

In vivo evaluation of copper-64-labeled monooxo-tetraazamacrocyclic ligands

Xiankai Sun^{a,1}, Joonyoung Kim^a, Arthur E. Martell^{b,*}, Michael J. Welch^a, Carolyn J. Anderson^{a,*}

^aMallinckrodt Institute of Radiology, School of Medicine, Washington University, St. Louis, MO 63110, USA

^bDepartment of Chemistry, Texas A & M University, College Station, TX, 77842 USA

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Abstract

Copper-64 ($T_{1/2}=12.7$ h; β^+ : 0.653 MeV, 17.4%; β^- : 0.578 MeV, 39%) has applications in positron emission tomography (PET) imaging and radiotherapy, and is conveniently produced on a biomedical cyclotron. Tetraazamacrocyclic ligands are the most widely used bifunctional chelators (BFCs) for attaching copper radionuclides to antibodies and peptides due to their relatively high kinetic stability. In this paper, we evaluated three monooxo-tetraazamacrocyclic ligands with different ring sizes and oxo group positions. H1 [1,4,7,10-tetraazacyclotridecan-11-one], H2 [1,4,8,11-tetraazacyclotetradecan-5-one] and H3 [1,4,7,10-tetraazacyclotridecan-2-one] were radiolabeled with ⁶⁴Cu in high radiochemical yields under mild conditions. The three ⁶⁴Cu-labeled complexes are all +1 charged, as determined by their electrophoretic mobility. While they demonstrated >95% stability in rat serum out to 24 h, both biodistribution and microPET imaging studies revealed high uptake and long retention of the compounds in major clearance organs (e.g., blood, liver and kidney), which suggests that ⁶⁴Cu dissociated from the complexes in vivo. Of the three complexes, ⁶⁴Cu-2⁺, which has a cyclam backbone (1,4,8,11-tetraazacyclotetradecane), exhibited the lowest nontarget organ accumulation. The data from these studies may invalidate the candidacy of the monooxo-tetraazamacrocyclics as BFCs for copper radiopharmaceuticals. However, the data presented here suggest that neutral or negatively charged Cu(II) complexes of tetraazamacrocyclic ligands with a cyclam backbone (tetradecane) are optimal for copper radiopharmaceutical applications.

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1. Introduction

Copper-64 ($T_{1/2}=12.7$ h; β^+ : 0.653 MeV, 17.4%; β^- : 0.578 MeV, 39%) has shown its versatility in both positron emission tomography (PET) imaging [1–7] and radiotherapy [8–13], due to its decay characteristics and the ability for its large-scale production with high specific activity on a biomedical cyclotron [14]. In the development of copper radiopharmaceuticals, tetraazamacrocyclic ligands such as 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) have been traditionally preferred over acyclic chelators, such as diethylenetetraaminepentaacetic

acid (DTPA) and ethylenediaminetetraacetic acid (EDTA). Structural features of the macrocyclic Cu(II) complexes render them extra kinetic stability under biological conditions [1,15–17].

We demonstrated in our previous studies that for a series of ⁶⁴Cu-labeled azamacrocyclic complexes, the formal charge of the Cu-bifunctional chelator (BFC) complex influenced its biological behavior in normal rats [18], and in certain cases, the BFC had a significant effect on the clearance properties of the ⁶⁴Cu-BFC biomolecules [19,20]. While the positively charged complexes exhibited high accumulation in the kidney and liver out to 24 h postinjection, the neutral and negatively charged ones cleared rapidly and efficiently through kidneys [18].

In 1984, Kimura et al. [21] reported the synthesis of a monooxo-tetraazamacrocyclic (H2) and its coordination chemistry with Cu(II) ($\log K_{Cu-2}=13.00$, 0.2 M NaClO₄, 25°C). In 1992, Siegfried and Kaden [22] demonstrated that the dissociation rates of Cu(II) complexes of 1,4,8,11-

* Corresponding author. Tel.: +1 314 362 8427; fax: +1 314 362 9940.
E-mail address: andersoncj@wustl.edu (C.J. Anderson).

¹ Current address: University of Texas Southwestern Medical Center at Dallas E6.118, 5323 Harry Hines Boulevard, Dallas, Texas 75390.

* Deceased.

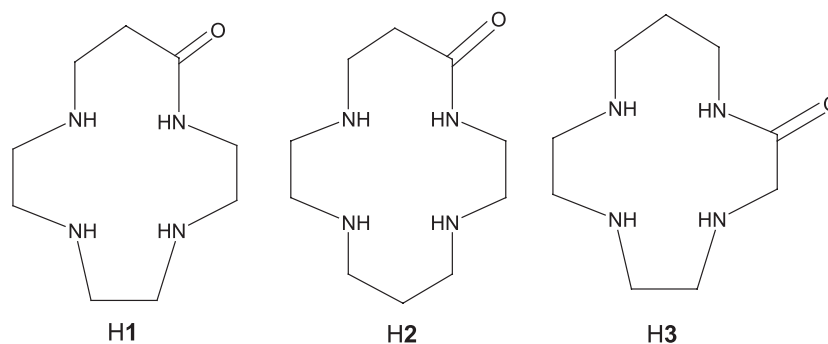


Fig. 1. The structures of H1 (1,4,7,10-tetraazacyclotridecan-11-one), H2 (1,4,8,11-tetraazacyclotetradecan-5-one) and H3 (1,4,7,10-tetraazacyclotridecan-2-one).

