Synthesis and preliminary biological evaluation of a ^{99m}Tc-chlorambucil derivative as a potential tumor imaging agent

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Technetium-99m-based radiopharmaceuticals have been used widely as diagnostic agents in the nuclear medicine. Chlorambucil (CLB) as one typical alkylating drug exhibits excellent inhibition effects against many human malignancies. In order to develop and explore a novel potential imaging agent for early diagnosis of tumors, tricarbonyl technetium-99m and rhenium complexes based on the tridentate ligand dipicolylamine (DPA) bound to the chlorambucil pharmacophore were designed and synthesized: ^{99m}Tc-DPA-CLB (**3**) and Re-DPA-CLB (**4**). HPLC analyses showed that the retention time of **3** and **4** was 13.5 and 13.6 min, respectively. Radiolabeling efficiency (RE) of the ^{99m}Tc-DPA-CLB tracer was 97% and the radiochemical purity (RCP) was larger than 95% after 6 h stored in phosphate buffered saline or human serum as observed by TLC and HPLC. Biodistribution studies in a mouse model of breast cancer showed ^{99m}Tc-DPA-CLB exhibited a favorable tumor affinity. The radiotracer cleared quickly in the first hour via hepatobiliary and renal routes of excretion, resulted in a very low background at 4 h p.i.. It had moderate uptake ratios of tumor to blood and tumor to muscle. These results suggested ^{99m}Tc-DPA-CLB might be a promising SPECT imaging agent for tumor diagnosis.

Keywords: tricarbonyl technetium/rhenium complex; dipicolylamine; chlorambucil; SPECT; tumor imaging

Introduction

Cancer is one of the leading causes of death all over the world. Early detection is one of the critical challenges for contemporary clinical cancer care. In particular, detection of small pre-malignant lesions and early stage primary tumors is crucial for effective cancer therapy. For this reason, development of highly specific and sensitive non-invasive molecular imaging methodologies has increased tremendously in recent decades, such as single photon emission computed tomography (SPECT) and positron emission tomography (PET). Many groups have also studied the in vivo detection of tumors using contrast agents appropriate for various imaging modalities.[1-3] There are single-modality tumor agents for PET, SPECT, MRI, CT, fluorescence or ultrasound, and there are also hybrid agents (these possess two or more signals within a single molecule/material) for PET-CT, PET-MRI, and etc. Methods have been developed for specific targeting in order to maximize the localization of ligands in the tumor and to minimize the uptake in the surrounding normal tissues to achieve high signal-to-noise ratios.[4,5]

Since the 1950s, technetium-99m-based radiopharmaceuticals have been important diagnostic agents in nuclear medicine, mainly because the radionuclide has favorable nuclear properties ($E\gamma$ = 142 keV, $t_{1/2}$ = 6.02 h) for imaging and from the convenient, reusable on-site ^{99}Mo/^{99m}Tc generator.[6] To date, many ^{99m}Tc-agents have been developed and achieved regulatory approval for routine clinical use.[7,8] Technetium(V) and (III) are the more common ionic forms for coordinating with ligands. The fac-$[^{99m}Tc(CO)_3]^+$ species is particularly attractive because of its ease of preparation via an aqueous-based kit, versatile coordination chemistry, and formation of kinetically inert complexes which can be conjugated with targeting biomolecules.[9] The lipophilic tridentate ligand 2,2′-dipicolylamine (DPA) has been employed as the chelating scaffold to bind with the *fac*-$[M^I(CO)_3]^+$ core that yields stable complexes in high efficiency.[10-12] For example, $[^{99m}Tc(CO)_3]^+$ labeled with DPA was found to be stable against trans-chelation by other ligands in vivo.[12]

It is known that the nitrogen mustard drugs, such as cyclophosphamide and mechlorethamine, share a similar reactivity to nucleophilic atoms at the N,N-bis(2-chloroethyl)-amine group. This electrophilic moiety can form covalent adducts with double-helical DNA, especially via reaction with N7 of guanines, either within the same strand (intrastrand) or between the two opposite DNA strands (interstrand).[13,14] Chlorambucil (CLB) is a chemotherapy drug classified within the nitrogen mustard group, its mechanism of action involves alkylation, and has been employed for the treatment of various malignancies.[15,16] Moreover, it has also been used as a template to design drugs for diagnosis and therapy of tumors.[17-22]

In this study, a novel SPECT tumor imaging agent was synthesized based on a design that combined the *fac*-$[^{99m}Tc(CO)_3]^+$ core, CLB and DPA. As a mimic of the ^{99m}Tc-complex, the rhenium analogue was also prepared using the same chemistry, and the purified complex was characterized by spectroscopic techniques. The in vitro stability, lipophilicity and in vivo biodistribution in the mouse xenograft model bearing a breast cancer cell line (MDA-MB-231) were studied for the ^{99m}Tc-DPA-CLB radiotracer.

Material and Methods

General

2,2'-Dipicolylamine was purchased locally (J&K Scientific Ltd; Shanghai; China), chlorambucil from another supplier (Sigma-Aldrich; Saint Louis; USA), and other reagents/solvents from elsewhere (Sinopharm Chemical Reagent Co. Ltd; Shanghai; China). All the chemicals were analytically pure and used without further purification. $Na^{99m}TcO_4$ was eluted from the ^{99}Mo/^{99m}Tc generator, which was obtained from the China Institute of Atomic Energy. The organometallic precursor $[Et_4N]_2[Re(CO)_3Br_3]$[9] and the radioactive precursor *fac*-$[^{99m}Tc(CO)_3(H_2O)_3]^{+}$[23] were prepared as reported.

