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Promising Bifunctional Chelators for Copper 64-PET imaging: Practical ⁶⁴Cu Radiolabeling and High In Vitro and In Vivo Complex Stability

Ningjie Wu¹, Chi Soo Kang¹, Inseok Sin¹, Siyuan Ren¹, Dijie Liu³, Varyanna C. Ruthengael³, Michael R. Lewis^{2,3}, and Hyun-Soon Chong^{1,*}

¹Department of Chemistry, Illinois Institute of Technology, Chicago, IL, USA

²Research Service, Harry S. Truman Memorial Veterans' Hospital, Columbia, MO, USA

³Department of Veterinary Medicine and Surgery, University of Missouri, Columbia, MO, USA

Abstract

Positron emission tomography (PET) using copper-64 is a sensitive and non-invasive imaging technique for diagnosis and staging of cancer. A bifunctional chelator that can present rapid radiolabeling kinetics and high complex stability with ⁶⁴Cu is a critical component for targeted PET imaging. Bifunctional chelates 3p-*C*-NE3TA, 3p-*C*-NOTA, and 3p-*C*-DE4TA were evaluated for complexation kinetics and stability with ⁶⁴Cu *in vitro* and *in vivo*. Hexadentate 3p-*C*-NOTA and heptadentate 3p-*C*-NE3TA possess a smaller TACN-based macrocyclic backbone, while nonadentate 3p-*C*-DE4TA is constructed on a larger CYCLEN-based ring. The frequently explored chelates of ⁶⁴Cu, octadentate *C*-DOTA and hexadentate *C*-NOTA were also comparatively evaluated. Radiolabeling kinetics of bifunctional chelators with ⁶⁴Cu was assessed under mild conditions. All bifunctional chelates instantly bound to ⁶⁴Cu in excellent radiolabeling efficiency at room temperature. *C*-DOTA was less efficient in binding ⁶⁴Cu than all other chelates. All ⁶⁴Cu-radiolabeled bifunctional chelates remained stable in human serum without any loss of ⁶⁴Cu for 2 days. When challenged by an excess amount of EDTA, ⁶⁴Cu complexes of 3p-*C*-NE3TA and 3p-*C*-NOTA were shown to be more stable than ⁶⁴Cu-*C*-DOTA and ⁶⁴Cu-*C*-DE4TA. 3p-*C*-NE3TA and 3p-*C*-NOTA displayed comparable *in vitro* and *in vivo* complex stability to ⁶⁴Cu-*C*-NOTA. *In vivo* biodistribution result indicates that the ⁶⁴Cu-radiolabeled complexes of 3p-*C*-NOTA and 3p-*C*-NE3TA possess excellent *in vivo* complex stability, while ⁶⁴Cu-3p-*C*-DE4TA was dissociated as evidenced by high renal and liver retention in mice. The results of *in vitro* and *in vivo* studies suggest that the bifunctional chelates 3p-*C*-NOTA and 3p-*C*-NE3TA offer excellent chelation chemistry with ⁶⁴Cu for potential PET imaging applications.

Introduction

Positron emission tomography (PET) is a nuclear imaging technique that has been used for diagnostics and staging of various cancers [1–3]. Highly energetic photons released from positron-electron annihilation process make acquisition of PET images in high resolution

*To whom correspondence should be addressed. Chong@iit.edu, Phone: 312-567-3235, Fax: 312-567-3494. Mailing address: 3101 S. Dearborn St, LS 182, Department of Chemistry, Illinois Institute of Technology, Chicago, IL, 60616.

