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Copper-64 radiolabeling and biological evaluation of bifunctional chelators for radiopharmaceutical development☆

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ABSTRACT

Introduction: The development of novel bifunctional chelates for attaching copper-64 to biomolecules has been an active area of research for several years. However, many of these ^{64}Cu-chelates have poor in vivo stability or harsh radiolabeling conditions.

Methods: In this study, two triazacyclononane analogs; *C*-NE3TA (4-carboxymethyl-7-[2-(carboxymethyl-amino)-3-(4-nitro-phenyl)-propyl]-[1,4,7]triazo-nan-1-yl-acetic acid) and *N*-NE3TA (4-carboxymethyl-7-[2-[carboxymethyl-(4-nitro-benzyl)-amino]-ethyl]-[1,4,7]triazonan-1-yl-acetic acid) were evaluated for their labeling efficiency with ^{64}Cu at room temperature and evaluated in vitro and in vivo. In vitro studies included complexation kinetics with Cu(II) using a spectrophotometric method and rat serum stability, while the in vivo biodistribution was evaluated using SCID mice.

Results: *C*-NE3TA and *N*-NE3TA were labeled at >95% efficiency up to ~3.4 Ci/μmol. Both *C*-NE3TA and *N*-NE3TA formed complexes with Cu(II) almost immediately, with the Cu(II) complexation by *C*-NE3TA being faster than the formation of Cu(II)-*N*-NE3TA. Both ^{64}Cu-*N*-NE3TA and ^{64}Cu-*C*-NE3TA were 96.1% and 90.5% intact after 48 h incubation in rat serum, respectively. This is compared to ^{64}Cu complexes of the control chelators, *p*-NH_2-Bn-DOTA and *p*-NH_2-Bn-NOTA, with 93.9% and 97.9% retention of ^{64}Cu in the complex, respectively. In vivo evaluation of ^{64}Cu-*N*-NE3TA and ^{64}Cu-*C*-NE3TA demonstrates good clearance from normal tissues except for the liver, where 59% and 51% of the radioactivity is retained at 24 h compared to 1 h for ^{64}Cu-*N*-NE3TA and ^{64}Cu-*C*-NE3TA, respectively. This compares to 78% and 3% retention for ^{64}Cu-*p*-NH_2-Bn-DOTA and ^{64}Cu-*p*-NH_2-Bn-NOTA.

Conclusions: These studies demonstrate that while *N*-NE3TA and *C*-NE3TA appear to be superior chelators for ^{64}Cu than *p*-NH_2-Bn-DOTA, they are not better than *p*-NH_2-Bn-NOTA. Nevertheless, it may still be interesting to evaluate these chelators after conjugation to biomolecules.

1. Introduction

^{64}Cu ($t_{1/2} = 12.7$ h, $I_{\beta+} = 17\%$, $I_{\beta-} = 39\%$, $I_{EC} = 43\%$, $E_{max} = 0.656$ MeV) has become an attractive radionuclide in the development of a wide range of radiopharmaceuticals for positron emission tomography (PET) and radiotherapy due its half-life, beta emission, and the ability to produce it on large scale with high specific activity [1,2]. The intermediate half-life of ^{64}Cu makes it a good candidate for PET evaluation of peptide and protein interactions with their cellular targets [3,4]. The increased use of ^{64}Cu in PET and targeted radiotherapy has highlighted the need for bifunctional chelators (BFCs) that form copper complexes with high stability. Chemically reactive functional groups in BFCs can be coupled to various bioactive molecules such as peptides, nanoparticles, and proteins.

Earliest reported BFCs were derived from well established non-macrocyclic chelating agents *e.g.* ethylenediamine tetraacetic acid (EDTA). However, Cu complexes of non-macrocyclic chelators such as EDTA and diethylenetriamine pentaacetic acid (DTPA) have been shown to be unstable in vivo although the complexes are thermodynamically stable [5,6]. In response to the kinetic instability of acyclic chelators, macrocyclic BFC derivatives of 1,4,7,10-tetra-azacylcododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (DOTA) and 1,4,8,11-tetraazacyclotetra decane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (TETA) derivatives have been developed for complexing with ^{64}Cu. These macrocyclic Cu(II)