tetraazacyclotetradecan-5-one (H2) and two dioxo-tetraazamacrocyclics in an acidic solution determined by stopped-flow spectrophotometry were influenced by both the ring size and the number of oxo groups. Two years later, they also published the crystal structure of Cu(II)-2, which indicates that the protonation of the oxo group plays an important role in the acid dissociation [23]. In 2001, Martell et al. reported the synthesis of 1,4,7,10-tetraazacyclotridecan-11-one (H1) and 1,4,7,10-tetraazacyclotridecan-2-one (H3), and the thermodynamic stability constants of their Cu(II) complexes ($\log K_{\text{Cu-1}}=10.58$; $\log K_{\text{Cu-3}}=11.09$, 0.1 M KCl, 25°C) [24].

To better understand the relationship between the structure and clearance properties of radiolabeled complexes, we further evaluated ^{64}Cu -labeled complexes of H1, H2 and H3 (Fig. 1) in normal rats. We previously reported that dioxo-tetraazamacrocyclic ligands form neutral complexes with Cu(II) upon loss of the nitrogen-bound protons of two amide groups; it was therefore assumed that monooxo-tetraazamacrocyclics form +1 charged complexes with Cu(II) [25]. One of the goals of the studies in this paper was to determine the charge of the Cu(II)-monooxo-tetraazamacrocyclic complexes.

MicroPET imaging is currently emerging as a noninvasive method to evaluate PET radiopharmaceuticals in small animal models using smaller numbers of animals than required by biodistribution studies [5,26]. In this work, microPET was employed to evaluate the *in vivo* behavior of the ^{64}Cu -labeled complexes in parallel to the traditional biodistribution method.

2. Materials and methods

2.1. Reagents and instrumentation

H1 and H2 were synthesized by a modified published method [21,24]. H3 was prepared as previously described by a Richman–Atkins tosylamide procedure [24,27]. Copper-64 was prepared on the Washington University Medical School Cyclotron CS-15 cyclotron by the $^{64}\text{Ni}(p,n)^{64}\text{Cu}$ nuclear reaction at a specific activity of 50–200 mCi/ μg [14]. Water was distilled and then deionized (18 M Ω /cm 2) by passing through a Milli-Q water filtration system

(Millipore, Bedford, MA, USA). Ammonium acetate was purchased from Fluka Chemie (Buchs, Switzerland). Cyclen (1,4,7,10-tetraazacyclododecane) was purchased from Strem Chemicals (Newburyport, MA, USA), and DO2A [1,4,7,10-tetraazacyclododecane-1,7-di(acetic acid)] was prepared as previously described [28]. Rat serum was purchased from Sigma (St. Louis, MO, USA). C18 silica gel thin-layer chromatography (TLC) plates (Waters, KC18F, 60 Å, 200 μm) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Radio-TLC was performed using a Bioscan 200 imaging scanner (Bioscan, Washington, DC USA). Radioactivity was counted with a Beckman Gamma 8000 counter containing a NaI crystal (Beckman Instruments, Irvine, CA, USA).

Complex charge was determined by electrophoresis using a Helena Laboratories electrophoresis chamber (Beaumont, TX, USA) with Sephaphore III cellulose polyacetate strips (Gelman Sciences, Ann Arbor, MI, USA) presoaked in 0.1 M ammonium acetate buffer, pH 7.0 [18,25,29]. The strips were developed using a Bio-Rad model 1000/500 power supply (Richmond, CA, USA) at a constant current of 10 mA and a power of 2 W for 120 min. The ^{64}Cu -cyclen and DO2A complexes, known to have overall charges of 2+ and neutral, were used as standards [18]. The strips were analyzed using the Bioscan 200 imaging scanner to determine the migration of radioactivity and overall charge of the complexes.

2.2. Preparation of ^{64}Cu -labeled complexes

Ligand solutions (5 mM in 0.1 M ammonium acetate, pH 6.5) were prepared by dissolving the ligand solids in 0.1 M ammonium acetate buffer. To 100 μl of a ligand solution, ^{64}Cu chloride (1.0 μl in 0.1 M HCl, 1–2 mCi) was added. The formation of the three complexes was complete within 2 h at 75°C as determined by radio-TLC on silica plates eluting with 1:1 methanol/10% ammonium acetate. Freshly prepared ^{64}Cu -labeled cyclen and DO2A complexes were employed as electrophoresis standards [18].

2.3. Determination of partition coefficients

The partition coefficients ($\log P$) of the ^{64}Cu (II) complexes were determined by adding 4 μl of the ^{64}Cu -

labeled complex to a solution containing 500 μl of octanol and 500 μl of water ($n=10$). The resulting solutions were then shaken for 2 h at room temperature. From each of the 10 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 the counts in the water fraction. An average log P value was obtained from the 10 samples [18,25,29,30].

2.4. Serum stability

In vitro serum stability experiments were conducted by adding 10 μl of ^{64}Cu -labeled complexes to 500 μl of rat serum. The solutions were incubated at 37°C, and samples were analyzed by radio-TLC at 10, 30 and 60 min, and 2, 4 and 24 h postadministration to rat serum.

