Synthesis and biodistribution of a novel ^{99m}Tc nitrido radiopharmaceutical with proline dithiocarbamate as a potential tumor imaging agent

Miao Liu · Xiao Lin · Xiaoqing Song · Yang Cui · Peiwen Li · Xuebin Wang · Junbo Zhang

Abstract In the present study, proline dithiocarbamate (PRODTC) ligand was radiolabeled with the $[^{99m}Tc \equiv N]^{2+}$ core successfully to obtain the ^{99m}TcN-PRODTC complex with high radiochemical purity. No decomposition of the complex at room temperature was observed over a period of 6 h. Its partition coefficient indicated that it was a hydrophilic complex. The electrophoresis results showed that the complex was negative. The biodistribution of ^{99m}TcN-PRODTC in mice bearing S 180 tumor showed that the complex accumulated in the tumor with a certain uptake. The tumor/blood and tumor/muscle ratios reached 2.19 and 4.54 at 2 h post-injection, suggesting it would be a promising candidate for tumor imaging.

Keywords Tumor imaging · L-proline · Dithiocarbamate · $[^{99m}TcN]^{2+}$ core

Introduction

[^{18}F]fluorodeoxyglucose [^{18}F]FDG has been the most widely used radiotracer for tumor imaging. However, [^{18}F]FDG can not distinguish tumor from inflammatory tissue or normal brain tissues. Amino acids play an important role in the growth of tumor cells, providing a good opportunity for their use in tumor imaging. The tumor imaging by using radiolabeled amino acids is less influenced by inflammatory processes, suggesting they can overcome the limitation displayed by [^{18}F]FDG [1]. Currently, many amino acids derivatives radiolabeled with positron emitters such as [^{11}C]methionine ([^{11}C]MET) [2, 3], L-3-[^{18}F]fluoro-α-methyl-L-tyrosine ([^{18}F]FMT) [4, 5], O-(2-[^{18}F]-fluoroethyl)-L-tyrosine ([^{18}F]FET) [6–8] and Cis-4-[^{18}F]fluoro-L-proline(Cis-[^{18}F]FPro) [9] have been developed for positron emission tomography (PET). However, a cyclotron is essential to produce these radionuclides, thus restricting their wide use in clinical practice. By comparison, ^{99m}Tc has been the isotope of choice for development of novel radiopharmaceuticals owing to its short half-life, optimal γ-energy, inexpensive cost and diverse coordination chemistry. Therefore, achieving ^{99m}Tc labeled amino acids derivatives as single photon emission computed tomography (SPECT) probes is considered to be of great interest. Recently, several ^{99m}Tc labeled amino acids derivatives as tumor imaging agents have been reported [10–12]. Among them, ^{99m}Tc-diethylenetriamine-pentaacetic acid-bis(methionine) (^{99m}Tc-DTPA-bis(Met)) shows the most promising character to be a tumor imaging agent. However, its chemical structure is poorly defined due to its complicated coordination structure. The preparation of novel ^{99m}Tc labeled amino acids derivatives is still considered to be necessary and highly challenging.

The $[^{99m}TcN]^{2+}$ core has been found to complex well with ligands containing sulfur atoms, as in dithiocarbamates. Recently, some novel ^{99m}TcN labeled dithiocarbamate complexes for infection and tumor imaging have been reported by our group [13–15]. L-proline is a natural amino acid and its molecular structure has an active amine group, thus making it possible to react with carbon disulfide in NaOH solutions to produce the corresponding dithiocarbamate. This background encouraged us to prepare the ^{99m}TcN-PRODTC complex by ligand-exchange reaction

M. Liu · X. Lin · X. Song · Y. Cui · P. Li · X. Wang · J. Zhang (✉)
Key Laboratory of Radiopharmaceuticals, Ministry of Education, College of Chemistry, Beijing Normal University, Beijing 100875, People's Republic of China
e-mail: zhjunbo@bnu.edu.cn

with ^{99m}TcN intermediate to find a potential good tumor imaging agent. In this study, the synthesis and biological evaluation of the ^{99m}TcN-PRODTC complex as a potential agent to target tumor are reported for the first time.

Experimental

Materials and methods

L-proline was purchased from J&KCHEMICA, China. Succinic dihydrazide (SDH) kit was obtained from Beijing Shihong Pharmaceutical Center, Beijing Normal University, China. All other chemicals were of reagent grade and were used without further purification. ^{99}Mo/^{99m}Tc generator was obtained from the China Institute of Atomic Energy (CIAE). IR spectrum was obtained with an AVA-TAR 360 FT-IR spectrometer using KBr pellets. NMR spectrum was recorded on a 500 MHz Bruker Avance spectrophotometer. ESI–MS spectrum was recorded on a LC–MS Shimadzu 2010 series. HPLC analysis was carried out with a reversed-phase column (Kromasil 100-5C, 250 4.6 mm), Shimadzu SCL-10AVP series.