feasible using a molecular probe in a picomolar range [3]. Fluorine-18 ($t_{1/2} = 110$ min) has been extensively explored as a positron probe for PET imaging, and ^{18}F -fluoro-2-deoxy-glucose (^{18}F -FDG) is the most frequently used drug for PET imaging of cancer [1–3]. However, the short half-life of ^{18}F and synthetic challenges in preparation of the ^{18}F -radiolabeled molecular tracers remain limitations for broad applications of ^{18}F -PET imaging. Among metallic radionuclides for PET imaging, copper-64 ($t_{1/2} = 12.7$ h; $E_{\max}^{\beta^+} = 0.655$ MeV; $E_{\max}^{\beta^-} = 0.573$ MeV; $E_{\max}^{\gamma} = 0.511$ MeV) is of great interest due to its favorable physical properties [4–6]. ^{64}Cu can be readily produced in large quantities using a cyclotron and has longer half-life ($t_{1/2} = 12.7$ h) than fluorine-18, and its β^- emission can be utilized for radiotherapy. The coordination chemistry of copper has been well studied and applied for the design of adequate chelates for efficient radiolabeling with ^{64}Cu [7–10]. Macrocyclic chelates including DOTA (1,4,7,10-tetraazacyclododecane- N,N',N'',N''' -tetraacetic acid), TETA (1,4,8,11-tetraazacyclotetradecane- N,N',N'',N''' -tetraacetic acid), and CB-TE2A (1,4,8,11-tetraazabicyclo[6.6.2]hexadecane-4,11-diacetic acid) have been evaluated for ^{64}Cu -based PET imaging [7–9]. However, the currently available chelates of ^{64}Cu present limitations for practical use in PET applications including preparation of ^{64}Cu -radiolabeled chelates under harsh radiolabeling conditions and premature dissociation of ^{64}Cu from the complexes *in vivo* [7,9–13]. NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid) is a TACN (1,4,7-Triazacyclononane)-based hexadentate chelate that is known to form a stable complex with ^{64}Cu *in vitro* and *in vivo* [14]. We previously reported NE3TA (7-[2-(carboxymethyl)amino]ethyl]-1,4,7-triazacyclononane-1,4-diacetic acid)-based heptadentate chelates containing both the macrocyclic TACN backbone and a pendant arm. The acyclic binding moiety appended to the TACN backbone was proposed to enhance complexation kinetics of the chelates by rapidly binding ^{64}Cu , which can be subsequently solvated by the donors in the TACN backbone to form a stable complex. The bifunctional NE3TA analogues rapidly formed a stable complex with ^{64}Cu [15–17]. The bifunctional chelates *C*-NE3TA (7-[2-(carboxymethyl)amino]-3-(4-nitrophenyl)propyl]-1,4,7-triazacyclononane-1,4-diacetic acid) and *N*-NE3TA (7-[2-[(carboxymethyl)amino]-2-(4-nitrophenyl)methyl]-1,4,7-triazacyclononane-1,4-diacetic acid) were shown to have superior *in vivo* stability than ^{64}Cu -*C*-DOTA complex.[15] Both *C*-NE3TA and *N*-NE3TA conjugated to a tumor targeting biomolecule (transferrin) rapidly bound to ^{64}Cu at room temperature [18]. *N*-NE3TA-transferrin conjugate was slightly more efficient in binding ^{64}Cu than *C*-NE3TA-transferrin conjugate. ^{64}Cu -radiolabeled *N*-NE3TA-transferrin conjugate remained in human serum for 48 h and displayed rapid blood clearance and increasing tumor uptake in PC-3 tumor bearing mice over a 24 h period [18].

In this study, the potential bifunctional chelates 3p-*C*-NOTA, 3p-*C*-NE3TA, and 3p-*C*-DE4TA (Figure 1) along with the standard chelates *C*-NOTA and *C*-DOTA were comparatively evaluated for radiolabeling kinetics and *in vitro* and *in vivo* complex stability with ^{64}Cu . TACN-based chelates 3p-*C*-NOTA and 3p-*C*-NE3TA contains six and seven coordinating groups, respectively. 3p-*C*-DE4TA with nine donors is constructed on relatively larger macrocyclic cyclen (1,4,7,10-tetraazacyclododecane) backbone. All of the chelates studied contain a longer propyl chain which connects the chelating backbone to the functional group (para-nitro-phenyl) for conjugation to a biomolecule. In contrast, *C*-NOTA

and C-DOTA (2-(4-nitrobenzyl)-1,4,7,10-tetraazacyclotetradecane-1,4,7,10-tetraacetic acid) contains a methyl spacer. The relatively long alkyl spacer of the present chelates was proposed to reduce steric hindrance in metal complexation of the chelate conjugated to a protein. The bifunctional chelates were evaluated for radiolabeling kinetics with ^{64}Cu , and *in vitro* stability of ^{64}Cu -radiolabeled complexes was evaluated in human serum and excess EDTA solution. *In vivo* stability of ^{64}Cu -radiolabeled complexes was also assessed by conducting a biodistribution study in mice.