2.5. Biodistribution studies

All animal studies were performed in compliance with guidelines set by the Washington University Animal Studies Committee. Copper-64-labeled complexes in 0.1 M ammonium acetate were diluted with saline. Mature female Sprague–Dawley rats ($n=4$ per time point) weighing 180–200 g were anesthetized with isoflurane and injected with ca. 10–15 μCi of activity via the tail vein. The injected volume of activity per rat was 100–150 μl . The rats were anesthetized prior to sacrifice (by decapitation) at each time point. Organs of interest were removed, weighed and counted. Standards were prepared and counted along with the samples to calculate the percent injected dose per gram (%ID/g) and percent injected dose per organ (%ID/organ).

2.6. MicroPET evaluation

The microPET imaging studies were performed using the microPET-R4 rodent scanner (Concorde Microsystems, Knoxville, TN). The scanner provides a 10 \times 8-cm field of view, and the scanner is currently capable of an axial and transaxial resolution of 2 mm, with an absolute sensitivity of 900 counts per second per μCi [31]. Images were reconstructed using Fourier rebinning followed by two-dimensional filtered back projection [5,26]. Normal mature Sprague–Dawley rats weighing 180–200 g were anesthetized with 1–2% vaporized isoflurane and injected with ca. 500–1000 μCi of activity in 150 μl saline via the tail vein. At specific time points, the rats were reanesthetized and then

immobilized in a supine position on custom-built support beds with attached anesthetic gas nose cones for data collection. For time points up to 4 h pi, the imaging collection time was 10 min; at 24 h pi, the imaging collection time was 20 min. The regions of interest (ROIs) were quantified by viewing these areas in the selected tissues and averaging the activity concentration over the contained voxels. Region of interest analysis software consisted of two programs: Analyze AVW 3.0 (Biomedical Imaging Resource, Mayo Foundation, Rochester, MN) and a viewing application program developed in-house by R. Laforest (Washington University School of Medicine, St. Louis, MO) in International Data Language (Research Scientific, Boulder, CO). The correlations between the data of biodistribution and microPET imaging analysis were evaluated by Pearson product moment correlation coefficients calculated by Excel (Microsoft Office XP).

3. Results

3.1. Radiochemistry

Radiolabeling. The reaction conditions, TLC conditions and ^{64}Cu -labeling yield are presented in Table 1. At the no-carrier-added (NCA) level, H1, H2 and H3 were successfully labeled with ^{64}Cu after 1–2 h at 75°C in 0.1 M ammonium acetate (pH 6.5) in high radiochemical purity (>95%).

Copper-64-labeled **1**, **2** and **3** were analyzed by electrophoresis to determine the charge of the complexes (Table 1). The migration distances of the ^{64}Cu complexes were compared with the migration distances of ^{64}Cu -cyclen (+2 charged) and ^{64}Cu -DO2A (neutral). At pH 7.0 (0.1 M ammonium acetate), the ^{64}Cu -cyclen standard migrated with a distance of 29.4 mm in the direction of the cathode, while the ^{64}Cu -DO2A standard migrated 2.4 mm in the direction of the anode. The slight migration of the ^{64}Cu -DO2A standard toward the anode was due to electro-osmotic transport [32] and is consistent with its neutral charge. Under the same experimental conditions, ^{64}Cu -**1**, **-2** and **-3** migrated with distances of 21.0, 18.4 and 21.5 mm, respectively, in the direction of the cathode, suggesting a +1 charge for all three complexes in the ammonium acetate buffer, pH 7.0.

Table 1
Formation of ^{64}Cu (II)-labeled **1**, **2** and **3** under NCA conditions

Ligand	Reaction conditions	RCP (%)	R_f	Log P	Electrophoresis d (mm) ^a
H1	0.1 M NH_4OAc , pH 6.5, 75°C, 1–2 h	97.4	0.73	-1.37 ± 0.24	21.0
H2		98.8	0.68	-1.52 ± 0.24	18.4
H3		98.9	0.68	-2.35 ± 0.36	21.5
Cyclen	0.1 M NH_4OAc , pH 6.5, RT, 10 min	>98	0.12	n.d.	29.4
DO2A	0.1 M NH_4OAc , pH 6.5, RT, 1 h	100	0.17	n.d.	–2.4

In all reactions, 1 μl $^{64}\text{CuCl}_2$ was added to the ligand solution. The radio-TLC conditions were silica gel plates as the stationary phase eluted with 1:1 methanol/10% ammonium acetate. Under these conditions, free ^{64}Cu (in the forms of acetate and chloride) remained at the origin. RCP: radiochemical purity; n.d.: not determined.

^a The migration distance in the direction of the cathode (–).

The octanol–water partition coefficients or log P values of ^{64}Cu -1, -2 and -3 were determined to be -1.37 ± 0.24 , -1.52 ± 0.24 and -2.35 ± 0.36 , respectively, suggesting they were all hydrophilic complexes (Table 1). In vitro serum stability experiments showed by radio-TLC that ^{64}Cu -1, -2 and -3 complexes remained greater than 95% intact in rat serum out to 24 h postadministration. These data demonstrated in vitro stability of the ^{64}Cu complexes and suggested that they were worthy of in vivo investigation.