Elemental analysis was carried out using an analyzer (Vario EL III; Elementar; Germany). Electron spray ion mass spectra (ESI-MS) were determined using a Waters Platform ZMD4000 LC/MS (Waters, USA). FT-IR spectra (4000-400 cm^{-1}) were recorded on an IR Fourier transform spectrophotometer using KBr pellets (Bruker Vector 22 FT-IR spectrophotometer; Bruker; Germany). Nuclear magnetic resonance spectrometers (^{13}C-NMR;

Bruker DRX-400; Bruker; Germany) and (^{1}H-NMR; Bruker DRX-400; Bruker; Germany) were used to obtain spectra of samples dissolved in $CDCl_3$ or CD_3OD, and the chemical shift value was referenced to tetramethylsilane. Chromatography paper (Xinhua; Shanghai; China) was used for thin layer chromatography (TLC) analysis. The high performance liquid chromatography (HPLC) system was equipped with a pump (Waters 1525 HPLC; Waters; USA), connected to reverse phase column (RP-C18; 4.6 × 250 mm; 10 um; Elite Analytical Instrument Company; Dalian; China), a UV detector (2487 dual wavelength absorbance; Waters; USA) and a radioactivity detector (Radiomatic 610TR; Perkin Elmer; MA; USA) which were operated by software programs (Breeze Software; NY; USA) and (proFSA; Perkin Elmer; USA). The flow rate for HPLC analysis was 1.0 mL/min, and the mobile phase was gradient conditions: A: H_2O + 0.1% TFA, B: CH_3CN + 0.1% TFA; 0-3 min 20% B, 3-35 min 20-80% B, 35-45 min 80-20% B. Radioactivity was counted using a counter (Packard-multi-prias; Perkin Elmer; USA).

BALB/c nude mice (18-20 g; 4-6 week old; female; SLAC Laboratory Animal Co. Ltd; Shanghai; China) were used for animal experiments. Mice were housed with free access to food and water and allowed ample time to acclimatize before the experiments. All procedures and animal protocols were approved by the Animal Care and Ethnics Committee of Jiangsu Institute of Nuclear Medicine.

Synthesis of the ligand DPA-CLB

Preparation of 4-(4-[bis(2-chloroethyl)amino]phenyl)butanoyl chloride (1). Chlorambucil (3.03 g, 0.01 mol) was dissolved in CH_2Cl_2 (75 mL). Then the solution was treated dropwise with thionyl chloride ($SOCl_2$) (30 g, 0.25 mol) and stirred for 2 h at room temperature. The reaction mixture was concentrated in rotary evaporator (RE-2000B; Shanghai Yarong Biochemical Instrument Plant; Shanghai; China) and finally compound **1** was obtained as black grease and used immediately in the next step without additional purification to avoid hydrolysis.

Preparation of 4-(4-[bis(2-chloroethyl)amino]phenyl)-N,N-bis(pyridin-2-ylmethyl) butanamide (2). Compound **1** (0.636 g, 2.0 mmol) was dissolved in CH_2Cl_2 (10 mL). While stirring at room temperature, DPA (0.398 g, 2.0 mmol) in CH_2Cl_2 (20 mL) was added and then triethylamine (0.2 mL). After stirring for 1 h water (20 mL) was added to quench the reaction, the organic layer was separated and washed with HCl (1 N, 2 × 10 mL), saturated NaCl solution (2 × 10 mL) and then water (3 × 10 mL), respectively. The organic layer was dried over anhydrous Na_2SO_4, and then the solvent was removed by rotary evaporator. Finally, compound **2** was obtained as brown viscous oil after purification by silica gel chromatography with an eluent of ethyl acetate:ethanol [8:1] (R_f = 0.6). The first fraction (120 mL) of the product was collected. HPLC analysis showed a single peak (t_R = 13.6; purity: 98.6%). The combined yield of two steps was 72%.

Anal. Calcd for $C_{26}H_{30}Cl_2N_4O$: C, 64.33; H, 6.23; N, 11.54%. Found: C, 64.24; H, 6.41; N, 11.38%. ESI-MS, m/z (%): 485.2(100) = $[M+H]^+$. ^{1}H-NMR (400 MHz, $CDCl_3$, ppm) δ: 8.59 (d, 2H, *J* = 4.8 Hz, α-CH, Py), 7.69 (q, 2H, *J* ＝ 8.0 Hz, γ-CH, Py), 7.14-7.34 (m, 4H, β, δ-CH, Py), 7.07 (d, 2H, *J* = 8.8 Hz, H-Ar), 6.61 (d, 2H, *J* = 8.4 Hz, H-Ar), 4.80 (s, 2H, N-CH_2-Py), 4.70 (s, 2H, N-CH_2-Py), 3.73 (m, 4H, *J* ＝ 6.8 Hz, Cl-CH_2), 3.64 (m, 4H, *J* ＝ 6.8 Hz, Ar-N-CH_2), 2.58 (t，2H, *J* ＝7.6 Hz, $COCH_2$), 2.47 (t, 2H, *J* ＝ 7.6 Hz, Ar-CH_2)，2.02 (m, 2H, CH_2). IR (KBr, cm^{-1}): 3448 (m, $\nu_{C\text{-}H,}$ Ar), 2929 (m, $\nu_{C\text{-}H}$), 1646 (s, $\nu_{C=O}$), 1570 (m, ν_{ph}), 1434 (s, $\nu_{C\text{-}N}$), 1097 (w, $\nu_{C\text{-}Cl}$), 750 (m, ν_{ph}), 654(w, $\nu_{C\text{-}H}$).

