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Novel Synthetic Ligands for Targeted PET Imaging and Radiotherapy of Copper

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Abstract

Novel ligands, NBEA, NBPA, NETA, NE3TA, and NE3TA-Bn were synthesized and evaluated as potential chelators of copper radioisotopes for use in targeted positron emission tomography (PET) imaging or radiation therapy. The new ligands were radiolabeled with ⁶⁴Cu, and *in vitro* stability of the radiolabeled complexes was assessed in rat serum. Serum stability results suggest that among the ligands tested, NETA, NE3TA, and NE3TA-Bn form stable complexes with ⁶⁴Cu.

Introduction

Among the available copper radioisotopes, ⁶⁴Cu ($t_{1/2} = 12.7$ h, $E_{\max}^{\beta+} = 656$ keV; $E_{\max}^{\beta-}$ is proven to be effective for use in positron emission tomography (PET) imaging and targeted radiation therapy applicable to many types of cancer.^{1–2} Bifunctional ligands that possess both binding moieties of Cu(II) and a functional group for conjugation to a targeting moiety are required for the modalities. Research efforts have been directed towards the development of optimal bifunctional ligands that can rapidly form stable complexes with the short-lived ⁶⁴Cu while being conjugated to a targeting moiety, either peptide or antibody, to provides an efficient way of generating stable and safe copper radioisotope-labeled drugs for cancer therapy and imaging.³

Recently, we have developed a structurally novel ligand, NETA, and its analogues.⁴ NETA features a parent macrocyclic ligand with a flexible acyclic multidentate pendant arm. DOTA (and DTPA are the frequently explored chelators of biologically important metals for biomedical applications. In general, the acyclic ligands such as DTPA possess rapid complex formation kinetics, but weak binding to the metal, whereas the less dynamic macrocyclic ligands such as DOTA display strong binding to the metal, but with relatively slower complex formation kinetics. The idea of designing NETA was to integrate the advantage of both the macrocyclic and acyclic frameworks, i.e., both thermodynamic stability and favorable formation kinetics. The bimodal octadentate NETA was found to effectively bind the metallic radionuclides for targeted radiotherapy such as ⁹⁰Y, ¹⁷⁷Lu, and ^{205/6}Bi.^{4–5} Given the capability of octadentate DOTA to tightly bind Cu(II) *in vitro* and *in vivo*,² we wanted to explore complexation of NETA and NETA analogues, NE3TA and NE3TA-Bn with Cu(II). NE3TA

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contains four amines and three carboxylates as potential donor groups. NE3TA-Bn is a heptadentate ligand with a benzyl group which can be further modified for conjugation to a targeting moiety. Hexadentate NBEA and NBPA possess three amines, two carboxylates, and a hydroxyl group as the donor groups. Our hypothesis for the design of NBEA and NBPA was that the size-fit between the macrocyclic cavity in NBEA and NBPA and the ionic radius of Cu(II)⁶ might provide enhanced radioisotope complex stability while producing a neutral Cu (II) complex that would have an advantage of less protein interaction and a potentially more favorable *in vivo* tissue distribution. NBPA possesses a longer propylene bridge between one of the amino groups and the hydroxyl group compared to the analogous ethylene bridged ligand, NBEA.

The structures of the novel ligands, NETA, NE3TA, NE3TA-Bn, NBEA, and NBPA are shown in Scheme 1. These ligands are not functionalized for conjugation to a targeting peptide or antibody. Preparation of the non-functionalized parent ligands is generally relatively straightforward as compared to the bifunctional analogues wherein introduction of an element of asymmetry frequently complicates synthetic efforts. The generally valid assumption is that the bifunctionalization of the ligands usually makes little difference in their metal binding ability. *In vitro* serum stability is a useful and qualitative screening procedure for identifying unstable metal complexes of non-functionalized chelates by assessment of their potential to maintain stable complexes without dissociation of the metal ion *in vitro*.^{4a} The radiolabeled non-functionalized chelates that demonstrate acceptable *in vitro* stability qualify for preparation of their respective bifunctional analogues for further evaluation post-conjugation to targeting molecules.