3.2. Biodistribution studies

The biodistribution results of ^{64}Cu -labeled 1, 2 and 3 are presented as ^{64}Cu activity measured in blood, liver and kidney (Table 2 and Fig. 2). All three ^{64}Cu -labeled complexes showed similar blood uptake at 30 min postinjection (pi) (^{64}Cu -1: 5.257 ± 1.447 %ID/blood; ^{64}Cu -2: 4.028 ± 0.430 %ID/blood; ^{64}Cu -3: 4.612 ± 0.250 %ID/blood), but their blood clearance properties were different. Copper-64-1 exhibited significant accumulation in the blood at 2 h pi (7.002 ± 0.932 %ID/blood) with no significant clearance out to 24 h pi (6.681 ± 0.923 %ID/blood). The blood level of ^{64}Cu -3 remained constant from 30 min to 2 h pi (4.336 ± 0.284 %ID/blood), but from 2 to 24 h pi, it increased (5.926 ± 1.273 %ID/blood). Of the three complexes, ^{64}Cu -2 cleared the blood much more rapidly and efficiently out to 2 h, but remained constant after that time point (0.919 ± 0.082 %ID/blood at 2 h pi, 1.051 ± 0.166 %ID/g at 24 h pi). The three ^{64}Cu -labeled complexes showed significantly different uptake in the liver at 30 min pi (^{64}Cu -1: 14.368 ± 4.998 %ID/liver; ^{64}Cu -2: 5.901 ± 0.932 %ID/liver; ^{64}Cu -3: 12.436 ± 1.303 %ID/liver), and they cleared slowly out to 24 h pi. Of the three complexes, ^{64}Cu -2 exhibited the lowest uptake and most efficient clearance. The complexes of ^{64}Cu -1 and ^{64}Cu -3 behaved similarly with respect to kidney uptake and excretion. ^{64}Cu -2 exhibited the lowest kidney uptake at 30 min pi (1.761 ± 0.118 %ID/kidney) and cleared over time (0.963 ± 0.184 %ID/kidney at 2 h pi, 0.391 ± 0.121 %ID/kidney at 24 h pi). No significant lung, spleen, heart or brain uptake was observed for the three complexes.

3.3. MicroPET imaging studies

The microPET images of the three ^{64}Cu -labeled complexes (2 h pi) are shown in Figs. 3 and 4. The imaging intensity of liver and kidneys corresponds well with the

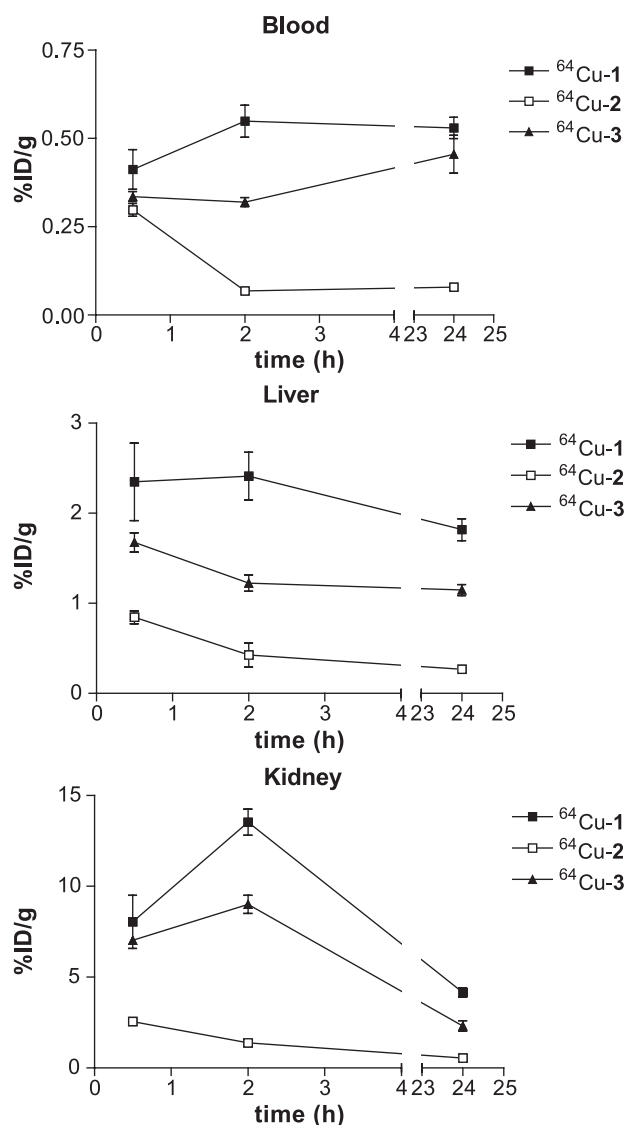


Fig. 2. Biodistribution data for the three ^{64}Cu -labeled complexes represented as radioactivity measurement in blood, liver and kidney. Data are present in %ID/g vs. time ($n=4$ for each time point).

biodistribution results (Table 2 and Fig. 2). Of the three compounds, ^{64}Cu -2 showed the lowest radioactivity concentration in both organs. Due to the low uptake, other organs are not clearly visualized in the images.

The time activity curves of the three ^{64}Cu -labeled complexes in the liver and kidney shown in Fig. 5 were obtained from the ROI analysis of static microPET images.