Preparation of ^{99m}Tc/Re complexes

The technetium-99m-labeled complex (**3**) was prepared according to the following procedure. To a solution of *fac*-$[^{99m}Tc(H_2O)_3(CO)_3]^+$ (500 μL, pH = 6) in water in a glass vial (10 mL), was added DPA-CLB (**2**) in ethanol (5 g/L; 100 μL) under a blanket of carbon monoxide gas. The reaction was heated to 75 ^oC for 30 min and then cooled to room temperature in water. The radiolabeling efficiency (RE) and radiochemical purity (RCP) of the complex were measured by TLC and HPLC. Two developing solvents, acetonitrile/methanol = 2/1 (V/V) and ethyl acetate/methanol = 10/1 (V/V), were employed for the TLC analysis.

The rhenium-complex **4** was synthesized according to the following procedure. $(Et_4N)_2[Re(CO)_3Br_3]$ (55 mg, 0.08 mmol) and the ligand DPA-CLB (**2**) (48 mg, 0.1 mmol) were dissolved in methanol (3 mL) and then stirred at room temperature (25 ^oC) for 1.5 h. Then the reaction mixture was concentrated in vacuum and the residue was purified by silica gel chromatography with an eluent of ethyl acetate:ethanol:triethylamine [8:1:0.1] (R_f = 0.4). The gray powder product was obtained (yield: 75%). HPLC analysis showed a single peak (t_R = 13.6; purity: 99.4%).

Anal. Calcd for $C_{29}H_{31}C_{l2}N_4O_4Re$: C, 46.03; H, 4.13; N, 7.40%. Found: C, 45.91; H, 4.26; N, 7.35%. ESI-MS, m/z (%): 756.21(100) = $[M+H]^+$. ^{1}H-NMR (400 MHz, CD_3OD, ppm) δ: 8.82 (d, 2H, *J* = 6.4 Hz, α-CH, Py), 7.84-7.88 (td, 2H, $J_{1,2,3}$ = 1.2 Hz, γ-CH, Py), 7.58 (d, 2H, *J* = 8.0 Hz，β-CH, Py), 7. 31 (t, 2H, *J* = 6.4 Hz, δ-CH, Py), 6.94 (m, 2H, H-Ar), 6.56 (m, 2H, H-Ar), 4.72 (s, 4H, N-CH_2-Py), 3.61 (m, 4H, Cl-CH_2), 3.25 (m, 4H, Ar-N-CH_2), 2.43 (t, 2H, *J* ＝ 7.2 Hz, Ar-CH_2), 2.20 (t, 2H, *J* ＝ 7.2 Hz, CH_2 C=O), 1.22 (m, 2H, $CH_2CH_2CH_2$). ^{13}C-NMR (100 MHz, CD_3OD, ppm) δ: 194.68 ($Re(CO)_3$), 174.28 (C=O), 160.30, 151.29, 139.65, 124.96, 122.866 (Py), 144.169, 137.223, 129.64, 111.76 (Ph), 61.62 (CH_2-Py), 52.69 (CH_2-Cl), 51.70 (N-CH_2), 40.18 (Ph-CH_2), 33.10 (-CH_2C=O), 26.13 ($CH_2CH_2CH_2$). IR (KBr, cm^{-1}): 3443 (m, $\nu_{C\text{-}H,}$ Ar), 2922 (m, $\nu_{C\text{-}H}$), 2027 (s, ν_{CO}), 1928 (s, ν_{CO}), 1888 (s, ν_{CO}), 1610 (m, $\nu_{C=O}$), 1580 (m, ν_{ph}), 1414 (m, $\nu_{C\text{-}N}$), 1090 (br, $\nu_{C\text{-}Cl}$), 798 (m, ν_{ph}), 615 (w, $\nu_{C\text{-}H}$).

In vitro stability assays

The *in vitro* stability of the ^{99m}Tc-DBA-CLB (**3**; 3.7 MBq) diluted in saline (100 μL), was evaluated in the presence of phosphate buffered saline (PBS; 0.1 M; pH = 7; 1 mL), and also in human serum (1 mL) separately. Then the solutions were incubated (K37 multifunctional incubator, Hangzhou Allsheng Instruments Company; Hangzhou; China) at 37 ^oC. Samples (50 uL) were removed from each incubating solution at t = 0, then every hour for 6 hours, and analyzed for %RCP by HPLC.