Experimental

Instruments and materials

Analytical HPLC was performed on an Agilent 1200 equipped with a diode array detector ($\lambda = 254$ and 280 nm), thermostat set at 35°C , and a Zorbax Eclipse XDB-C18 column (4.6×150 mm, $80 \text{ \AA}$). Ultrapure ammonium acetate was purchased from Aldrich. Ultrapure HCl was purchased from Fisher Scientific. Radio-TLC was performed on a TLC scanner (Bioscan, B-FC-1000). ^{64}Cu in the chloride form (specific activity of $248 \text{ mCi}/\mu\text{g}$) was obtained from Washington University (Saint. Louis, MO).

Complexation formation kinetics of chelates with ^{64}Cu

Bifunctional chelates 3p-C-NOTA, 3p-C-NE3TA, 3p-C-DE4TA were prepared as previously reported [19]. C-DOTA were purchased from Macrocyclics (Dallas, TX). C-NOTA in the form of NH_2 was prepared by the method reported in the literature [20]. All HCl solutions were prepared from ultrapure HCl (Fisher Scientific). For metal-free radiolabeling, plasticware including pipette tips, tubes, and caps was soaked in 0.1 M HCl overnight and washed thoroughly with Milli-Q ($18 \text{ M}\Omega$) water, and air-dried overnight. Ultrapure ammonium acetate was used to prepare buffer solutions (0.25 M , $\text{pH } 5.5$). 0.25 M NH_4OAc buffer solution ($\text{pH } 5.5$) using 0.1 M and 1 M HCl solution was prepared and chelexed, shaken overnight at room temperature, and filtered through $0.22 \mu\text{m}$ filter (Corning) prior to use. The stock solution of the radioisotopes ($0.013 \mu\text{L}$, 9 mCi) was diluted to $125 \mu\text{L}$ by adding 0.05 M HCl solution ($112 \mu\text{L}$). TLC plates (6.6×1 cm or 6.6×2 cm, Silica gel 60 F_{254} , EMD Chemicals Inc., #5554-7) with the origin line drawn at 0.6 cm from the bottom were prepared. To a buffer solution (0.25 M NH_4OAc , $\text{pH } 5.5$) in a capped microcentrifuge tube (1.5 mL) was sequentially added a solution of the chelator in the buffer ($30 \mu\text{g}$) and ^{64}Cu in 0.05 M HCl ($90 \mu\text{Ci}$). The total volume of the resulting solution was $60 \mu\text{L}$. The reaction mixture was agitated on the thermomixer (Eppendorf, 5355) set at $1,000$ rpm at room temperature for 30 min. The labeling efficiency was determined by TLC eluted with 20 mM EDTA/ 0.15 M NH_4OAc as the mobile phase. A solution of radiolabeled complexes ($2.0 \mu\text{L}$) was withdrawn at the designated time points (1 min, 10 min, and 30 min), spotted on a TLC plate, and then eluted with the mobile phase. After completion of elution, the TLC plate was warmed and dried on the surface of a hot plate maintained at 35°C and scanned using TLC scanner (Bioscan, B-FC-1000). Bound and unbound copper-64 appeared $30\sim 40$ mm ($R_f = 0.54$) and $48\sim 55$ mm ($R_f = 0.94$).