The new ligands NBEA and NBPA are efficiently synthesized starting from **1** and **2**, respectively. We previously reported the efficient and convenient synthetic route to the starting materials **1** and **2** from the readily available ditosylate^{4b} using a convenient cyclization and detosylation process.⁷ The base-promoted reaction of **1** or **2** with *tert*-butylbromoacetate afforded alkylated ligand **3** or **4** in good yield (~50%).⁸ Removal of *tert*-butyl groups in **3** and **4** was efficient with TFA (room temperature, 4 h) to provide NBEA and NBPA in high yields (> 90%) without any further purification.⁹ Synthesis of NE3TA and NETA, and NE3TA-Bn involves a coupling reaction between two precursor molecules, a pre-alkylated amino ethyl bromide and a bisubstituted 1,4,7-triazatricyclononane (tacn) derivative. The key coupling step was very efficiently achieved while minimizing formation of polyalkylated byproducts.⁵

The radiolabeling reactions of the new ligands with ⁶⁴Cu were performed at elevated temperatures to ensure complete complex formation.¹⁰ At the no-carrier-added (NCA) level, all ligands were successfully labeled with ⁶⁴Cu in quantitative radiochemical yields (100%) as determined by radio-TLC. The ligands displayed rapid labeling reaction kinetics with ⁶⁴Cu. The ⁶⁴Cu complexes of NE3TA, NETA, NE3TA-Bn, NBEA, and NBPA possess the respective R_f values of 0.63, 0.61, 0.53, 0.62 and 0.59 (Table 1. R_f2). The ⁶⁴Cu-labeled complexes were freshly prepared for their serum stability evaluation, which was assessed by measuring the transfer of radionuclide from the complex to serum proteins over 24 h.¹¹ The results determined by radio-TLC are shown in Table 1. The stability of the complexes is comparable to that of ⁶⁴Cu-DOTA, which was used as a positive control. DOTA is known to bind Cu(II) using the four nitrogens in the macrocyclic ring and two pendant carboxylate oxygens as demonstrated by a X-ray crystallography.²

Among the ligands tested, ⁶⁴Cu-labeled complexes of NETA, NE3TA, NE3TA-Bn were stable in rat serum for 24 h with no measurable loss of radioactivity. However, the ⁶⁴Cu complexes of NBPA and NBEA appear to be less stable in rat serum. Significant amounts of ⁶⁴Cu dissociated from the NBPA and NBEA complexes (⁶⁴Cu-NBPA: 29%; ⁶⁴Cu-NBEA: 54%. See R_f1 values in Table 1) in 24 h as determined by radio-TLC. It appears that the serum stability

of the Cu(II) complexes has some dependency on the length of the carbon chain between the pendant donor groups and the macrocyclic ring. It is interesting to note that NBPA possessing the hydroxyl group connected to the amino group via a longer propyl chain that can form six-membered chelate ring with the metal is more effective in holding ^{64}Cu compared to NBEA. The serum stability data suggest that the bidentate aminocarboxylate groups in NETA, NE3TA, and NE3TA-Bn more effectively serve as the donor group, tightly holding ^{64}Cu in serum than the monodentate hydroxyl group in NBEA and NBPA, and that the introduction of a benzyl group into one of the tertiary amines in the side arm (NE3TA-Bn) does not impact the complex stability. That no measurable loss of radioactivity from ^{64}Cu -NE3TA-Bn was recorded out to 24 h (nearly 2 half lives of ^{64}Cu) demonstrates the potential of utilizing the NE3TA-Bn backbone as a basis for bifunctional chelators for targeted PET imaging or radiation therapy.