Determination of octanol/water partition coefficient

The octanol/water partition coefficient of ^{99m}Tc-DPA-CLB (**3**) was determined at two different pH values of 7.0 and 7.4 by measuring its distribution in n-octanol and PBS, respectively. A sample of **3** (100 μL) was added to an immiscible liquid containing PBS (900 μL; pH 7.0 or 7.4) and n-octanol (1 mL), then after 2 min vigorous vortex (XH-C vortex, Nanjing Eastmai Science and Technology Instrument Co., Ltd.; Nanjing; China), the mixture was incubated for 30 min at room temperature. Centrifugation (PJ-TDL-40B, Wuxi Ruijiang Analysis Instrument Co. Ltd., Wuxi; China) at 4000 g for 5 min ensured complete separation of the organic and the aqueous layers. An aliquot (100 uL) from each layer was measured with a γ counter. The partition coefficient was calculated using the following formula: log *P* = log (octanol counts/water counts). The reported value is the average obtained from three independent measurements.

Biodistribution studies in tumor-bearing mice

In vivo biodistribution of ^{99m}Tc-DBA-CLB (**3**) was evaluated in the mouse xenograft model bearing breast cancer cell MDA-MB-231 (Cell Bank of the Chinese Academy of Sciences; Shanghai; China). The breast cancer cells MDA-MB-231 (1×10^6) in phosphate buffered saline (0.2 mL) were injected subcutaneously into the left front leg of the mice. Ten or fifteen days after inoculation, thirty five mice with the tumor size in the range of 0.5-1.0 cm in diameter divided into seven groups were used to study the biodistribution of ^{99m}Tc-DBA-CLB (**3**) in various organs. Each mouse per group was intravenously administered with the radiotracer (100 μL, 37 kBq) via the tail vein using an insulin syringe. The mice were anesthesized (2% isoflurane in oxygen at a flow rate of 2 L/min) and sacrificed at 10, 30, 60, 120 and 240 min post injection. Tissue samples of interest were collected and weighed, such as bone (femur), muscle from leg, heart, liver, spleen, lung, kidney, uterus, ovary, intestines, stomach, and brain as well as 200 μL blood taken from the carotid artery. The radioactivity in each sample was measured by a γ counter. The radiotracer uptake in different organs was calculated and expressed as the percent uptake of the injected dose per gram of organ using the formula %ID/g = 100 × [organ counts/(organ weight x whole body counts)]. And the uptake ratios of tumor to muscle and to blood were calculated

from the %ID/g values. The results were expressed as mean values of five independent experiments.

Results and discussion

Synthesis and radiolabeling

From the commercially available starting material chlorambucil, suitable functionalization with a bifunctional chelating group 2,2'-dipicolylamine was possible in a two-step procedure involving the activation of the carboxyl group and the formation of an amide (Scheme 1). Briefly, the corresponding acid chloride of chlorambucil (**1**) was prepared with excess thionyl chloride, and further reaction with 2,2'-dipicolylamine in the presence of triethyl amine in CH_2Cl_2 afforded the compound DPA-CLB (**2**). The chemical syntheses were convenient and the overall yield of the product was good. The DPA moiety in DPA-CLB (**2**) contained three N-donor atoms for coordination with the $[M^{VII}(CO)_3(H_2O)_3]^+$ core to form stable octahedral complexes, and the rhenium complex was characterized by the elemental analysis, MS and $^1H/^{13}C$-NMR. The results obtained were in agreement with the expected chemical structure.

Taking advantage of the stability of ^{99m}Tc-tricarbonyl reagent and the adducts derived from it, $[^{99m}Tc(CO)_3(H_2O)_3]^+$ was hence selected as a precursor of choice for the radiolebeling step. The radiolabeled complex **3** was prepared by the ligand-exchange reaction of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ and DPA-CLB in aqueous ethanol solvent in 97.3% RE within 20 minutes. Furthermore, it is acknowledged that the development of technetium-labeled complexes as radiopharmaceuticals has been facilitated by the use of rhenium, the group VIIB congener of technetium. Rhenium chemistry is similar to technetium-99m chemistry and therefore non-radioactive metal complexes are often used as mimics of ^{99m}Tc-analogues, with the practical advantage that larger scale syntheses can yield quantities of product for structural characterization.[23-25] In this study, the rhenium-complex **4** was synthesized in sufficient quantity from DPA-CLB (**2**) in 75% yield via Scheme 1, it was characterized by elemental analysis and a mix of different spectroscopic methods. NMR analysis of the Re complex in CD_3OD allowed the specific binding mode of the precursor to be deduced. The

aromatic portion of ^{1}H-NMR spectrum showed that two pyridyl units were equivalent and shifted downfield with respect to the free precursor. The chemical shift of α-CH was 8.59 ppm in the ligand precursor molecule (**2**) and 8.82 ppm for the product rhenium-complex (**4**). This downfield shift of hydrogens between two molecules was evidence that neighboring nitrogen atoms coordinated to the transition metal. The chemical shift of the methylene neighboring pyridyl is 4.72 ppm, while they are 4.70 and 4.80 ppm in the precursor. The results indicated that the two pyridyl nitrogen atoms are symmetrical at the octahedron coordination geometry of the Re metal. Interestingly, the ^{13}C data of the complex revealed that there were only two Re-CO peaks (~195 ppm), due to the simple overlap or those two CO groups were magnetically equivalent because of the mirror symmetry.