***In vitro* serum stability of ^{64}Cu -radiolabeled complexes**

Human serum was purchased from Gemini Bioproducts (#100110). ^{64}Cu -radiolabeled complexes were prepared by reaction of the chelators (30 μg) with ^{64}Cu (90 μCi) in 0.25M NH_4OAc buffer (pH 5.5) for 1 h at room temperature or 90 $^\circ\text{C}$ (for *C*-DOTA). The total volume of the final solution was 10 μL . The labeling efficiency of the radiolabeled complexes were found to be ~100% as determined by TLC. The freshly prepared radiolabeled complexes were directly used for serum stability studies without further purification. ^{64}Cu -radiolabeled complex (~75 μCi , ~10 μL) was added to human serum (90 μL) in a microcentrifuge tube. ^{64}Cu -3p-*C*-DE4TA were prepared by reaction of the chelators (10 μg) with ^{64}Cu (30 μCi) in 0.25M NH_4OAc buffer (pH 5.5) for 1 h at room temperature. The total volume of the final solution was 20 μL . The labeling efficiency of the radiolabeled complexes were found to be ~100% as determined by TLC. ^{64}Cu -radiolabeled complex (~7 μCi , ~20 μL) was added to human serum (60 μL) in a microcentrifuge tube. The stability of ^{64}Cu -radiolabeled complexes in human serum was evaluated at 37 $^\circ\text{C}$ for 2 days using TLC and HPLC (solvent A: 0.1% TFA in H_2O , solvent B: 0.1% TFA in CH_3CN , 0–100% B/15 min, flow rate: 1 mL/min). ^{64}Cu -EDTA complex was eluted early (t_{R} = ~2.5 min), while ^{64}Cu -radiolabeled complexes of the chelators have retention time at 8~8.3 min.

EDTA Challenge

^{64}Cu -radiolabeled complexes were prepared by reaction of each chelator (10 μg) with ^{64}Cu (30 μCi) in 0.25M NH_4OAc buffer (pH 5.5) for 1 h at room temperature. The total volume of the resulting solution was 20 μL . The freshly prepared radiolabeled complexes were directly used for EDTA challenge experiment without further purification. A solution of the ^{64}Cu -radiolabeled complex containing each chelator (1 mM) in 0.25M NH_4OAc buffer was mixed with a solution of EDTA (100 mM, H_2O , pH 5.0) at a 100-fold molar excess. The resulting mixture was incubated for 24 h at 37 $^\circ\text{C}$. The stability of ^{64}Cu -radiolabeled complexes in the solution was evaluated using TLC (20 mM EDTA in 0.15M NH_4OAc). Bound and unbound radioisotope appeared 30~40 mm (R_{f} = 0.54) and 48~55 mm (R_{f} = 0.94) from the bottom of the TLC plate, respectively. Stability of the complexes was also evaluated at 25 h time point by HPLC (solvent A: 0.1% TFA in H_2O , solvent B: 0.1% TFA in CH_3CN , 0–100% B/15 min, flow rate: 1 mL/min). ^{64}Cu -EDTA complex was eluted early (t_{R} = ~2.5 min). ^{64}Cu -radiolabeled complex of 3p-*C*-NE3TA, 3p-*C*-NOTA, 3p-*C*-DE4TA, *C*-NOTA (in the NH_2 form), and *C*-DOTA have the respective retention time (t_{R} = 8.2, 8.0, 7.8, 4.2, 7.5 min).

Biodistribution

All animal experiments were conducted in accordance with the guidelines established by the Animal Care and Use Committee of the University of Missouri and the Harry S. Truman Memorial Veterans' Hospital Subcommittee for Animal Studies. Six to eight week old CF-1 mice were obtained from Charles River Laboratories and housed one week prior to the studies. An aliquot of ^{64}Cu -radiolabeled complex (60 μCi) was prepared as described above and intravenously injected via the tail vein in phosphate-buffered saline (100 μL). At 1 h, 4 h and 24 h post-injection, mice were sacrificed and blood, liver, kidney, muscle and bone were collected, weighed and counted in a gamma counter. The radioactivity from each tissue/organ was decay-corrected by a known aliquot of the injected dose, and the percent-injected

dose per gram of tissue (% ID/g) was calculated. Values are presented as mean $\pm$ SD for each group of 3 mice.

