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Transferrin conjugates of triazacyclononane-based bifunctional NE3TA chelates for PET imaging: Synthesis, Cu-64 radiolabeling, and in vitro and in vivo evaluation

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ABSTRACT

Three different polyaminocarboxylate-based bifunctional NE3TA (7-[2-[carboxymethyl)amino]ethyl]-1,4,7-triazacyclononane-1,4-diacetic acid) chelating agents were synthesized for potential use in copper 64-PET imaging applications. The bifunctional chelates were comparatively evaluated using transferrin (Tf) as a model targeting vector that binds to the transferrin receptor overexpressed in many different cancer cells. The transferrin conjugates of the NE3TA-based bifunctional chelates were evaluated for radiolabeling with ^{64}Cu. In vitro stability and cellular uptake of ^{64}Cu-radiolabeled conjugates were evaluated in human serum and prostate (PC-3) cancer cells, respectively. Among the three NE3TA–Tf conjugates tested, *N*-NE3TA–Tf was identified as the best conjugate for radiolabeling with ^{64}Cu. *N*-NE3TA–Tf rapidly bound to ^{64}Cu (>98% radiolabeling efficiency, 1 min, RT), and ^{64}Cu–*N*-NE3TA–Tf remained stable in human serum for 2 days and demonstrated high uptake in PC-3 cancer cells. ^{64}Cu–*N*-NE3TA–Tf was shown to have rapid blood clearance and increasing tumor uptake in PC-3 tumor bearing mice over a 24 h period. This bifunctional chelate presents highly efficient chelation chemistry with ^{64}Cu under mild condition that can be applied for radiolabeling of various tumor-specific biomolecules with ^{64}Cu for potential use in PET imaging applications.

1. Introduction

Positron emission tomography (PET) is a non-invasive and sensitive nuclear imaging technique that uses two highly energetic photons (511 keV) produced from interaction of positrons emitted from decay of radionuclides and electrons [1,2]. Among the clinically available positron-emitting radionuclides, ^{18}F ($t_{1/2}$ = 110 min) has been successfully applied for PET imaging, and ^{18}F-2-fluoro-2-deoxyglucose (^{18}F-FDG) is one of the most frequently used tracers for detection of tumors [3]. However, the short half-life of ^{18}F presents a limitation for its broad application in imaging of cancers, particularly for biomolecules with slow tumor accumulation such as antibodies. Copper-64 ($t_{1/2}$ = 12.7 h) with a relatively longer half-life and useful decay property ($I_{\beta+}$ = 17%, $I_{\beta-}$ = 39%, I_{EC} = 43%, $\beta^{+}{}_{max}$ = 0.656 MeV) is an attractive metallic radionuclide for PET imaging [4]. Research efforts have been made to develop clinically viable bifunctional chelating agents for radioimmuno-PET imaging applications of ^{64}Cu using tumor-specific antibodies or peptides [4–6].

Macrocyclic polyaminocarboxylate-based chelating agents 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 development of ^{64}Cu-based PET imaging agents [4–6]. However, DOTA, TETA, and CB-TE2A are not optimal chelating agents of ^{64}Cu for PET applications due to in vivo dissociation of ^{64}Cu-radiolabeled complex or harsh radiolabeling conditions [7–9].

Recently, we reported NE3TA (7-[2-[carboxymethyl)amino]ethyl]-1,4,7-triazacyclononane-1,4-diacetic acid) and bifunctional versions of NE3TA (*N*-NE3TA and *C*-NE3TA, Fig. 1) as potential chelating agents of ^{64}Cu [10–11]. The heptadentate NE3TA analogues rapidly bound to ^{64}Cu, and ^{64}Cu-radiolabeled complexes of the chelates remained inert in serum [11]. Copper-64-radiolabeled complexes of *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 lower uptake

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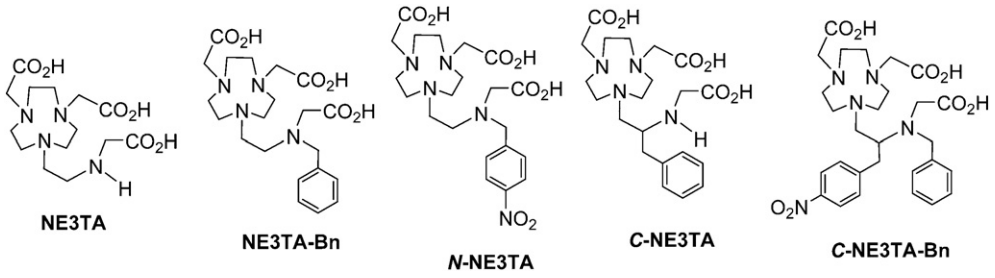

Fig. 1. Structure of NE3TA and bifunctional NE3TA ligands for targeted PET imaging application.

in normal organs and more rapid blood and muscle clearance and superior in vivo stability than the ^{64}Cu–*C*-DOTA complex [10].

