Research Article

Synthesis and Evaluation of Amino Acid-Based Radiotracer 99mTc-N4-AMT for Breast Cancer Imaging

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Purpose. This study was to develop an efficient synthesis of 99mTc-O-[3-(1,4,8,11-tetraazabicyclohexadecane)-propyl]-α-methyl tyrosine (99mTc-N4-AMT) and evaluate its potential in cancer imaging. Methods. N4-AMT was synthesized by reacting N4-oxalate and 3-bromopropyl AMT (N-BOC, ethyl ester). In vitro cellular uptake kinetics of 99mTc-N4-AMT was assessed in rat mammary tumor cells. Tissue distribution of the radiotracer was determined in normal rats at 0.5–4 h, while planar imaging was performed in mammary tumor-bearing rats at 30–120 min. Results. The total synthesis yield of N4-AMT was 14%. Cellular uptake of 99mTc-N4-AMT was significantly higher than that of 99mTc-N4. Planar imaging revealed that 99mTc-N4-AMT rendered greater tumor/muscle ratios than 99mTc-N4. Conclusions. N4-AMT could be synthesized with a considerably high yield. Our in vitro and in vivo data suggest that 99mTc-N4-AMT, a novel amino acid-based radiotracer, efficiently enters breast cancer cells, effectively distinguishes mammary tumors from normal tissues, and thus holds the promise for breast cancer imaging.

1. Introduction

18F-fluorodeoxy-glucose (FDG), an 18F-labeled glucose analog, is the most common radiotracer for positron emission tomography (PET) in cancer diagnosis [1]. However, FDG-PET has several limitations in practice, for example, FDG cannot distinguish tumor tissues from inflammatory or normal brain tissues. Therefore, 18F-labeled amino acid-based radiotracers have been reported as an alternative, which is based on the fact that tumor cells take up and consume more amino acids to maintain their sustained fast growth. Among those radiotracers, 18F-labeled alpha-methyl tyrosine (AMT) has shown high tumor uptake and great ability to differentiate tumor tissue from inflammatory sites in brain tumors and squamous cell carcinoma [2]. 18F-AMT enters the tumor cells via L-type amino acid transporters (LAT), which is the only system that can transport large neutral amino acids with aromatic rings [3]. LAT, especially its subtype LAT1, was reported to be highly expressed in many cancer cell lines and positively correlates with tumor growth [4, 5]. So far, 18F-AMT is the most suitable amino acid transporter-targeting radiotracer for tumor imaging, regardless of low synthesis yield and requirement of an on-site cyclotron to produce 18F.

Although PET has emerged as an advanced imaging tool for cancer diagnosis, only limited facilities around the world can afford complete armamentarium of PET and cyclotron for the local production of short-lived positron-emitting radionuclides such as 11C and 18F. Therefore, mature technologies, that is, single photon emission computed tomography (SPECT) or its combination with computed tomography (CT), still play important and irreplaceable roles in nuclear imaging area [6]. The most common radionuclide for SPECT is technetium-99m (99mTc, $t_{1/2} = 6.02$ h), which is produced by in-house generator and does not require the cyclotron [7]. Due to their similar chemistry, the diagnostic radioisotope 99mTc and the therapeutic radioisotope rhenium-188 (188Re) could be labeled to the same ligand, which leads to the diagnostic/therapeutic matched pair. Unlike most of the cyclotron-produced radionuclides that utilize the covalent chemistry for labeling, 99mTc requires a “chelator” to conjugate the radionuclide with the target ligand. The nitrogen,
oxygen, and sulfur mixtures, have been shown to be stable chelators for 99mTc such as N4 (e.g., DOTA, cyclam-14), N2S2 (e.g., MAG-3), N2S2 (e.g., ECD), N3S3, S3 (e.g., sulfur colloid), diethylentriamine pentaacetic acid (DTPA), O2S2 (e.g., DMSA), and hydrazinonicotinamide (HYNIC) [8–13].