Results and Discussion

The new chelates 3p-*C*-NE3TA, 3p-*C*-NOTA, and 3p-*C*-DE4TA were evaluated for complexation with ^{64}Cu . Among the known bifunctional chelators of ^{64}Cu , *C*-DOTA and *C*-NOTA were selected for the present study on comparative evaluation for radiolabeling with ^{64}Cu at room temperature. *C*-NOTA rapidly forms a stable complex with ^{64}Cu and possesses superior radiolabeling kinetics and complex stability with ^{64}Cu than *C*-DOTA [14,15]. *C*-NOTA analogues have been extensively explored for development of ^{64}Cu -based radiopharmaceuticals [21–23]. Radiolabeling efficiency of the chelates in binding ^{64}Cu was measured at room temperature in duplicate using TLC and HPLC (Table 1). The new chelates 3p-*C*-NE3TA, 3p-*C*-NOTA, and 3p-*C*-DE4TA instantly bound to ^{64}Cu at room temperature and pH 5.5, and radiolabeling was almost complete at the beginning of the measurement (>99% radiolabeling efficiency, 3 $\mu\text{Ci}/\mu\text{g}$ specific activity, 1 min, RT). As expected, *C*-NOTA was extremely rapid in binding ^{64}Cu , while *C*-DOTA display slower radiolabeling kinetics when compared to all other chelates studied (Table 1). 3p-*C*-NETA, 3p-*C*-NOTA, and *C*-NOTA completely bound to Cu-64 at 30 min as evidenced by both TLC and HPLC analysis. However, radiolabeling of *C*-DOTA with ^{64}Cu was incomplete at 30 min time point. The noted difference in Cu-64 radiolabeling efficiency of *C*-DOTA measured by TLC (95.4%) and HPLC (88.6%) seems to be related to formation of less stable ^{64}Cu -*C*-DOTA complexes that can be dissociated during TLC and HPLC analysis.

The ^{64}Cu -radiolabeled chelates were further evaluated for complex stability in human serum and excess EDTA solution. Copper-64 complexes of 3p-*C*-NOTA, 3p-*C*-NE3TA, 3p-*C*-DE4TA, and *C*-NOTA were readily prepared by reaction of each chelator with ^{64}Cu for 1 h at room temperature (pH 5.5, 100% radiolabeling efficiency, TLC). However, complete radiolabeling of *C*-DOTA with ^{64}Cu required significantly longer time (5 h) at room temperature. Therefore, ^{64}Cu -*C*-DOTA for the *in vitro* complex stability studies was prepared from reaction of *C*-DOTA with ^{64}Cu for 1 h at 90 °C.

Human serum stability studies of ^{64}Cu -radiolabeled chelates were performed to determine if the complexes remained stable without loss of ^{64}Cu (37 °C, pH 7). Stability was assessed by measuring the transfer of ^{64}Cu from the complex to serum proteins over the course of 2 days using TLC and HPLC (Table 2 and Supporting Information). All ^{64}Cu -radiolabeled chelates remained stable in human serum without ^{64}Cu being released into the serum over 2 days. Stability of ^{64}Cu -radiolabeled complexes was further evaluated by challenging the complexes with EDTA at 100-fold molar excess (Table 3, Figure 2, and Supporting Information). ^{64}Cu -3p-*C*-NOTA, ^{64}Cu -3p-*C*-NE3TA, and *C*-NOTA remained intact in the EDTA solution for 24 h of incubation. The ^{64}Cu complex of 3p-*C*-DE4TA with nine donors was not able to hold ^{64}Cu stably, and a significant amount of ^{64}Cu was transchelated from the chelate to EDTA over a 24 h incubation time (~55%). Copper-64-*C*-DOTA released >5% of ^{64}Cu into the EDTA solution.