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complexes are kinetically more inert than Cu(II)-EDTA and Cu(II)-DTPA. However, they still have issues with regards to in vivo stability and slow complex formation rates [7] which can result in high uptake of activity by nontarget tissues such as the liver [8,9]. Cross-bridged cyclams offer improved kinetic stability to prevent in vivo transchelation reactions compared to DOTA and TETA [7,10,11]. However, ligands of this type require harsh conditions such as high temperatures to incorporate ^{64}Cu. Thus, labeling antibodies and certain peptides is unfavorable with cross-bridged cyclams. Previous studies have shown the potential use of 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) as a BFC for labeling Cu-64 [12,13]. NOTA forms a 6-coordinate distorted prismatic complex with Cu(II) by coordinating the lone pairs of the three nitrogen atoms and the three carboxylate groups of the chelator [14]. The previous work by Kukis et al. with ^{67}Cu showed less than 1% loss of the radioactivity per day in human serum from a functionalized NOTA wherein one carboxylate group was functionalized for conjugation to a biomolecule [12]. Further, Prasanphanich et al. showed that a peptide conjugated to NOTA [15] displayed considerably lower accumulation of radioactivity in the liver and kidney compared to the ^{64}Cu-DOTA-labeled peptide [8,16,17]. Therefore, the evaluation of novel triazacyclononane-based BFCs that can form stable ^{64}Cu-complexes is warranted. Chong et al. have synthesized several triazacyclononane-derived BFCs and evaluated them in vitro and in vivo as the chelators of ^{153}Gd, ^{90}Y, or ^{177}Lu [18–27]. These studies showed high in vitro serum stability and good in vivo biodistribution of the radiolabeled complexes [26–28]. 2,2′-(7-((carboxymethyl)amino)ethyl)-1,4,7-triazonanane-1,4-diyl) diacetic acid (NE3TA) was previously radiolabeled with ^{64}Cu and the complex showed high stability in rat serum [24]. The bifunctional versions of NE3TA, (4-carboxymethyl-7-[2-(carboxymethyl-amino)-3-(4-nitro-phenyl)-propyl]-[1,4,7]triazonan-1-yl-acetic acid) (*C*-NE3TA) and (4-carboxymethyl-7-[2-[carboxymethyl-(4-nitro-benzyl)-amino]-ethyl]-[1,4,7]triazonan-1-yl-acetic acid) (*N*-NE3TA), were synthesized (Fig. 1) [23,27]. *C*-NE3TA and *N*-NE3TA differ in that the 4-nitro-phenyl group that will be used for conjugation to proteins is attached either to the nitrogen (*N*-NE3TA) or a carbon (*C*-NE3TA) of the amino ethyl side chain. This small change was made to determine how it would affect the complexation with copper. In this work, we demonstrate the efficient and high specific activity radiolabeling of *C*-NE3TA and *N*-NE3TA with ^{64}Cu at room temperature. The complex kinetics of *C*-NE3TA and *N*-NE3TA with Cu(II) were determined and the ^{64}Cu radiolabeled *C*-NE3TA and *N*-NE3TA were evaluated for in vitro stability and compared to the known Cu(II) chelators, 2-(4-aminobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (*p*-NH_2-Bn-DOTA) and S-2-(4-aminobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (*p*-NH_2-Bn-NOTA). Biodistribution studies were also performed in normal SCID mice to evaluate the tissue uptake and clearance of the ^{64}Cu-radiolabeled NE3TA analogues and compare results to that of ^{64}Cu-DOTA and ^{64}Cu-NOTA BFCs.