With the encouraging data on chelation chemistry of bifunctional NE3TA analogues with ^{64}Cu, we proceeded with evaluation of the bifunctional NE3TA-based chelates for targeted PET imaging using transferrin as a model tumor targeting vector. Transferrin (Tf) binds to ferric iron (Fe^{3+}) in circulation and transports the iron into cells and tissues. When the transferrin binds to transferrin receptor (TfR), the Tf–TfR complex is transported into the cell via endocytosis. TfR is highly expressed on some cancers including cervical, colon, prostate, and breast cancers [12–13]. Due to its elevated expression level in cancer cells, TfR has been used as a therapeutic target for cancer treatment and diagnosis. Particularly, holotransferrin (Holo-Tf) in the form of its iron complex has high binding affinity to TfR and has been used as a targeting moiety of therapeutics for cancers with high expression of TfR [14].

We herein report preparation, characterization, and evaluation of transferrin conjugates of three bifunctional NE3TA analogues for targeted PET applications of ^{64}Cu. The NE3TA–transferrin (Tf) conjugates were prepared, characterized, and evaluated for radiolabeling kinetics with ^{64}Cu. The corresponding ^{64}Cu-radiolabeled NE3TA–Tf conjugates were further evaluated for complex stability in human serum and binding affinity to TfR using PC-3 cancer cells, as well as in vivo biodistribution in PC-3 xenograft-bearing mice.

2. Experimental

2.1. Instruments and analyses

^{1}H and ^{13}C NMR spectra were obtained using a Bruker 300 instrument, and chemical shifts are reported in ppm on the δ scale relative to TMS or solvent. Fast atom bombardment (FAB) or Electrospray ionization (ESI) high resolution mass spectra (HRMS) were obtained on JEOL double sector JMS-AX505HA mass spectrometer (University of Notre Dame, IN). Size-exclusion HPLC (SE-HPLC) chromatograms were obtained on Agilent 1200 equipped with a diode array detector and an in-line IN/US γ-Ram Model 2 radiodetector (Tampa, FL), fitted with BioSep-SEC S 3000 column (Phenomenex, Torrance, CA). All absorbance measurements for the protein concentration and ligand protein ratio were obtained on an Agilent 8453 diode array spectrophotometer equipped with an 8-cell transport system (designed for 1-cm cells). Arsenazo III (AAIII, 2,2-(1,8-dihydroxy-3,6-disulfonaphthylene-2,7-bisazo) bis-benzenearsonic acid), copper atomic absorption standard solution, and diferric (holo) transferrin were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. *C*-DOTA analogues were purchased from Macrocyclics (Dallas, TX).

2.2. 2-{[(4-Aminophenyl)methyl]({2-[4,7-bis(carboxymethyl)-1,4,7-triazonan-1-yl]ethyl})-amino}acetic acid (**2**)

4 M HCl/dioxane (6 mL) was added to **1** [15] (39 mg, 62.9 μmol) in a flask was cooled with an ice bath. After the addition was complete, the reaction mixture was gradually warmed to room temperature and allowed to stir overnight at room temperature. Ethyl ether (50 mL) was added to the reaction mixture with vigorous stirring, and the resulting slurry was kept in the freezer for 2 h. The precipitate was collected and washed with ethyl ether, and immediately dissolved in water, and the resulting solution was lyophilized to provide pure salt **2** (34 mg, 85%) as a light yellow solid. ^{1}H NMR (D_2O, 300 MHz) δ 2.97 (m, 4H), 3.21 (m, 6H), 3.33 (s, 4H), 3.48 (t, J = 5.4 Hz, 2H), 3.93 (s, 4H), 3.99 (s, 2H), 4.46 (s, 2H), 7.40 (d, J = 6.4 Hz, 2H), 7.59 (d, J = 6.4 Hz, 2H). ^{13}C NMR (D_2O, 75 MHz) δ 48.4 (t), 48.7 (t), 49.8 (t), 49.9 (t), 51.1 (t), 51.3 (t), 53.9 (t), 56.1 (t), 58.5 (t), 124.1 (d), 129.5 (s), 131.8 (s), 133.2 (d), 168.7 (s), 171.2 (s); HRMS (Positive ion ESI) Calcd for $C_{14}H_{26}N_4O_6$ [M − $BnNH_2$ + H]$^+$ m/z 347.3874, Found: [M − $BnNH_2$ + H]$^+$ m/z 347.1958.