The *in vivo* stability of ^{64}Cu -radiolabeled chelates was evaluated by a biodistribution study using non-tumor bearing CF-1 mice (i.v. injection, $n = 3$). After injection of ^{64}Cu -radiolabeled chelates, radioactivity accumulation in harvested organs and blood was measured at 1 h, 4 h, and 24 h post-injection (Figure 3 and Supporting Information). ^{64}Cu -3p-*C*-NE3TA almost completely disappeared from the blood and all organs at 24 h post injection. The level of the radioactivity accumulated in the blood and in the organs was negligible ($<0.2\%$ ID/g) at 24 h post injection. The highest uptake of the tracer was observed in the kidney at 1 h post-injection (3.0% ID/g). The liver (2.5% ID/g at 1 h) cleared quickly, having less than 0.1% ID/g remaining at 24 h. No considerable accumulation of the tracer was detected in blood, muscle, and bone during the period of study. Copper-64-3p-*C*-NOTA displayed its highest uptake in the liver at 1 h (3.6% ID/g). However, the tracer rapidly disappeared by 24 h ($<0.6\%$ ID/g). The radioactivity retained in the kidney at 1 h (1.1% ID/g) cleared out by 24 h. Uptake of radioactivity in the blood and in the other organs was not significant at any time point. Significant radioactivity was released from ^{64}Cu -3p-*C*-DE4TA and accumulated in liver and kidney at 1 h (14.3% and 8.2%, respectively), and the radioactivity was retained in these organs at 24 h (11.1% and 5.7%, respectively). Blood, bone, and muscle had low levels of the tracer at 24 h (1.5%, 1.4%, and 0.5%, respectively). The biodistribution data indicate that 3p-*C*-NOTA and 3p-*C*-NE3TA cleared well *in vivo* but ^{64}Cu -3p-*C*-DE4TA showed substantially higher uptake and retention in the key organs. The instability of the DE4TA complex to EDTA challenge leads us to believe that the chelate dissociated *in vivo*.

Cu(II) with the prevalent oxidation state of +2 has a small ionic radius (73 pm) for coordination number 6 [24]. The borderline hard and soft Cu(II) is reported to adopt five or six coordination and display high affinity for nitrogen, oxygen, or sulfur donors such as aromatic nitrogens, thiol, amide, aminocarboxylate, and hydroxyl group [7,25,26].

The macrocyclic chelate NOTA with three nitrogens and three oxygen donors forms a stable hexacoordinate complex [25,27]. Hexa-coordination of Cu(II) with the four nitrogens in the cyclen backbone and two pendant carboxylate oxygens in DOTA produced Cu(II)-DOTA complex with high thermodynamic stability [28]. Copper-64-NOTA was reported to have more favorable complex stability and resistance to transchelation than ^{64}Cu -DOTA *in vivo* [15,21–23,29]. It is speculated that the higher kinetic stability of ^{64}Cu -NOTA relative with ^{64}Cu -DOTA stems from more favorable size-fit of Cu(II) to the smaller ring TACN.

The complexation radiolabeling and EDTA challenge data confirm that *C*-DOTA with a larger macrocyclic cavity and higher denticity forms less stable complex with Cu(II) than the TACN-based hexadentate *C*-NOTA. It should be noted that ^{64}Cu -*C*-DOTA complex for the present studies of *in vitro* stability was prepared from radiolabeling of *C*-DOTA with ^{64}Cu at 90 °C for 1 h, while all other chelates were well labeled with ^{64}Cu at RT. Under the harsh radiolabeling condition, *C*-DOTA was expected to form thermodynamically more stable complex as reaction of *C*-DOTA at room temperature could produce less stable intermediate ^{64}Cu -*C*-DOTA complexes [30]. However, ^{64}Cu -*C*-DOTA was found to be less stable than *C*-NOTA and other TACN-based chelates 3p-*C*-NOTA and 3p-*C*-NE3TA when challenged by excess EDTA.