Quality control

The RCP of *fac*-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ and ^{99m}Tc-DPA-CLB was checked by TLC and HPLC, respectively. In the developing solvent of acetonitrile/methanol (V/V = 2/1), *fac*-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ moved to the solvent front (R_f = 0.9-1.0), while ^{99m}TcO$_2$·nH$_2$O and Na^{99m}TcO$_4$ remained at the origin (R_f = 0-0.1) in comparison with the corresponding reference materials. When TLC developed by ethyl acetate/methanol (V/V = 10/1), the R_f value for ^{99m}Tc-DPA-CLB (**3**) was about 0.6-0.7, while ^{99m}TcO$_2$·nH$_2$O, Na^{99m}TcO$_4$ and *fac*-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ remained at the origin (R_f = 0-0.1). In two kinds of developed solvent systems, most of the radioactivity moved to the front and the RCP of ^{99m}Tc-DPA-CLB (**3**) was higher than 95%. About 2% *fac*-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ and 3% ^{99m}Tc-pertechnetate along with ^{99m}TcO$_2$ were determined to be the impurities.

The radiotracer **3** was analyzed by the radio-HPLC, as shown in Figure 1. It was observed that the retention time of [^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ was 4.5 min, while that of ^{99m}Tc-DPA-CLB was found to be ~13.5 min (Figure 1a). The reference complex (**4**) was checked by HPLC with t_R = 13.6 min and high purity (Figure 1b). The retention time of ^{99m}Tc-labelled complex was almost identical with that of the corresponding Re complex (**4**). According the HPLC analysis, the radiolabeling yield of the ^{99m}Tc-labelled complex was 96%. About 4% impurities were observed at ~3.8 min and ~4.5 min for ^{99m}Tc-pertechnetate and

fac-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$, respectively. The HPLC results were consistent with TLC analysis, indicating that the quality control methods were feasible and efficient for the radiotracer. Since the freshly prepared radiotracer was used without further purification for both in vitro and in vivo studies, the RCP was identical to the RE.

In vitro stability and lipophilicity

In vitro stabilities of the radiotracer **3** in PBS (pH = 7.0) and human serum were both studied and analyzed by radio-HPLC. As shown in Figure 2, after 6 h of incubation, more than 95% of the complex still remained intact according to the HPLC analysis. No obvious decomposition products or reoxidation to pertechnetate were detected during the period of study, demonstrating that the chemical stability of the radiotracer was high enough for further biological investigation as a molecular imaging agent.

The partition coefficient log*P* of ^{99m}Tc-DPA-CLB (**3**) was determined to be 2.34 ± 0.48, suggesting that it was hydrophobic rather than hydrophilic. In comparison with the radiolabeling precursor **2** (clog *P* = 3.47), the positive charge of the tricarbonyl moiety increased the polarity and aqueous solubility of the overall complex (**3**). The pyridine nitrogen atom lone pairs contributed to stabilizing the electropositive metal, and resulted in an integral molecule for six hours at room temperature in media in vitro.[12,26]

Biodistribution in tumor-bearing mice

In vivo biodistribution results of the radiotracer **3** were summarized in Figure 3 and Table 1, in which the initial tumor uptake of 1.2% ID/g accumulated to 1.5% ID/g after 60 minutes and this level was retained for an hour until its gradual decline to 1.2% ID/g at 4 hours post injection. The complex was excreted quickly via hepatobiliary and renal route. Hence, the liver and the kidney had higher radioactivities than other organs, especially in the first hour. Moreover, it was cleared from cancer-free organs very fast, thus leading to a very low background at 4 h p.i.. For example, its blood clearance decreased from 2.1% ID/g at 10 min p.i. to 0.6% ID/g at 30 min p.i., and then to 0.2% ID/g at 4 h p.i.; and the liver clearance

decreased from 15.5% ID/g at 10 min p.i. to 12.9% ID/g at 60 min p.i., and then to 5.6% ID/g at 4 h p.i.. On the whole, the radiotracer **3** had modest uptake ratios of tumor-to-blood (T/B) and tumor-to-muscle (T/M) after 1 h (3.27 ± 0.35 and 2.44 ± 0.46, respectively), which increased up to 5.22 ± 0.63 and 7.18 ± 0.80, respectively, at 4 h p.i.. ^{99m}Tc-pertechnetate is characteristically taken up by the stomach in mammals. In this study the stomach uptake in mice was found to be low to the extent of 0.3-0.7% ID/g over 1-4 hours, indicating no significant decomposition or metabolism of the complex to ^{99m}Tc-pertechnetate occurred *in vivo*. This result concurs with the stability of the radiotracer in serum at 37 ^{o}C *in vitro*. As reported in a previous study,[20] another chlorambucil derivative was synthesized by conjugating a NNO chelating group [N-(3-(dimethylamino)propyl)amido]acetic acid to chlorambucil, which was further labeled with $^{99m}Tc(CO)_3$ successfully. The *in vivo* biological studies of the reported $^{99m}Tc(CO)_3$-labeled chlorambucil derivative showed increasing uptake in the stomach with the time (increasing from 3.07% ID/g at 1 h to 17.7% ID/g at 3 h), indicating that this radiotracer took place an *in vivo* decomposition even though it was very stable (98% RCP) *in vitro* experimental study under 37 ^{o}C incubation in PBS at pH 7.0 as well as in human serum.[20] The difference might originate from the different tridentate chelating moieties. In our study, the tridentate ligand 2,2′-dipicolylamine (DPA) was chosen as the chelating scaffold, which exhibited favorable complexation property and kinetic stability with the *fac*-$[M^{I}(CO)_3]^{+}$ core.[27,28] As a whole, the ^{99m}Tc-DPA-CLB radiotracer (**3**) has a superior stability and favorable tumor uptake with good T/NT uptake ratios, which strongly suggests that it has potential for tumor imaging.