Table 2

The biodistribution of ^{64}Cu -labeled 1, 2 and 3 complexes in normal Sprague–Dawley rats

	30 min			2 h			24 h		
	Blood	Liver	Kidney	Blood	Liver	Kidney	Blood	Liver	Kidney
^{64}Cu -1	5.257 ± 1.447	14.368 ± 4.998	5.771 ± 2.157	7.002 ± 0.932	14.521 ± 2.585	9.457 ± 1.289	6.681 ± 0.923	11.877 ± 1.106	2.891 ± 0.283
^{64}Cu -2	4.028 ± 0.430	5.901 ± 0.932	1.761 ± 0.118	0.919 ± 0.082	2.900 ± 0.147	0.963 ± 0.184	1.051 ± 0.166	1.850 ± 0.189	0.391 ± 0.121
^{64}Cu -3	4.612 ± 0.250	12.436 ± 1.303	5.611 ± 0.321	4.336 ± 0.284	8.646 ± 1.005	7.075 ± 0.829	5.926 ± 1.273	8.606 ± 0.309	1.735 ± 0.457

Data are presented as measured radioactivity in blood, liver and kidney (%ID/organ \pm S.D., $n=4$).

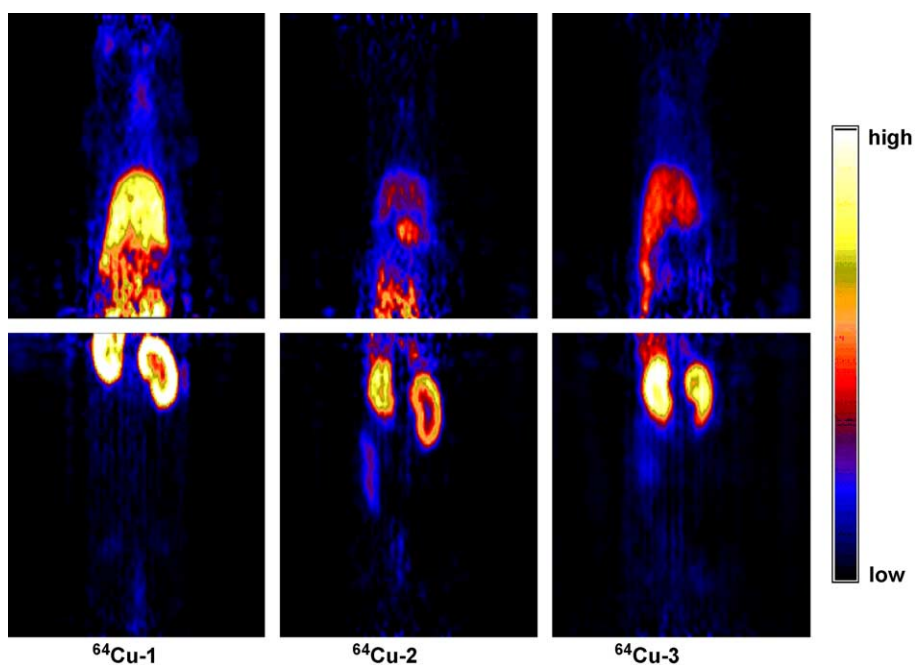


Fig. 3. Coronal microPET images of the three ^{64}Cu -labeled complexes at 2 h pi in normal mature Sprague–Dawley rats anesthetized with 1–2% vaporized isoflurane and injected with ca. 500–1000 μCi of activity in 150 μl saline via the tail vein. The image collection time was 10 min.

The concentrations of ^{64}Cu -1, ^{64}Cu -2 and ^{64}Cu -3 in rat liver at 15 min pi were 17.64 ± 1.56 $\mu\text{Ci}/\text{ml}$, 4.82 ± 0.22 $\mu\text{Ci}/\text{ml}$ and 11.07 ± 0.97 $\mu\text{Ci}/\text{ml}$, respectively, and decreased over time to 3.81 ± 0.50 $\mu\text{Ci}/\text{ml}$, 0.39 ± 0.02 $\mu\text{Ci}/\text{ml}$ and 1.97 ± 0.11 $\mu\text{Ci}/\text{ml}$ at 24 h pi, respectively. Low liver uptake and efficient clearance was observed again for ^{64}Cu -2. In the kidneys, the accumulation of ^{64}Cu -1 and ^{64}Cu -3 increased out to 2 h pi (39.75 ± 1.57 and 28.71 ± 3.59 $\mu\text{Ci}/\text{ml}$, respectively, at 15 min pi; 49.41 ± 1.59 and 28.48 ± 3.72 $\mu\text{Ci}/\text{ml}$, respectively at 2 h pi), then their concentrations

decreased to a very low level at 24 h pi (7.44 ± 0.24 and 2.83 ± 0.07 $\mu\text{Ci}/\text{ml}$, respectively). As in the liver, ^{64}Cu -2 exhibited low uptake in kidney and efficient clearance (12.58 ± 2.22 $\mu\text{Ci}/\text{ml}$ at 15 min pi; 5.41 ± 0.51 $\mu\text{Ci}/\text{ml}$ at 2 h pi; 0.82 ± 0.07 $\mu\text{Ci}/\text{ml}$ at 24 h pi). Because of the poor microPET image contrast, ROI analysis was not conducted in other organs. As shown in Figs. 3 and 4, the microPET images correlate with biodistribution results (Table 2 and Fig. 2). This is further confirmed by the comparable trends of the time activity curves (Figs. 2 and 5) obtained by