Here, we report the synthesis of precursor O-[3-(1,4,8,11-tetraazacyclotetradecan)-propyl]-α-methyl tyrosine (N4-AMT) and its radiolabeling with 99mTc. In vitro cellular uptake kinetics and planar scintigraphic imaging of 99mTc-N4-AMT were also evaluated.

2. Materials and Methods

All chemicals of analytical grade and solvents of HPLC grade for compound synthesis were purchased from Sigma-Aldrich (St. Louis, MO). 1H-, 13C-NMR spectra were performed on Bruker 300 MHz spectrometer in CDCl3, CD2OD, and D2O. Tetramethylsilane was used as an external standard. Chemical shifts were reported in δ (ppm) and J values in hertz. Sodium pertechnetate (Na99mTcO4) was obtained from Mallinckrodt (Houston, TX).

2.1. Synthesis of Precursor N4-AMT

2.1.1. α-Methyl Tyrosine Ethylester 2. The synthetic strategies for precursor N4-AMT are demonstrated in Figure 1. Thionyl chloride (10 mL; 137.42 mmol) was added to a solution of α-methyltyrosine 1 (10.00 g; 51.22 mmol) in anhydrous ethanol (60 mL) at 0°C, and then heated at 78°C for 4 h while stirring. After cooling, the reaction mixture was reduced to 20 mL, and then 10 mL of triethylamine was added into it. The mixture was poured into 100 mL of water and extracted with chloroform. The combined organic layers were dried over MgSO4 and evaporated. The crude product was recrystallized in acetone: ethanol to yield white crystals of N1,N4-dioxylyl-1,4,8,11-tetraazabicyclotetradecane (N1,N4-cyclooxamide) (0.56 mL; 6.18 mmol) was added and continued heating over night. The ethanol was removed under reduced pressure and replaced with 40 mL of ethyl acetate, washed with water, and dried over MgSO4. After removal of the solvent, it was purified by silica gel column chromatography (hexane: ethylacetate 2:1), giving 0.83 g (2.17 mmol, 71% yield) of the product as clear yellow oil. 1H-NMR (CDCl3) δ = 7.01 (d, 2H, J = 6.3 Hz), 6.81 (d, 2H, J = 6.6 Hz), 4.20 (2d, 2H, J = 5.1 Hz, J = 5.1 Hz), 4.12 (2t, 2H, J = 15.0 Hz), 3.86 (t, 2H, J = 10.2 Hz), 3.16 (dd, J = 13.5 Hz, J = 12.9 Hz), 2.06 (m, 2H), 1.54 (s, 3H), 1.47 (s, 9H), 1.31 (t, 3H, J = 12.3 Hz) ppm.

2.1.2. N-t-Butoxycarbonyl-α-Methyl Tyrosine Ethylester 3. Compound 2 (2.09 g; 9.36 mmol) was dissolved in 40 mL of anhydrous DMF under nitrogen and treated with triethylamine (40.32 mmol, 78.75%). 1H-NMR (CDCl3) δ = 7.02 (d, 2H, J = 8.4 Hz), 6.70 (d, 2H, J = 8.4 Hz), 4.22 (dd, 2H, J = 7.2 Hz, J = 7.8 Hz), 3.14 (dd, 2H, J = 13.5 Hz, J = 13.5), 1.42 (s, 3H), 1.33 (t, 3H, J = 16.2 Hz) ppm. 13C-NMR δ = 175.47, 156.36, 130.66, 126.39, 114.87, 61.05, 58.65, 45.29, 24.08, 13.07 ppm, MS: m/z = 224.23 [M]+.