Conclusion

A new analogue of the anticancer drug chlorambucil was synthesized after covalent bonding with the dipicolylamine chelating group. The resulting ligand DPA-CLB was successfully radiolabeled with $^{99m}Tc(CO)_3(H_2O)_3$ in high yield (>95% RE), and the resulting ^{99m}Tc-DPA-CLB complex was found to be stable in vitro and in vivo. Biodistribution studies in a mouse model of breast cancer showed rapid clearance from the bloodstream, with the major route of excretion by the hepatobiliary system. There was peak tumor uptake of 1.5% ID/g at 60 min pi and this gradually decreased to 1.2% ID/g after 4 hours. In a mouse model

of breast cancer, this agent gave a tumor/blood ratio increase from 3.3 to 5.2, and a tumor to muscle ratio from 2.4 to 7.2 over that time frame. The ^{99m}Tc-complex has the property of favorable tumor uptake and retention, fast clearance from background and good T/NT ratios, which is an advantage over another reported CLB analogue. The data presented so far suggests ^{99m}Tc-DPA-CLB (**3**) has potential as a tumor imaging agent. This laboratory intends to evaluate its application further.

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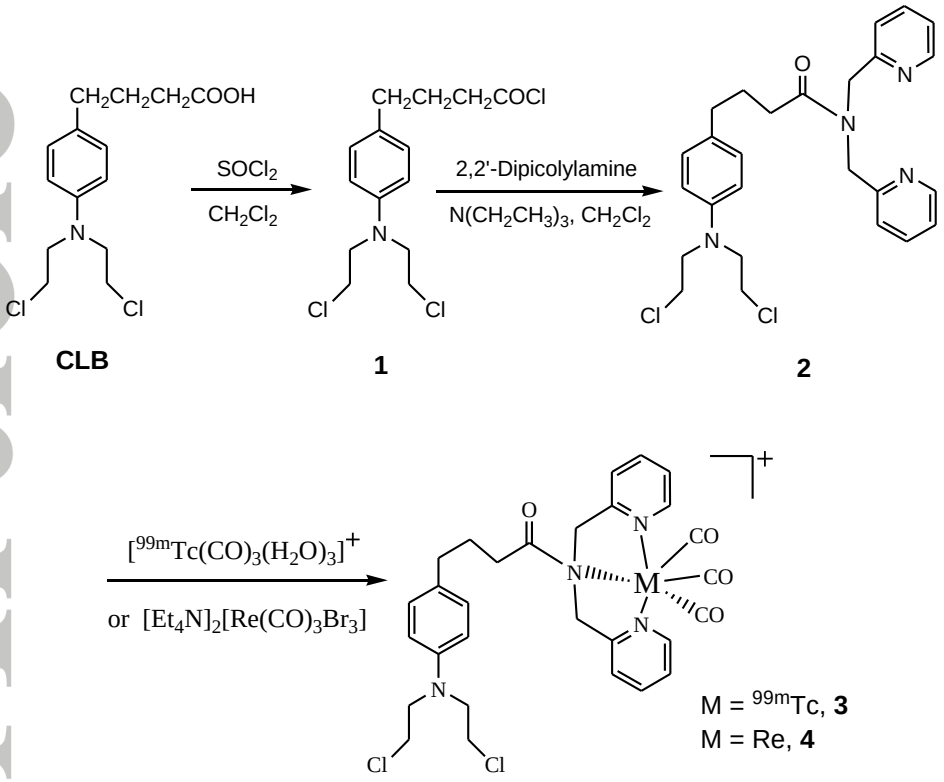

Scheme 1. Synthesis of the ligand DPA-CLB (**2**) and the complexes ^{99m}Tc-DPA-CLB (**3**), Re-DPA-CLB (**4**).