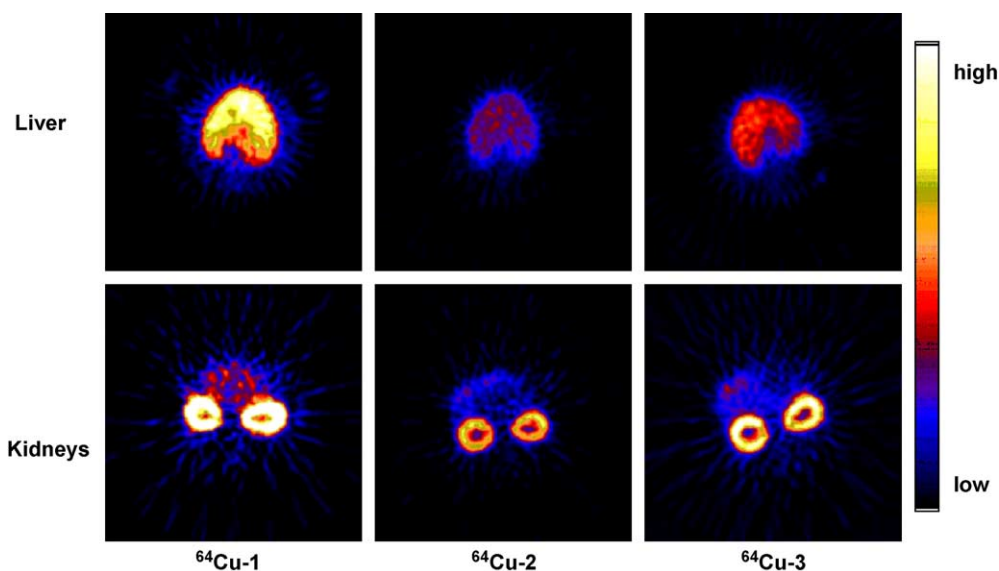


Fig. 4. Transaxial microPET images of the three ^{64}Cu -labeled complexes at 2 h pi in normal mature Sprague–Dawley rats anesthetized with 1–2% vaporized isoflurane and injected with ca. 500–1000 μCi of activity in 150 μl saline via the tail vein. The image collection time was 10 min.

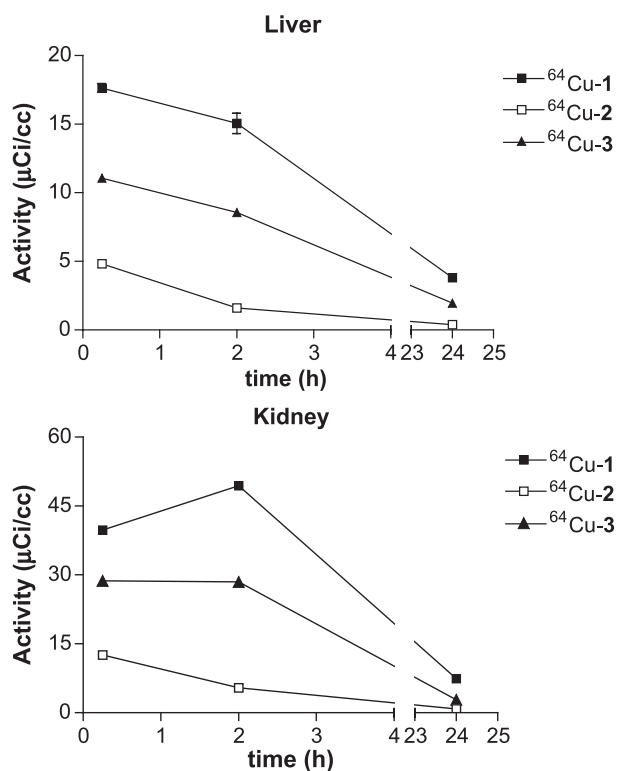


Fig. 5. Quantitative analysis results of static microPET images of the three ^{64}Cu -labeled complexes in the liver and kidney. Data are represented as time–activity curves.

quantitative analysis of microPET images and the biodistribution results.

4. Discussion

In this study, we evaluated three monooxo-tetraazamacrocyclic ligands differing in the ring size and the oxo group position in the tetraazamacrocyclic backbone. The purpose of this work was to determine the effects of ring size and charge of ^{64}Cu -labeled complexes on the *in vivo* and *in vitro* stability and biodistribution of the ^{64}Cu complexes. One of the goals was to determine if this class of tetraazamacrocyclic chelator could serve as BFCs for labeling copper radionuclides to biological molecules.

Both TETA (14-membered ring) and DOTA (12-membered ring) form 2– charged complexes with Cu(II). Currently, they are the most commonly used chelators for copper radionuclides. In previous studies, we found that neutral and negatively charged compounds exhibited lower liver uptake and rapid kidney clearance, whereas the positively charged compounds showed relatively higher retention in liver and kidneys in normal Sprague–Dawley rats [18,25,30]. While neutral and negatively charged compounds have been extensively evaluated, the three positively charged complexes previously studied exhibited a 2+ charge [18]. Monooxo-tetraazamacrocyclic ligands (H1, H2 and H3) are assumed to form 1+ complexes with Cu(II) upon loss of the nitrogen-bound proton of the

amide group [25]. Data presented here confirm this assumption.