In summary, novel ligands, NBEA, NBPA, NETA, NE3TA, and NE3TA-Bn have been evaluated as potential chelators of ^{64}Cu for targeted PET imaging or radiation cancer therapy. NBEA, NBPA, NETA, NE3TA, and NE3TA-Bn possess flexible carboxylate coordination groups, while NBEA and NBPA contain a hydroxyl pendant arm and form neutral complexes with Cu(II). The new ligands were efficiently synthesized and radiolabeled with ^{64}Cu , and *in vitro* stability of these radiolabeled complexes were evaluated in rat serum. *In vitro* studies indicate that NETA, NE3TA, and NE3TA-Bn complexes with ^{64}Cu stayed intact in rat serum for 2 days, while NBEA and NBPA compounds were not so effective in binding ^{64}Cu . The results suggest that NETA, NE3TA, and NE3TA-Bn are a new group of chelating agents for copper radiopharmaceuticals that may find applications in PET imaging or targeted radiation therapy. Exploration of the bifunctional versions of these new ligands is underway.

List of Potential Reviewers

We would like to recommend any of the following experts in the field as potential reviewers:

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5. The fully detailed synthesis and characterization of NE3TA and NE3TA-Bn is described elsewhere.
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8. General synthetic procedure to compound 3 and 4: To a suspension of alcohol 1 or 2 (1mmol) and K_2CO_3 (2 mmol) in CH_3CN (5 mL) under argon was added dropwise *tert*-butyl bromoacetate (2 mmol), and the resulting mixture was stirred at room temperature for 1h, followed by heating at 40 °C for 8h. The reaction mixture was allowed to cool gradually to ambient temperature and was filtered, and the filtrate was concentrated *in vacuo*. The residue was purified via column chromatography on silica gel (220–400 mesh) eluting with 8% CH_3OH in CH_2Cl_2 . Compound 3 or 4 was obtained as a waxy white solid: Compound 3 (Yield: 65%) ^1H NMR (300 MHz, $CDCl_3$) δ 4.51 (t, J = 6.5 Hz, 2H), 3.77 (t, J = 6.32 Hz, 2H), 3.45 (br, 8H), 3.2 (m, 6H), 2.99 (s, 2H), 1.27 (s, 18H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 170.82, 81.72, 61.72, 56.25, 54.67, 52.09, 47.91, 28.14. HRMS (positive ion FAB) Calcd for $C_{22}H_{42}O_6$: $[M + H]^+$ m/z 402.2692. Found: $[M + H]^+$ m/z 402.2968. Compound 4 (Yield: 50%) ^1H NMR (300 MHz, $CDCl_3$) δ 1.42 (s, 18H), 2.10–2.25 (m, 2H), 2.60–2.85 (m, 4H), 2.90–3.20 (m, 4H), 3.30–3.55 (m, 10H), 4.15 (t, 2H); ^{13}C NMR (300 MHz, $CDCl_3$) δ 28.15, 48.23, 51.58, 52.29, 53.61, 56.92, 64.24, 81.76, 170.84. HRMS (positive ion FAB) Calcd for $C_{23}H_{44}O_6$: $[M + H]^+$ m/z 416.3129. Found: $[M + H]^+$ m/z 416.3124.