In our previous studies, *C*-DOTA and *C*-NOTA were comparatively evaluated for *in vivo* complex stability with ^{64}Cu , and *C*-NOTA displayed superior *in vivo* stability than *C*-DOTA [15]. Copper-64-labeled *C*-DOTA was shown to have higher uptake in all tissues at all time points than ^{64}Cu -*C*-NOTA (1, 4, and 24 h post-injection, $n=4$, female SCID mice). Particularly, significant radioactivity was retained in the liver for ^{64}Cu -*C*-DOTA (78% at 24 h when compared to 1 h), while ^{64}Cu -*C*-NOTA displayed lower uptake in the liver at all time points (3% retention at 24 h when compared to 1 h) [15].

C-NOTA is built on a small macrocyclic cavity which is expected to be less dynamic in binding a metal than acyclic chelators [20,31]. 3p-*C*-NE3TA contains the same TACN backbone as NOTA [19]. In virtue of the acyclic bidentate arm, 3p-*C*-NE3TA was expected to form a complex with Cu(II) more rapidly. However, the predicted dynamic binding of 3p-*C*-NE3TA with Cu(II) using seven donors could form a less stable complex than *C*-NOTA. The *in vitro* and *in vivo* data indicate that 3p-*C*-NE3TA was extremely rapid in binding Cu(II) and formed a stable complex with Cu(II), and ^{64}Cu -3p-*C*-NE3TA was well tolerated in the EDTA challenge and produced excellent biodistribution profile in mice. ^{64}Cu -3p-*C*-NE3TA displayed rapid clearance from the liver and kidney and negligible retention in all organs at 24 time point. 3p-*C*-NE3TA displayed comparable chelation chemistry with ^{64}Cu to *C*-NOTA. As expected, the CYCLEN-based 3p-*C*-DE4TA with nine donors rapidly bound to Cu(II) but formed a complex with very low kinetic stability as evidenced by EDTA challenge and biodistribution data. Both *C*-DOTA and 3p-*C*-DE4TA are structured on the same macrocyclic backbone (CYCLEN). *C*-DOTA with 8 donors was found to form kinetically more inert complex with ^{64}Cu than 3p-*C*-DE4TA with 9 donors. ^{64}Cu -*C*-DOTA was more resistant to transchelation with EDTA than ^{64}Cu -3p-*C*-DE4TA. Higher retention of ^{64}Cu in the liver and kidney was observed with ^{64}Cu -3p-*C*-DE4TA (11.0% and 5.7% ID/g, respectively, Figure 3 and Supporting Information) when compared to ^{64}Cu -*C*-DOTA (3.8% and 2.4%, respectively) [15]. It appears that the enhanced kinetic stability of ^{64}Cu -*C*-DOTA relative to ^{64}Cu -3p-*C*-DE4TA is partly attributed to denticity of the chelates.

Conclusion

The bifunctional chelators 3p-*C*-NE3TA, 3p-*C*-NOTA, and 3p-*C*-DE4TA were evaluated for their potential use in ^{64}Cu -based PET imaging applications. All the chelators instantly and almost completely bound to ^{64}Cu at room temperature. The corresponding ^{64}Cu -radiolabeled chelates showed high *in vitro* serum stability, releasing no considerable radioactivity over 2 days. Copper-64 complexes of hexadentate (3p-*C*-NOTA) and heptadentate (3p-*C*-NE3TA) remained quite stable when challenged with EDTA. However, copper-64 complexes of *C*-DOTA (octadentate) and 3p-*C*-DE4TA (nonadentate) released a considerable amount of ^{64}Cu , which was gradually transchelated to EDTA. ^{64}Cu -3p-*C*-NOTA and ^{64}Cu -3p-*C*-NE3TA produced excellent biodistribution profiles and displayed low retention in all organs and blood over the entire period of the study. ^{64}Cu -radiolabeled 3p-*C*-DE4TA with nine donors was shown to undergo dissociation *in vivo* and have high retention in the liver. The *in vitro* and *in vivo* results suggest that 3p-*C*-NE3TA and 3p-*C*-NOTA possess superior chelation chemistry with ^{64}Cu relative to 3p-*C*-DEPTA and *C*-DOTA structured on the cyclen backbone and great promise for development of molecular PET imaging probes.