Of the three monooxo-tetraazamacrocyclic ligands, H2 had the highest thermodynamic stability constant with Cu(II) ($\log K_{\text{Cu-1}}=10.58$ [24], $\log K_{\text{Cu-2}}=13.00$ [21] and $\log K_{\text{Cu-3}}=11.09$ [24]). The conditional stability constants for dioxo-macrocyclic complexes of Cu(II) of the 13-membered ring ligands showed intermediate thermodynamic stability (pM values) between the 14- and 12-membered azamacrocyclic ligands, with the highest pM value for Cu(II)-1,4,8,11-tetraazacyclotetradecan-3,9-dione being 16 [33]. If compared with TETA and DOTA regarding the thermodynamic stability of their Cu(II) complexes ($\log K_{\text{Cu-TETA}}=21.9$, $\log K_{\text{Cu-DOTA}}=22.7$ [34]), it appears that the three monooxo macrocycles studied here, or the dioxo ligands, would not be useful as BFCs in copper radiopharmaceuticals. However, because of the labile nature of Cu(II) complexes, the thermodynamic stability is often not an accurate indicator of *in vivo* stability, which is more determined by kinetic stability of the complexes [17,29,30].

All of the three monooxo-tetraazamacrocyclic ligands were successfully labeled with ^{64}Cu under conditions shown in Table 1 in high radiochemical yields (>97%), although the thermodynamic stability constants of their Cu(II) complexes are more than 10 orders of magnitude lower than those of Cu(II)-cyclen and -cyclam complexes ($\log K_{\text{Cu-cyclen}}=24.8$ [35], $\log K_{\text{Cu-cyclam}}=28.09$ [27]). It appears that for these ^{64}Cu (II) complexes, there is no direct correlation between the radiolabeling efficiency and the thermodynamic stability. Interestingly, in previously published studies, the dioxo-analogues did not show comparable radiolabeling efficiency with ^{64}Cu under the same conditions as the monooxo ligands studied here [25]. As revealed by the crystal structure of Cu(II)-2, the Cu(II) sits in the center of an elongated octahedron in which the four nitrogen atoms of the macrocycle occupy the equatorial coordination sites and the oxygen atoms of solvent molecules occupy the axial positions [23]. The significant difference of radiolabeling efficiency between the dioxo and monooxo ligands may be explained by the fact that the introduction of two oxo groups to the tetraazamacrocyclic backbone renders a more constrained structure requiring extra energy to make the four nitrogen atoms coplanar to coordinate Cu(II). In the monooxo-ligands, this structural constraint is partially removed by the replacement of an oxo group with a methylene carbon.

The negative $\log P$ values (Table 1) reflect the hydrophilic nature of the three complexes. While $^{64}\text{Cu-1}$ and -2 exhibited similar hydrophilicity, $^{64}\text{Cu-3}$ is significantly more hydrophilic. This is probably due to the difference in the position of the oxo group, but further investigation is needed. The electrophoresis results of the three ^{64}Cu -labeled complexes (Table 1) indicated that they were all positively charged in ammonium acetate buffers of pH 7.0. Compared to the migration distances of $^{64}\text{Cu-cyclen}^{2+}$ (29.4 mm) and neutral $^{64}\text{Cu-DO2A}$ (–2.4 mm) to

the direction of cathode, the migration distances of $^{64}\text{Cu-1}$ (21.0 mm), $^{64}\text{Cu-2}$ (18.4 mm) and $^{64}\text{Cu-3}$ (21.5 mm) suggest that the complexes are likely +1 charged. However, the net charge on a complex may not necessarily be quantified by its migration distance in the electrophoresis experiments, because different complex ions may have different ionic mobility that is related to their individual radius, ionic atmosphere and charge [36].

The *in vivo* comparison of ^{64}Cu -labeled complexes of H1, H2 and H3 in the blood, liver and kidneys is shown in Fig. 2 and Table 2. As shown in the coronal microPET images (Fig. 3), the organs showing the greatest uptake are liver and kidneys. We previously reported that ^{64}Cu will bind to proteins in the liver, such as superoxide dismutase and metallothionein [37,38]. Determining blood clearance is important, since highly stable ^{64}Cu complexes will clear rapidly from the blood, whereas dissociating ^{64}Cu will bind to proteins such as albumin and remain in the blood circulation [30,38]. The change of ring size and the oxo group position significantly altered the biodistribution of the ^{64}Cu complexes. Of the three complexes, $^{64}\text{Cu-2}$ demonstrated the most efficient clearance through blood, liver and kidney, $^{64}\text{Cu-1}$ exhibited the longest retention in the organs, while $^{64}\text{Cu-3}$ was intermediate in both uptake and clearance. One explanation is that H2 has the same backbone as cyclam and TETA. The rat biodistribution data of this type of +1 charged ^{64}Cu complexes are consistent with what was previously reported for the ^{64}Cu -labeled azamacrocyclic complexes, in that positively charged ^{64}Cu complexes had higher accumulations in the liver and kidneys of normal rats [18]. A comparison of rat biodistribution data of ^{64}Cu -labeled azamacrocyclic complexes with different formal charges is shown in Table 3. With respect to blood and liver clearance, the $^{64}\text{Cu-TETA}^{2-}$ showed the most optimal performance, followed by the neutral compound $^{64}\text{Cu-3,9-DOC}$ (where 3,9-DOC=1,4,8,11-tetraazacyclotetradecane-3,9-dione), $^{64}\text{Cu-2}^+$ and $^{64}\text{Cu-cyclam}^{2+}$. For the kidney clearance, the neutral complex was most optimal, followed by $^{64}\text{Cu-TETA}^{2-}$, $^{64}\text{Cu-2}^+$ and $^{64}\text{Cu-cyclam}^{2+}$. Although the kidney clearance of $^{64}\text{Cu-2}^+$ is similar to that of $^{64}\text{Cu-DOTA}^{2-}$ at 24 h postinjection, none of the three monooxo-tetraazamacrocyclic ligands demonstrated promising evidence that they were improved BFCs over TETA or DOTA, which are currently widely used for applications for copper