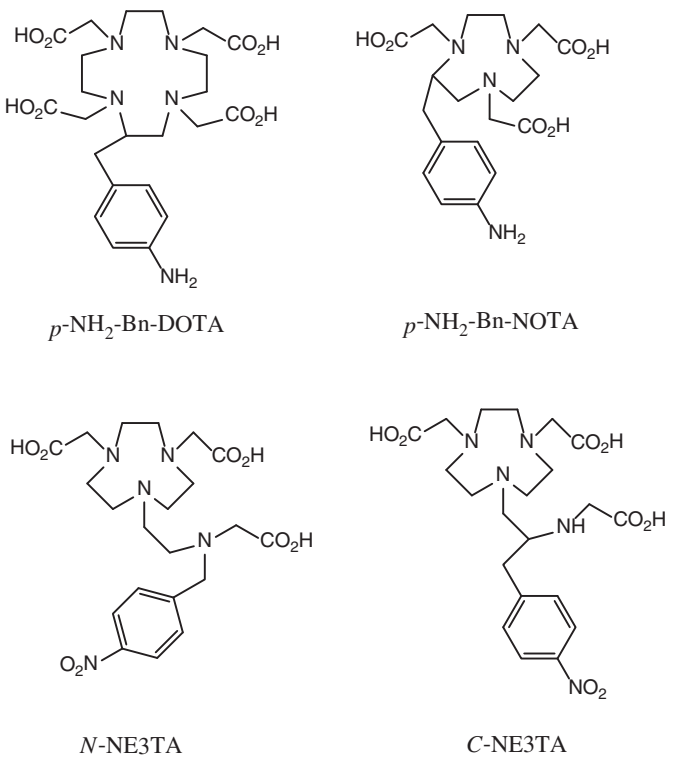

Fig. 1. Structure of bifunctional chelators (BFCs).

2. Material and methods

2.1. Reagents and Analyses

Reaction vials were acid washed and all solvents and reagents were used as purchased without further purification. Milli-Q water was chelexed before use. *p*-NH_2-Bn-NOTA and *p*-NH_2-Bn-DOTA were purchased from Macrocyclics, Inc. (Dallas, TX). Waters C18 silica gel TLC plates (KC18F, 60 Å) were purchased from Fisher Scientific (Pittsburgh, PA). Radio-TLCs were developed with 10% NH_4OAc: MeOH (3:7) and analyzed using a Bioscan 200 imaging scanner (Bioscan, Inc., Washington, DC). Copper-64 was prepared on CS-15 cyclotron at Washington University Medical School, St. Louis, MO according to the previous reported method [2]. Radioactivity was counted with a Beckman Gamma 8000 counter containing a NaI crystal (Beckman Instruments, Inc., Irvine, CA). $^{64}CuCl_2$ was diluted with a 10-fold excess of 0.1 M ammonium acetate (NH_4OAc), pH ~5.5 for radiolabeling. High-performance liquid chromatography (HPLC) purification (radiosynthetic and nonradiosynthetic) was performed by Phenomenex C-18 Luna 5 μm, 10×250 mm^2 analytical column with a mobile phase of water (0.1% TFA) (solvent A) and acetonitrile (0.1% TFA) (solvent B). A linear gradient of 100% solvent A to 100% solvent B over 40 min at 2 mL/min was used. HPLC purification was performed in Agilent Technologies 1200 series HPLC equipped with a diode array detector. Radioactivity signals were detected by Beckman 8000 automated well- type gamma counter (Fullerton, CA) and UV absorbencies were monitored at 220 nm and 254 nm. All absorbance measurements for complexation formation kinetics were obtained on an Agilent 8453 diode array spectrophotometer equipped with a 8-cell transport system (designed for 1 cm cells).

2.2. ^{64}Cu radiolabeling to determine the maximum specific activity

The maximum specific activities were determined experimentally via titrating $^{64}CuCl_2$ in 0.1 M NH_4OAc (pH ~5.5) with the new BFCs, *N*-NE3TA and *C*-NE3TA. Briefly, for each chelator, six reaction vials were prepared in 0.1 M NH_4OAc (pH ~5.5) via dilution to give final BFC masses in the range 0.002 to 0.1 μg. 3.7 MBq (100 μCi) of ^{64}Cu in 0.1 M. NH_4OAc (pH ~5.5) was added to each vial and adjusted the final volume to 100 μL (final pH ~5.5) and vortexed for 10–15 seconds. The reactions were incubated on a rotator at 37 °C for 1 h. After incubation, 1 μL aliquots were withdrawn from reaction vials and analyzed by TLC (C-18) with a mixture of 10% NH_4OAc:MeOH (3:7) as a mobile phase for labeling percentage. All reactions were done in triplicate. The data were plotted as % labeling vs. amount of BFC reacted and the amount of mass required to achieve 50% labeling was then determined. This mass was then multiplied by 2 to obtain the minimal mass for 100% labeling and the maximum specific activity. For HPLC analysis, the BFCs were reacted with excess cold $CuCl_2$ under the same reaction conditions as mentioned above.