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Figure 1. Bifunctional Chelators of ⁶⁴Cu in preclinical evaluation for targeted PET imaging

Figure 2. HPLC chromatograms of ^{64}Cu -radiolabeled complexes in a solution of EDTA at 100-fold molar excess at 24 h post-incubation (eluent: 0.1% TFA in H_2O , 100% to 0%/0.1% TFA in ACN, 0% to 100%).

Figure 3. Biodistribution of ^{64}Cu -radiolabeled bifunctional chelates in non-tumor bearing CF-1 mice

Table 1

Evaluation of bifunctional ligands for radiolabeling efficiency (%) with ^{64}Cu (room temperature, 0.25M NH_4OAc , pH 5.5) using TLC and HPLC (parentheses).[†]

Time	Bound ^{64}Cu complex (%)				
	3p-C-NOTA	3p-C-NE3TA	3p-C-DE4TA	C-NOTA [*]	C-DOTA
1 min	99.7 ± 0.2	99.9 ± 0.0	99.6 ± 0.3	99.8 ± 0.1	92.4 ± 0.7
10 min	99.9 ± 0.1	99.8 ± 0.1	99.9 ± 0.1	99.8 ± 0.1	94.6 ± 0.4
30 min	100.0 ± 0.1 (99.1 ± 0.8)	100.0 ± 0.1 (100.0 ± 0.0)	100.0 ± 0.1	99.9 ± 0.1 (100.0 ± 0.1)	95.4 ± 0.1 (88.6 ± 0.4)

[†] Radiolabeling efficiency was measured in duplicate;

^{*} C-NOTA in the NH_2 form

Evaluation of ⁶⁴Cu-radiolabeled complexes for *in vitro* stability in human serum (37 °C, pH 7) using TLC and HPLC (parentheses). ⁺

Table 2

Bound ⁶⁴ Cu complex (%)					
Time	3p-C-NE3TA	3p-C-NOTA	3p-C-DE4TA	C-NOTA *	C-DOTA
0 h	99.9 ± 0.1 (99.8 ± 0.3)	99.9 ± 0.1 (99.8 ± 0.1)	99.9 ± 0.0 (99.9 ± 0.1)	99.9 ± 0.0 (100.0 ± 0.0)	99.8 ± 0.1 (100.0 ± 0.0)
24 h	99.8 ± 0.3 (99.9 ± 0.1)	100.0 ± 0.1 (99.9 ± 0.1)	100.0 ± 0.0 (99.5 ± 0.3)	99.8 ± 0.1 (99.9 ± 0.2)	99.8 ± 0.2 (99.7 ± 0.1)
48 h	100.0 ± 0.1 (99.9 ± 0.1)	99.9 ± 0.0 (99.9 ± 0.2)	99.9 ± 0.1 (100.0 ± 0.0)	99.9 ± 0.1 (99.9 ± 0.1)	100.0 ± 0.1 (99.6 ± 0.1)

⁺ bound ⁶⁴Cu complex (%) was measured in duplicate;

* C-NOTA in the NH₂ form

Table 3
EDTA challenge of chelators with ^{64}Cu (37 °C, 0.25M NH_4OAc , pH 5.5) using TLC and HPLC (parentheses).[†]

Bound ^{64}Cu complex (%)					
Time	3p-C-NE3TA	3p-C-NOTA	3p-C-DE4TA	C-NOTA *	C-DOTA
0 h	99.9 ± 0.1	100.0 ± 0.1	99.9 ± 0.2	99.9 ± 0.0	99.9 ± 0.0
1 h	99.9 ± 0.1	100.0 ± 0.1	98.0 ± 0.8	99.9 ± 0.1	99.6 ± 0.0
24 h	99.2 ± 0.1 (97.4 ± 0.4)	99.6 ± 0.4 (98.0 ± 0.2)	56.5 ± 1.1 (50.5 ± 0.9)	99.7 ± 0.0 (99.0 ± 0.2)	97.0 ± 0.5 (94.3 ± 0.4)

[†] bound ^{64}Cu complex (%) was measured in duplicate;

* C-NOTA in the NH_2 form