radiopharmaceuticals. We recently reported a new group of tetraazamacrocyclic ligands with nonadjacent nitrogen bridged by CH_2CH_2 that exhibited superior *in vivo* behavior to their TETA/DOTA analogues [30,38]. They were designed to be capable of adopting a “clam-shell” conformation having all four nitrogen lone pairs convergent upon a cleft (*in, in* at the bridgehead nitrogen) so that Cu(II) is well-protected from external attack [39]. Interestingly, of the cross-bridged tetraamines, the one (CB-TE2A: 4,11-bis-(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane) that showed the most optimal results has the same cyclam backbone (1,4,8,11-tetraazacyclotetradecane) as H2, which was shown in this study to be the most optimal in terms of blood, liver and kidney clearance. These data imply that the cyclam backbone is favorable for the BFC design of copper radiopharmaceuticals.

For studies involving expensive transgenic rodent models, *in vivo* evaluation by microPET imaging is greatly preferred to traditional dissection protocols, because the noninvasive technique allows longitudinal studies in the same living subjects. The microPET imaging studies were performed to see if microPET evaluation of ^{64}Cu complexes could yield comparable results to the conventional biodistribution approach. A Pearson test was performed to evaluate the correlation between the data obtained from conventional biodistribution studies and quantitative analysis of microPET images. For all three compounds, a significant correlation was found between the biodistribution and microPET data in both kidney and liver ($^{64}\text{Cu-1}$ in kidney: $r=0.92$; $^{64}\text{Cu-1}$ in liver: $r=0.96$; $^{64}\text{Cu-2}$ in kidney: $r=1.00$; $^{64}\text{Cu-2}$ in liver: $r=1.00$; $^{64}\text{Cu-3}$ in kidney: $r=0.96$; $^{64}\text{Cu-3}$ in liver: $r=0.80$). These data ascertain the powerful role of microPET in small animal imaging studies. However, microPET also shares a common drawback as other imaging modalities: blood concentrations are difficult to accurately determine. When blood clearance information is required, the conventional biodistribution method is preferred for acquiring the data.

5. Conclusions

In summary, three monooxo-tetraazamacrocyclic ligands were successfully radiolabeled with ^{64}Cu in high radiochemical purity under mild conditions. Their biological behavior was evaluated by conventional biodistribution studies and a microPET imaging approach. Both *in vivo* evaluations yielded comparable results with nearly perfect Pearson product moment correlation coefficients (r), which validated the role of microPET in the noninvasive *in vivo* evaluation of these radiopharmaceuticals. Based on electrophoretic mobility, the three ^{64}Cu -labeled complexes appear to be +1 charged. These complexes exhibited higher uptake and longer retention in blood, liver and kidney as compared to $^{64}\text{Cu-TETA}$. Of the three complexes, $^{64}\text{Cu-2}$ showed the most optimal biodistribution result in normal rats, with kidney clearance comparable to $^{64}\text{Cu-DOTA}$. However,

Table 3

Comparative rat biodistribution data for ^{64}Cu -labeled complexes of H2, 3,9-DOC (14), cyclam, TETA and DOTA [18] at 24 h postinjection

Ligand	Cu(II) complex formal charge	Blood	Liver	Kidney
H2	+1	1.051±0.166	1.850±0.189	0.391±0.121
3,9-DOC	0	0.23±0.05	0.70±0.10	0.097±0.03
Cyclam	+2	2.11±0.39	10.5±1.3	4.5±1.25
TETA	-2	0.21±0.05	0.49±0.11	0.21±0.03
DOTA	-2	0.58±0.19	1.05±0.16	0.54±0.08

Data are presented as %ID/organ±S.D. (3,9-DOC: 1,4,8,11-tetraazacyclotetradecane-3,9-dione).

ligand **2** is not as good as either TETA or DOTA that has been traditionally used as BFCs in the applications of copper radiopharmaceuticals. These data, together with the results of our previous studies, suggest that neutral or negatively charged Cu(II) complexes of tetraazamacrocyclic ligands with a cyclam backbone (tetradecane) are optimal for radiopharmaceutical applications.

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