2.1.3. N-t-Butoxycarbonyl-O-[3-Hydroxypropyl]-α-Methyl Tyrosine Ethylester 4. Sodium metal (0.09 g; 14.02 mmol) was dissolved in 30 mL of anhydrous ethanol with stirring under nitrogen. Compound 3 (1.00 g; 3.09 mmol) was dissolved in 50 mL of anhydrous ethanol and treated with sodium ethoxide solution and refluxed for 2.5 h at 70°C. 3-Bromopropan-1-ol (0.56 mL; 6.18 mmol) was added and continued heating over night. The ethanol was removed under reduced pressure and replaced with 40 mL of ethyl acetate, washed with water, and dried over MgSO4. After removal of the solvent, it was purified by silica gel column chromatography (hexane: ethylacetate 2:1) giving 0.83 g (2.17 mmol, 71% yield) of the product as clear yellow oil. 1H-NMR (CDCl3) δ = 7.01 (d, 2H, J = 6.3 Hz), 6.81 (d, 2H, J = 6.6 Hz), 4.20 (2d, 2H, J = 5.1 Hz, J = 5.1 Hz), 4.12 (2t, 2H, J = 15.0 Hz), 3.86 (t, 2H, J = 10.2 Hz), 3.16 (dd, J = 13.5 Hz, J = 12.9 Hz), 2.06 (m, 2H), 1.54 (s, 3H), 1.47 (s, 9H), 1.31 (t, 3H, J = 12.3 Hz) ppm. 13C-NMR δ = 174.01, 171.91, 157.73, 154.35, 131.07, 128.65, 114.14, 79.39, 65.65, 61.54, 60.40, 60.24, 40.79, 32.01, 28.39, 23.58, 14.15 ppm. MS: m/z = 381.033 [M]+.

2.1.4. N-t-Butoxycarbonyl-O-[3-Br-Propyl]-α-Methyl Tyrosine Ethylester 5. A solution of 4 (5.44 g; 16.82 mmol) and 1,3-dibromopropane (136.74 g; 677.30 mmol) in 100 mL acetonitrile was purged with nitrogen for 15 min. Potassium carbonate (226.22 g; 163.69 mmol) was added to the reaction mixture, the mixture was then refluxed for 12 h at 75°C. After the removal of solvents and excessive reagents under reduced pressure, the residue was dissolved in chloroform, washed with water, and dried in anhydrous magnesium sulfate. A yellow liquid was purified by silica gel flash chromatography (hexane: ethylacetate, 2:1) furnishing 3 (3.80 g; 90.15 mmol, 64.69% yield). 1H-NMR (CDCl3) δ = 7.02 (d, 2H, J = 9.00 Hz), 6.82 (2d, 2H, J = 9.00 Hz), 4.23 (2d, 2H, J = 9.00 Hz), 4.09 (t, 2H, J = 6.00 Hz), 3.61 (t, 2H, J = 12.00 Hz), 3.32, 3.16 (d, 2H, J = 15.00, 12.00 Hz), 2.34 (dd, J = 24.00 Hz), 1.54 (s, 3H), 1.47 (s, 9H), 1.32 (t, 3H, J = 15.00 Hz) ppm. 13C-NMR δ = 173.96, 157.63, 154.32, 131.09, 128.78, 114.18, 77.48, 65.24, 61.51, 60.35, 40.80, 30.00, 28.40, 23.60, 14.19 ppm. MS: m/z = 446.3 [M]+.

2.1.5. N3, N4-Dioxyl-1,4,8,11-Tetraazabicyclotetradecane (N3,N4-Cyclooxamide) 6. 1,4,8,11-tetraazacyclotetradecane (cyclam) (15.00 g; 74.88 mmol) was dissolved in 150 mL of anhydrous ethanol, and diethyl oxalate (10.94 g; 74.88 mmol) was added. The reaction mixture was refluxed 18 h at 75°C. The solvent was rotary evaporated, and the crude product was recrystallized in acetone: ethanol to yield white crystals of N3,N4-dioxyl-1,4,8,11-1,5,8,12-tetraazabicyclotetradecane (N3,N4-cyclooxamide) (0.56 mL; 6.18 mmol) was added and continued heating over night. The ethanol was removed under reduced pressure and replaced with 40 mL of ethyl acetate, washed with water, and dried over MgSO4. After removal of the solvent, it was purified by silica gel column chromatography (hexane: ethylacetate 2:1) giving 0.83 g (2.17 mmol, 71% yield).
1.24 (m, 2H)) ppm. 13C-NMR δ = 158.55, 49.92, 49.38, 47.73, 44.13, 25.42 ppm. MS: m/z = 255.33 [M]+.