2.3. 2-({2-[4,7-Bis(carboxymethyl)-1,4,7-triazonan-1-yl]ethyl}[(4-isothiocyanatophenyl)-methyl]amino)acetic acid (**3**)

To a solution of **2** (20.1 mg, 31.7 μmol) in water (0.3 mL) was added $CSCl_2$ in $CHCl_3$ (39.4 μL). The resulting mixture was stirred at room temperature for 4 h. The aqueous layer was transferred to a round bottom flask, and the $CHCl_3$ layer was washed with water (1 mL × 2). The combined aqueous layers were lyophilized to provide compound **3** (*N*-NE3TA–NCS) as a yellow solid (20 mg, 99%). ^{1}H NMR (D_2O, 300 MHz) δ 2.97 (m, 4H), 3.02–3.36 (m, 12H), 3.44 (t, J = 5.4 Hz, 2H), 3.93 (s, 4H), 3.98 (s, 2H), 4.36 (s, 2H), 7.29 (d, J = 6.1 Hz, 2H), 7.47 (d, J = 6.1 Hz, 2H). HRMS (Positive ion ESI) Calcd for $C_{22}H_{32}N_5O_6S$ [M + H]$^+$ m/z 494.2073, Found: [M + H] + m/z 494.2098.

2.4. 2-{[1-(4-Aminophenyl)-3-[4,7-bis(carboxymethyl)-1,4,7-triazonan-1-yl]propan-2-yl](benzyl)amino}acetic acid (**5**)

To a solution of **4** [16] (14.5 mg, 20.2 μmol) in ethanol (8.0 mL) was added dry 10% Pd/C (3.9 mg) under Argon gas. The reaction mixture was subjected to hydrogenolysis (20 psi) for 14 h at room temperature. The resulting mixture was filtered via Celite® bed and washed thoroughly with ethanol. The filtrate was concentrated *in vacuo* to provide compound **5** as a light yellow solid (14 mg, 96%). ^{1}H NMR (D_2O, 300 MHz) δ 2.35–2.66 (m, 1H), 2.69–3.28 (m, 18H), 3.50 (s, 2H), 3.56–3.97 (m, 8H), 4.21 (s, 1H), 7.18–7.49 (m, 9H). ^{13}C NMR (D_2O, 75 MHz, DEPT-135) δ 45.0 (t), 48.5 (t), 50.1 (t), 50.9 (t), 51.9 (t), 55.9 (t), 57.6 (t), 61.0 (d), 62.3 (t), 123.5 (d), 123.7 (d), 129.4 (d), 130.0 (d), 130.1 (d), 130.6 (d), 130.8 (d). HRMS (Negative ion ESI) Calcd for $C_{28}H_{38}N_5O_6$ [M − H]$^+$ m/z 540.6312, Found: [M − H]$^+$ m/z 540.2823.

2.5. 2-[benzyl({1-[4,7-bis(carboxymethyl)-1,4,7-triazonan-1-yl]-3-(4-isothiocyanatophenyl)propan-2-yl})amino]acetic acid (**6**)

To a solution of **5** (10 mg, 13.8 μmol) in water (100 μL) was added 1 M $CSCl_2$ in $CHCl_3$ (16.6 μL). The resulting mixture was stirred at room temperature for 4 h. The aqueous layer was transferred to a round bottom flask, and the $CHCl_3$ layer was washed with water (2 × 1 mL). The combined aqueous layers were lyophilized to provide compound **6** as a pale yellow solid (9.2 mg, 91%). ^{1}H NMR (D_2O, 300 MHz) δ 2.35–2.66 (m, 1H), 2.69–3.28 (m, 18H), 3.50 (s, 2H),