal of coulombic charge. The fourth protonation constant is extremely small, and a very high proton concentration would be required for protonation.

Thermodynamic stability constants for the complexes are presented in Table 3. Cyclam has a slightly higher stability constant with Cu(II) than does cyclen. The two-carbon difference in macrocycle size allows for a better fit of Cu(II) in the tetraaza cavity of cyclam. The tetra acetate derivatives of cyclen and cyclam are DOTA and TETA, respectively. The stability constants of DOTA and TETA with Cu(II) are nearly identical, differing less than 1 order of magnitude. The additional coordinating groups on DOTA appear to provide an environment similar to TETA, both binding Cu(II) well. DO2A, which structurally is cyclen with two acetate groups, has a Cu(II) stability constant that is 3 to 9 orders of magnitude lower than any of the other macrocycles.

The significant variations in the log K values between the macrocyclic Cu(II) complexes does not explain the differences found in the biodistributions. Because of the lability of Cu(II) to ligand exchange, thermodynamic stability is generally not a pre-indicator of *in vivo* stability. Moi and co-workers (17) showed that Cu(II) complexes of bifunctional chelates based on ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA) rapidly dissociated in human serum and bound to albumin, even though the Cu(II) complexes had high thermodynamic stability constants ($\log K_{\text{Cu-EDTA}} = 18.7$; $\log K_{\text{Cu-DTPA}} = 21.4$). Cu(II) has been found to have much greater kinetic stability (and consequently greater serum stability) with macrocyclic chelates than with aliphatic ligands (5, 17). Kukis and co-workers (13) showed that there is differential biological stability between differ-

ent macrocyclic chelates, with the DOTA BFCs having greater serum stability than the TETA-based BFCs.

All the Cu(II) macrocyclic complexes evaluated here rapidly cleared the circulation, suggesting that they are all stable for the short time that they remain in the blood. A possible explanation for the higher uptake of Cu-cyclam, Cu-et-cyclam, and Cu-cyclen in the liver and kidneys is the instability of the complexes in these organs, leading to Cu(II) binding to proteins and retention of ^{64}Cu . Preliminary metabolism results show these compounds to be intact in the liver and kidneys (Jones-Wilson et al., unpublished results). Also, Rogers and co-workers (20) found that in rat liver there was some dissociation of Cu(II) from cyclam and 15aneN5-based BFC-MAb conjugates, but it occurred very slowly over 5 days. Radiocopper-labeled MAb fragments (MAb 1A3-F(ab')₂) with different macrocyclic BFCs have been shown to exhibit different levels of renal excretion. In the kidneys, antibody fragments conjugated to negatively charged BFC complexes (SCN-TETA, and BAT) were retained to a dramatically lesser extent in the kidney than antibody fragments bound to positively charged complexes of CPTA and PCBA. Metabolism experiments with ^{67}Cu -CPTA conjugated MAb 1A3-F(ab')₂ showed ^{67}Cu -CPTA- ϵ -lysine was the major metabolite produced in the kidney. These experiments showed that the nature of the bifunctional chelate has a profound effect on the biodistribution of large chelate-protein conjugates, as these large conjugates are metabolized in renal cells to low molecular weight ^{67}Cu -BFC-amino acid moieties. The results of Rogers et al. (20) suggest that the higher uptake of Cu-labeled cyclam, et-cyclam, and cyclen in the liver and kidneys is more likely owing to the positive charge rather than dissociation of Cu(II) from the complex.

The ^{64}Cu -labeled dodecane (12-member) and tetradecane (14-member) ring systems showed significantly different biodistributions. The addition of two carbons to generate the tetradecane backbone of cyclam increases the lipophilicity of the ^{64}Cu complex relative to cyclen (dodecane). The increased lipophilicity was reflected in the higher liver uptake and slower clearance of ^{64}Cu -cyclam at all times (Tables 4 and 5) relative to ^{64}Cu -cyclen. In fact, the percent injected dose of ^{64}Cu -cyclam in the liver increased with time. The addition of the ethylene bridge to generate et-cyclam also increased the lipophilicity of et-cyclam relative to cyclam. This was reflected in the increased liver uptake of ^{64}Cu -et-cyclam at all times.

Addition of four acetate groups to the dodecane and tetradecane backbones dramatically alters the biodistributions. The ^{64}Cu -DOTA, the tetra acetate derivative with the dodecane backbone, had substantial liver and kidney uptake. ^{64}Cu -TETA offers a sharp contrast to ^{64}Cu -DOTA. Initially, significant uptake was noted in the kidney, but the activity had completely cleared by 24 h. The liver activity was also clearing over time. The behavior of ^{64}Cu -TETA suggests that the radio-complex was cleared from the organ(s) and little metabolism occurred.

Within the two macrocycle classes examined, the biodistribution was dependent on the formal charge of the ^{64}Cu (II) complex. In particular, the differences in uptake and clearance of the positively and negatively charged radiopharmaceuticals from the clearance organs were most effected. Differences were more pronounced in the tetradecane macrocycle. ^{64}Cu -cyclam, with a net positive charge, cleared slowly from the liver, while ^{64}Cu -TETA, with a formal negative charge, was nearly completely cleared by 24 h. The clearance from the kidney showed similar behavior (Table 4). The ^{64}Cu (II) charge effect on the dodecane macrocycle system was not as dramatic as the tetradecane system. The net positively charged

complex, ^{64}Cu -cyclen, cleared the liver and kidney slowly compared to the net negatively charged complex ^{64}Cu -DOTA, but the differences were not as dramatic as with ^{64}Cu -TETA and ^{64}Cu -cyclam. The neutral complex, ^{64}Cu -DO2A, showed little retention in the liver and demonstrated efficient clearance through the kidney. The thermodynamic stability of ^{64}Cu -DO2A is lower than any of the other complexes; however, kinetically the two carboxyl groups may contribute to kinetic stability, as well as *in vivo* stability.

In conclusion, the biodistributions of six ^{64}Cu -labeled macrocycles were found to be dependent on both the macrocycle backbone and the formal charge of the complex. These data suggest there are potentially many factors that control the clearance of small molecules, with the charge playing one of the more important roles. Further studies to elucidate the role of charge relative to the macrocycle system are currently under investigation.

This work was supported by the NIH (R01 CA 42925 [M.J.W., A.E.M.] and F32 HL09271 [T.M.J.-W.]), DOE (DE-FG02-87ER60512 [M.J.W.]), and a grant from the Robert A. Welch Foundation (AT-584 [A.D.S.]). The authors would like to thank Elizabeth Sherman, Margaret Morris, and Lynne Jones for excellent technical assistance.

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