2.1.6. N-t-Butoxycarbonyl-O-[3-(N4,N4-Dioxyl-1,4,8,11-Tetraazabicyclotetradecane)-Propyl]-α-Methyl Tyrosine Ethylester 7. Compound 6 (1.00 g; 3.93 mmol) was dissolved in 20 mL of anhydrous DMF and treated with a solution of N-t-Butoxycarbonyl-O-[3-Br-propyl]-α-methyl tyrosine ethylester 5 (1.74 g; 3.93 mmol) in 40 mL of DMF under nitrogen atmosphere. The reaction mixture was heated to reflux for 18 h at 75°C and then allowed to cool down to room temperature. The solvent was removed in vacuo. The residue was dissolved in chloroform (30 mL), washed with water and 1 M Na2CO3 (12 mL), and then separated for the organic layer. It was dried with anhydrous Magnesium sulfate, filtered, and evaporated to a residue. The crude compound was purified by silica gel column chromatography (chloroform: methanol 9 : 1). Yield: 1.00 g (1.63 mmol, 42.00%). 1H-NMR (D2O) δ = 7.14 (d, 2H, J = 9.0 Hz), 6.94 (d, 2H), 4.33 (m, 4H), 4.05 (t, 2H, J = 9.0 Hz), 3.85 (m, 2H), 3.71 (m, 2H), 3.26 (m, 13H), 2.07 (m, 6H), 1.58 (s, 3H), 1.33 (t, 3H, J = 15.0 Hz) ppm. 13C-NMR δ = 173.97, 158.65, 158.64, 158.06, 157.91, 154.31, 131.01, 128.34, 114.04, 79.38, 77.27, 66.01, 61.50, 60.26, 53.76, 52.76, 49.20, 48.41, 47.54, 46.15, 45.83, 44.50, 42.60, 40.84, 28.40, 26.39, 23.57, 23.36, 23.90, 14.14 ppm. MS: m/z = 517.67 [M]+.

2.1.7. O-[3-(N4,N4-Dioxyl-1,4,8,11-Tetraazabicyclotetradecane)-Propyl]-α-Methyltyrosine Ethylester 8. Compound 7 (0.25 g; 0.40 mmol) was dissolved in dichloromethane (10 mL), and trifluoroacetic acid (1.0 mL) was added to it. The solution was stirred over night at room temperature, and volatiles were removed in vacuo. The crude compound was purified by chromatography over silica gel (chloroform: methanol 9 : 1) as white solid. Yield: 0.20 g (0.39 mmol; 95.69%). 1H-NMR (CD3OD) δ = 7.14 (d, 2H, J = 9.0 Hz), 6.94 (d, 2H), 4.33 (m, 4H), 4.05 (t, 2H, J = 9.0 Hz), 3.85 (m, 2H), 3.71 (m, 2H), 3.26 (m, 13H), 2.07 (m, 6H), 1.58 (s, 3H), 1.33 (t, 3H, J = 15.0 Hz) ppm. 13C-NMR δ = 173.97, 158.65, 158.64, 158.06, 157.91, 154.31, 131.01, 128.34, 114.04, 79.38, 77.27, 66.01, 61.50, 60.26, 53.76, 52.76, 49.20, 48.41, 47.54, 46.15, 45.83, 44.50, 42.60, 40.84, 28.40, 26.39, 23.57, 23.36, 23.90, 14.14 ppm. MS: m/z = 517.63 [M]+.