9. General procedure for deprotection of *tert*-Butyl groups in 3 and 4: To either compound 3 or 4 (2 mmol) was added TFA (3mL) at room temperature. The reaction mixture was stirred for 3h. TFA was removed under *vacuo* and the residue obtained was treated with diethyl ether (10 mL). The precipitated product was filtered, washed with diethyl ether (30 mL) and immediately dissolved in water (5mL). The aqueous layer was washed with CH_2Cl_2 (3×10 mL) and concentrated under *vacuo* to obtain the desired product 5 or 6: (Compound 5: Yield 86%) ^1H -NMR(300 MHz, D_2O) δ 4.51(t, J = 6.5 Hz, 2H), 3.87 (s, J = 6.3 Hz, 4H), 3.75 (br, 6H), 3.54 (m, 4H), 3.22 (s, 4H); ^{13}C NMR (300 MHz, D_2O) δ 172.70, 62.44, 56.50, 56.21, 51.34, 50.31, 49.25. HRMS (positive ion FAB) Calcd for $C_{14}H_{26}O_6$: $[M + H]^+$ m/z 290.1716. Found: $[M + H]^+$ m/z 290.1715. (Compound 6: Yield 92%) ^1H NMR (300 MHz, $CDCl_3$) δ 2.05–2.20 (m, 2H), 3.13–3.25 (m, 4H), 3.26–3.44 (m, 6H), 3.45–3.60 (m, 4H), 3.70–3.85 (m, 4H), 4.05 (t, 2H); ^{13}C NMR (300 MHz, $CDCl_3$) δ 23.80, 49.22, 50.59, 50.98, 54.88, 56.89, 65.66, 173.13. HRMS (positive ion FAB) Calcd for $C_{15}H_{28}O_6$: $[M + H]^+$ m/z 304.1872. Found: $[M + H]^+$ m/z 304.1880.
10. General radiolabeling procedure: Nitric acid (10 – 20%) used for acid wash was prepared by diluting 70% nitric acid with mini-Q water (18 M ω -cm). The ammonium acetate buffer (0.4 M, pH 7.0) was pretreated with Chelex 100 resin (Bio-Rad, Hercules, CA) before use. Silica gel 60 F₂₅₄ plates were purchased from Merck & Co (Whitehouse Station, NJ). Copper-64 ($^{64}CuCl_2$ in 0.1N HCl) was purchased from Trace Life Sciences (Denton, TX). Radio-TLC analysis was performed on a Rita Star Radioisotope TLC Analyzer (Straubenhardt, Germany). Prior to labeling, all reaction vials were acid washed with 10–20% nitric acid overnight. To 100 μ L of each ligand solution (5 mM in 0.4 M NH_4OAc buffer, pH 7.0), 0.5 μ L of $^{64}CuCl_2$ (420–450 μ Ci) was added. The resulting solutions were incubated at 60 °C for 1 h in an Eppendorf thermomixer with 1,000 rpm. The radiochemical yields and purity were determined by radio-TLC (Raytest, VA) with silica gel plate as the static phase and 10% $NH_4OAc/MeOH$ = 1/1 (v/v) as the mobile phase. Under this TLC condition, free $^{64}Cu^{2+}$ or ^{64}Cu -associated proteins stay at the origin.
11. *In vitro* serum stability: To 100 μ L of rat serum, 10 μ L of each ^{64}Cu -labeled complex was added (n = 3 per complex). DOTA labeled with ^{64}Cu was used as a positive control. The resulting solutions were incubated at 37°C in a water-bath. At 1 h, 4 h, 24 h and 48 h after the complex addition to rat serum, the solutions were sampled and analyzed by radio-TLC.

Scheme 1.
Potential Ligands for use in PET imaging and Radiotherapy of Copper

Scheme 2.

Synthesis of new hexadentate ligands, NBEA and NBPA

Table 1*In Vitro* Serum Stability of ^{64}Cu -Labeled New Ligands in Rat Serum

Complex	Incubation Time	R _f 1	R _f 2	Purity (%)
^{64}Cu -NBEA	1 h	0.12	0.6	100 ± 0.0
	4 h		0.6	100 ± 0.0
	24 h		0.28	45.34 ± 9.8
^{64}Cu -NBPA	1 h	0.09	0.55	99.59 ± 0.72
	4 h		0.55	97.48 ± 4.4
	24 h		0.67	70.52 ± 6.54
^{64}Cu -NE3TA	1 h		0.60	100 ± 0.0
	4 h		0.60	100 ± 0.0
	24 h		0.58	100 ± 0.0
^{64}Cu -NE3T-Bn	1 h		0.39	100 ± 0.0
	4 h		0.41	100 ± 0.0
	24 h		0.37	100 ± 0.0
^{64}Cu -NETA	1 h		0.39	100 ± 0.0
	4 h		0.42	100 ± 0.0
	24 h		0.39	100 ± 0.0