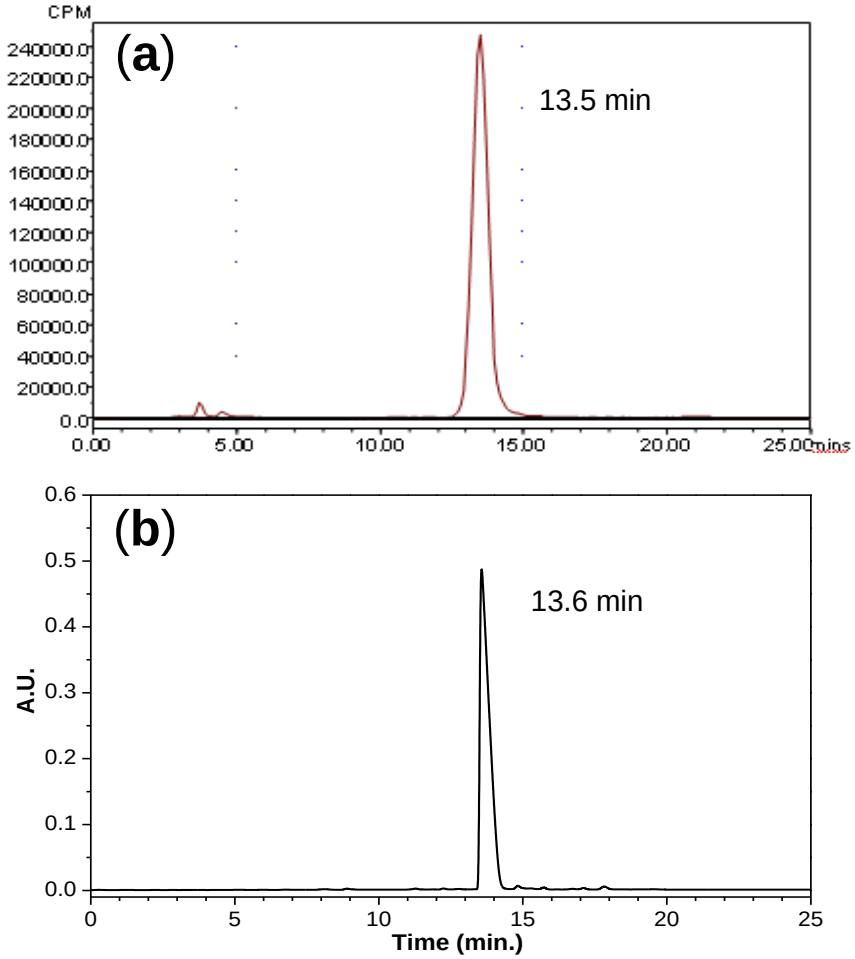

Figure 1. HPLC chromatogram of the radiotracer **3** (a, t_R=13.5 min) and the Re-complex **4** (b, t_R=13.6 min).

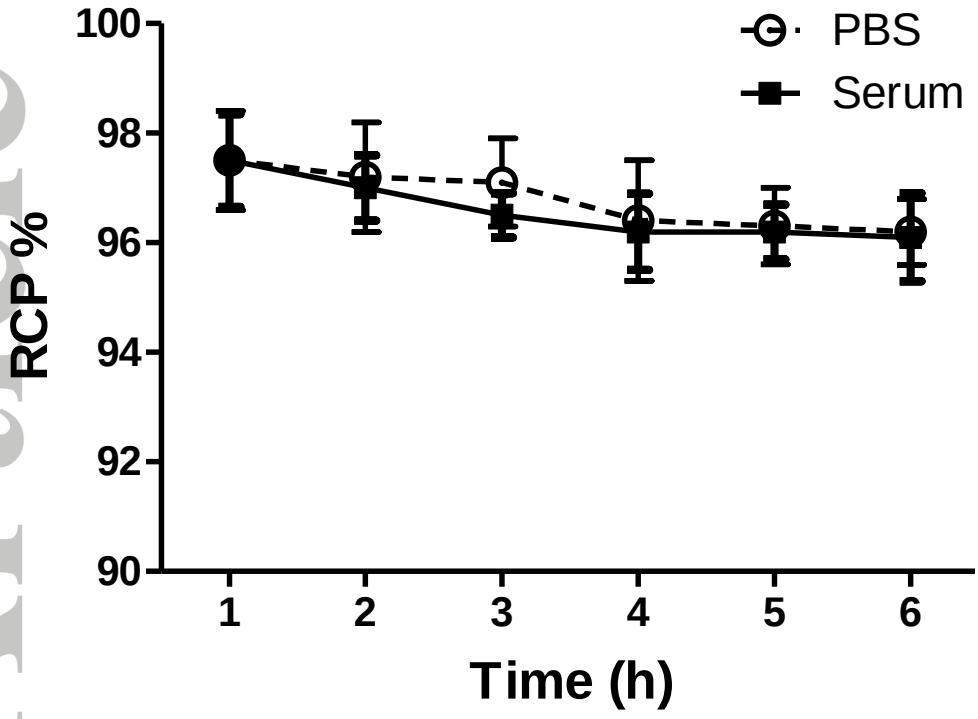

Figure 2. In vitro stability of the radiotracer **3** in PBS and human serum, respectively.

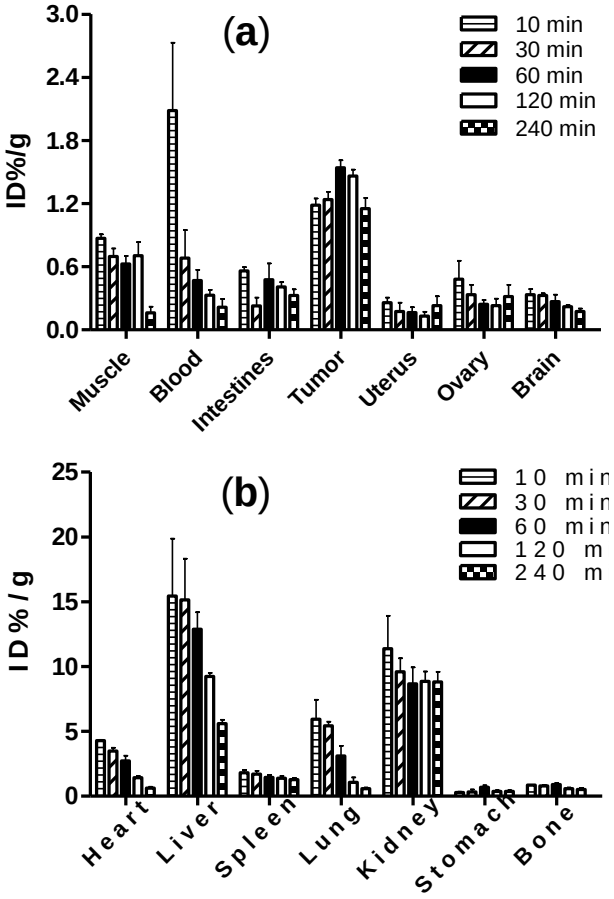

Figure 3. Biodistribution of the radiotracer **3** in the mouse model of breast cancer (n=5).