2.2. Radiosynthesis of 99mTc-N4-AMT. Radiolabeling of N4-AMT with 99mTc was performed in a standard manner [14]. Briefly, radiosynthesis of 99mTc-N4-AMT was achieved by adding a required amount of sodium pertechnetate into a vial containing precursor N4-AMT and SnCl2 (100 μg). Radiochemical purity was assessed by high-performance liquid chromatography (HPLC), equipped with NaI and UV detector (274 nm), and was performed using a C-18 reverse column with a mobile phase of acetonitrile : water (7 : 3) at a flow rate of 0.5 mL/min.
2.3. In Vitro Cellular Uptake of $^{99m}$Tc-N4-AMT. Rat mammary tumor cell line 13762 was obtained from American Type Culture Collection (Rockville, MD). The same cell line was used to create the animal model for in vivo evaluation. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO$_2$ in Dulbecco’s modified Eagle’s medium and nutrient mixture F-12 Ham (DMEM/F12; GIBCO, Grand Island, NY). Cells were plated to 6-well tissue culture plates ($2 \times 10^5$ cells/well) for two days before the study, and incubated with $^{99m}$Tc-N4-AMT (0.05 mg/well, 8 uCi/well) or $^{99m}$Tc-N4 chelator itself (0.025 mg/well, 8 uCi/well) for 15 min–4 h. After incubation, cells were washed with ice-cold PBS twice and detached by adding 0.5 mL of trypsin. Cells were then collected and the radioactivity was measured with gamma counter (Cobra Quantum; Packard, MN). Data was expressed in mean ± S.D. percent of cellular uptake (%Uptake).

2.4. In Vivo Tissue Distribution Studies. All animal work was carried out in the Small Animal Imaging Facility (SAIF) at University of Texas MD Anderson Cancer Center under the protocol approved by Institutional Animal Care and Use Committee (IACUC). Tissue distribution studies of $^{99m}$Tc-N4-AMT (study I, $n = 9$) or $^{99m}$Tc-N4 (study II, $n = 9$) were conducted by using normal female Fischer 344 rats (150 ± 25 g, $n = 18$) (Harlan Sprague-Dawley, Indianapolis, IN). For each radiotracer, nine rats were divided into three groups for three time intervals (0.5, 2, 4 h). The injection activity was 25 ± 0.5 μCi/rat intravenously. At each time interval, the rats were sacrificed, and the selected tissues were excised, weighed, and measured for radioactivity by gamma counter. Data from each sample were represented as the percentage of the injected dose per gram of tissue wet weight (%ID/g). Counts from a diluted sample of the original injection were used as the reference.

2.5. Planar Scintigraphic Imaging Studies of $^{99m}$Tc-N$_4$-AMT. Female Fischer 344 rats were inoculated subcutaneously with 0.1 mL of 13762 rat mammary tumor cell suspension ($10^5$ cells/rat) into the right posterior legs using 22-gauge needles. Imaging studies were performed 14 to 17 days after inoculation when tumors reached approximately 1 cm in diameter. The anesthetized rats were injected intravenously with $^{99m}$Tc-N4-AMT (0.3 mg/rat, 300 μCi/rat; $n = 3$) or with $^{99m}$Tc-N4 (0.15 mg/rat, 300 μCi/rat; $n = 3$), respectively. Planar scintigraphic images were obtained using M-CAM (Siemens Medical Solutions, Hoffman Estates, IL) equipped with a Low-Energy High-Resolution collimator at 30–120 min. The field of view was 53.3 cm × 38.7 cm. The intrinsic spatial resolution was 3.2 mm and the pixel size was from 19.18 mm (32 × 32, zoom = 1) to 0.187 mm (1024 × 1024, zoom = 3.2). Computer outlined regions of interest (ROI) (counts per pixel) of tumors, and normal muscle tissues at symmetric sites were used to calculate tumor-to-muscle (T/M) ratios.
3 was treated with 1, 3-dibromopropane and obtained as N-t-butoxycarbonyl-O-[3-bromopropyl]-α-methyl tyrosine ethylester 4. Acylation of 1,4,8,11-tetraazacyclo-tetradecane (cyclam) with diethyloxalate led to N1,N4-dioxalyl 1,4,8,11-tetraazacyclotetradecane (N1,N4-cyclamoxamide) 5. Under SN2 condition 4 and 5 were efficiently converted to alkylated compound N-t-butoxycarbonyl-O-[3-(N1,N4-dioxalyl-1,4,8,11-tetraazacyclotetradecane)-propyl]-α-methyl tyrosine ethylester 6. Exposure of 6 to trifluoroacetic acid in CH2Cl2 caused qualitative de-t-butoxycarboxylation to yield O-[3-(N1,N4-dioxalyl 1,4,8,11-tetraazacyclotetradecane)-propyl]-α-methyl tyrosine ethylester 7. 10 N NaOH in water at 75°C promoted the hydrolysis of ester to acid and a simultaneous deoxalation of 7 to yield the final compound O-[3-(1,4,8,11-tetraazacyclotetradecan)-propyl]-α-methyl tyrosine (N4-AMT) 8. The total synthesis yield was 14%, which can be adapted to industrial manufacturing. The structure and purity of the compounds at each step were validated by 1H- and 13C-NMR, mass spectra, and HPLC.

3.2. Radiosynthesis. As shown in Figure 2, precursor N4-AMT could be labeled with 99mTc successfully in a high radiochemical purity (>96%). The retention time of 99mTc-N4-AMT was 6.899 min. Given that 99mTc-N4-AMT is a kit-product and labeled without any further purification, the radiochemical yield was assumed to be identical to its radiochemical purity. In this study, we labeled amino acid α-methyltyrosine AMT with 99mTc using cyclam N4 as the chelator because of its stable chelating ability and fast renal clearance. 99mTc was selected as the radionuclide due to its favorable physical characteristics, suitable half-life, and cost-effectiveness.

3.3. In Vitro Cellular Uptake of 99mTc-N4-AMT. The cellular uptake kinetics of 99mTc-N4-AMT and 99mTc-N4 in rat breast tumor cell line 13762 is shown in Figure 3. There was a drastically increased uptake for 99mTc-N4-AMT in the tumor cells at 15–240 min, but not for 99mTc-N4 chelator. These findings suggest that by adding the amino acid AMT, 99mTc-N4-AMT can enter and accumulate into tumor cells effectively and rapidly. To further investigate the transport mechanisms of 99mTc-N4-AMT, the competitive inhibition study using various types of transporter inhibitors will be conducted in the future.

3.4. In Vivo Evaluation of 99mTc-N4-AMT. The result of the in vivo biodistribution studies in the normal Fischer rats at 0.5, 2, and 4 hours after intravenous administration of 99mTc-N4-AMT is shown in Table 1. Planar scintigraphic images of 99mTc-N4-AMT and 99mTc-N4 at 30, 60, and 120 min in breast tumor-bearing rats are shown in Figure 4. T/M ratios of 99mTc-N4-AMT were 2.3–4.0, whereas those of 99mTc-N4 were 1.9–2.5, respectively. Tumors could be clearly visualized by 99mTc-N4-AMT, but not by 99mTc-N4. In addition, the rat kidneys showed intense activity of 99mTc-N4-AMT in the planar images, which was consistent with the results from the in vivo biodistribution studies in the normal rats. This may be due to the nature of AMT, an inhibitor of tyrosine hydroxylase that cannot be excreted from kidneys and hence crystallized in the proximal tubules because of its poor solubility at the hydrogen ion concentrations of body fluids (pH 5–8) [15]. In the future, in vivo uptake blocking study using the unlabeled AMT will be performed to ascertain whether accumulation of 99mTc-N4-AMT in the kidney is attributed to AMT module.

4. Conclusion

In conclusion, efficient synthesis of N4-AMT was achieved. In vitro cellular uptake and in vivo imaging findings collectively suggest that 99mTc-N4-AMT is a potential radiotracer for breast cancer imaging. In compliance with the chelating
hereafter. Ranged beta emitters for internal radiotherapeutic purposes emitting radionuclides such as Gallium-68 or with short-ranged beta emitters for internal radiotherapeutic purposes hereafter.

**Authors’ Contribution**

Fan-Lin Kong and Mohammad S. Ali contributed equally to this work.

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