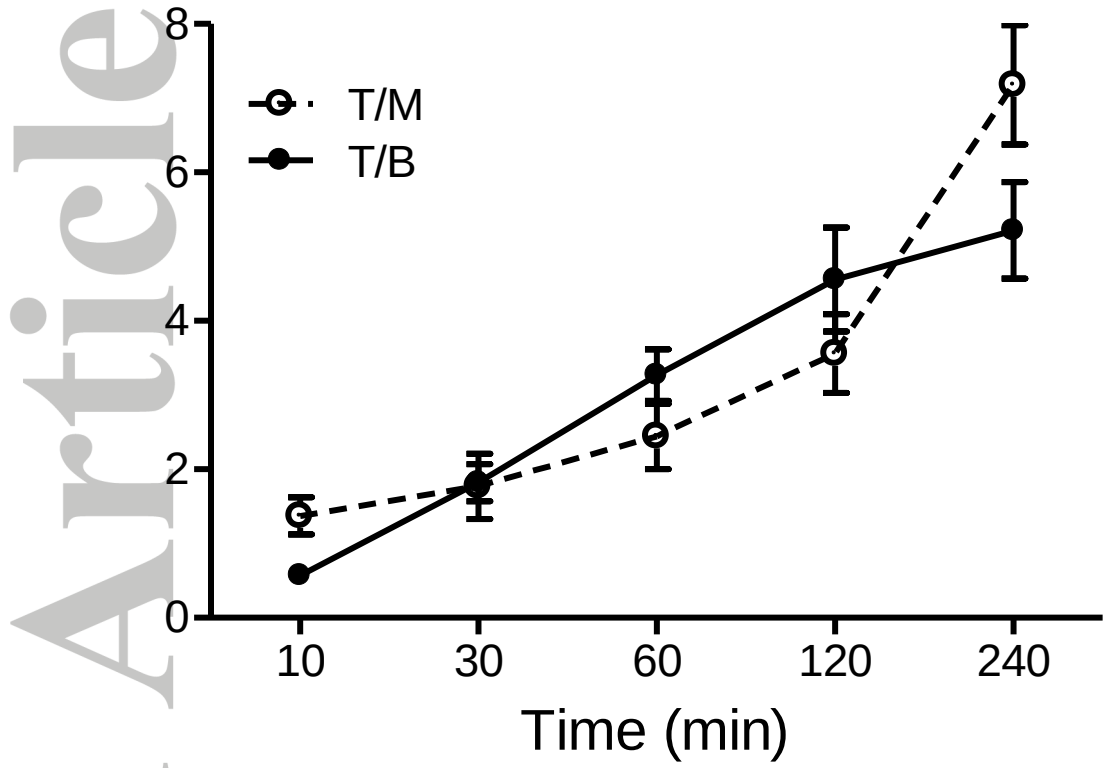

Figure 4. Uptake ratios of tumor/blood and tumor/muscle for the radiotracer **3**.

Table 1. Biodistribution of ^{99m}Tc-DPA-CLB (**3**) in the mouse model of breast cancer (mean ± SD, n=5, %ID/g)

Tissues	*Time after administration*				
	10 min	30 min	60 min	120 min	240 min
Tumor	1.19±0.06	1.24±0.07	1.54±0.07	1.46±0.06	1.15±0.10
Muscle	0.87±0.04	0.70±0.08	0.63±0.08	0.71±0.13	0.16±0.06
Blood	2.08±0.65	0.68±0.27	0.47±0.10	0.33±0.05	0.22±0.08
Intestines	0.56±0.04	0.23±0.08	0.48±0.15	0.41±0.05	0.33±0.06
Uterus	0.26±0.05	0.18±0.08	0.16±0.05	0.13±0.04	0.23±0.09
Ovary	0.48±0.18	0.33±0.10	0.24±0.04	0.23±0.07	0.32±0.11
Brain	0.33±0.05	0.33±0.02	0.27±0.07	0.22±0.02	0.17±0.03
Heart	4.29±0.05	3.49±0.25	2.71±0.41	1.42±0.13	0.61±0.11
Liver	15.45±4.42	15.15±3.16	12.88±1.32	9.24±0.28	5.59±0.31
Spleen	1.80±0.22	1.71±0.25	1.45±0.20	1.39±0.17	1.28±0.13
Lung	5.95±1.48	5.44±0.32	3.10±0.78	1.07±0.38	0.58±0.09
Kidney	11.38±2.54	9.60±1.04	8.67±1.28	8.86±0.76	8.82±0.79
Stomach	0.30±0.06	0.32±0.19	0.68±0.15	0.37±0.08	0.38±0.07
Bone (Femur)	0.85±0.05	0.79±0.08	0.89±0.10	0.57±0.09	0.51±0.14