Synthesis of proline dithiocarbamate (PRODTC)

The synthesis of PRODTC was carried out according to the literature [16]. Carbon disulfide (0.5 mL, 8.70 mmol) was dissolved in 5 mL anhydrous diethyl ether and cooled to 0 °C in an ice bath. L-Proline (1,000 mg, 8.70 mmol) and NaOH (692 mg, 17.3 mmol) were dissolved in 10 mL anhydrous methanol and added dropwise to the carbon disulfide solution. The mixture was stirred for 3 h in an ice bath. The solvent was removed and the resulting residue was triturated with diethyl ether. The white oil was dried in vacuo to give PRODTC as a pale yellow solid (1.12 g, 54.6 %).

Radiolabeling of ^{99m}TcN-PRODTC and quality control techniques

The preparation procedure for ^{99m}TcN-PRODTC and thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) analysis conditions are as follows. 1 mL of saline containing [^{99m}TcO$_4$]$^-$ (370 MBq) was added to a kit containing 0.05 mg of stannous chloride dihydrate, 5.0 mg of succinic dihydrazide (SDH), 5.0 mg of propylenediamine tetraacetic acid (PDTA). The mixture was kept at room temperature for 15 min. Then, 1.0 mg of PRODTC dissolved in 1.0 mL water was added and the reaction was allowed to stand for 20 min at room temperature. The TLC was performed on a polyamide strip and

eluted with saline and acetonitrile respectively. HPLC analysis was carried out with a reversed-phase column (Kromasil 100-5C, 250 × 4.6 mm), Shimadzu SCL-10AVP series, working at a flow rate of 1.0 mL/min. Water (A) and acetonitrile (B) mixtures were used as the mobile phase and the following gradient elution technique was adopted for the preparation (0 min 5 % B, 10 min 50 % B, 20 min 50 % B, 25 min 5 % B, 40 min 5 % B).

In vitro stability study

The stability of the complex was assayed by TLC analysis by measuring the RCP in the reaction mixture for 6 h at room temperature (25 °C). To evaluate the serum stability of ^{99m}TcN-PRODTC, 0.5 mL of ^{99m}TcN-PRODTC (18.5 MBq) was added to 1 mL human serum albumin (1 mg/mL) and incubated at 37 °C. The RCP of the complex was measured by TLC up to 6 h.

Human serum albumin binding assay

10 μL of ^{99m}TcN-PRODTC (370 KBq) was added in 100 μL human serum albumin (100 mg/mL) in the centrifuge tube. After the mixture was incubated at 37 °C for 2 h, the serum protein was precipitated by adding 1 mL trichloroacetic acid (250 mg/mL) to the mixture. The supernatant and precipitate were separated by centrifugation at 2,000g for 5 min. The radioactivities of both phases were measured separately. The above experimental procedure was repeated four times and the percentage of human serum protein binding was determined by the following equation: serum protein binding % = (cpm in precipitate)/(cpm in precipitate + cpm in supernatant) × 100 %.

Determination of the partition coefficient

The partition coefficient was determined by mixing the complex with an equal volume of 1-octanol and phosphate buffer (0.025 mol/L, pH 7.4) in a centrifuge tube. The mixture was vortexed at room temperature for 1 min and then centrifuged at 5,000g for 5 min. From each phase 0.1 mL of the aliquot was pipetted and counted in a well γ-counter. Each measurement was repeated three times. Care was taken to avoid cross contamination between the phases. The partition coefficient, P, was calculated using the following equation:

$$P = (cpm \, in \, octanol - cpm \, in \, background)/$$
$$(cpm \, in \, buffer - cpm \, in \, background)$$

Usually the final partition coefficient value was expressed as log P.

Paper electrophoresis

1 μL sample was spotted on a piece of Whatman 1 chromatography paper, saturated with 0.05 mol/L pH 7.4 phosphate buffer, in an electrophoresis bath. Across 15 cm of the strip, 150 V was applied for 1.5 h. The strips were dried, and the distribution of radioactivity on the strip was determined.

In vitro cell uptake

Cell uptake studies were carried out by using murine sarcoma S180 cell lines. ^{99m}TcN-PRODTC was incubated at 37 °C for 0.5–4.0 h with 1×10^6 S180 cells. After incubation, the cells were centrifuged at 2,000g for 5 min for pellet formation. The cell pellets were then washed three times. The radioactivity related to cell uptake was expressed in the mean percentages of radioactivity bound to tumor cell compared with the total radioactivity added after correcting for non-specific uptake (i.e. incubations without tumor cell added).

Biodistribution study

Biodistribution study was performed in Kunming male mice (weighing 18-20 g) bearing S180 tumor, which grew to a leg diameter of 10-15 mm. 0.1 mL of ^{99m}TcN-PRODTC (7.4×10^5 Bq) was injected via a tail vein and the injected radioactivity was measured with a well-type NaI(Tl) detector. The mice were sacrificed at 0.5, 2 and 4 h post-injection. The tumor, other organs of interest and blood were collected, weighed and measured for radioactivity. The results were expressed as the percent uptake of injected dose per gram of tissue (%ID/g). All biodistribution studies were carried out in compliance with the national laws related to the conduct of animal experimentation.

Results and discussion

Synthesis and radiolabeling

PRODTC was prepared by reacting L-proline with an equivalent amount of carbon disulfide in NaOH solutions. The reaction is schematically shown in Scheme 1. It was characterized by IR, ^{1}H NMR, ^{13}C NMR and ESI–MS spectroscopy. IR (KBr)/cm^{-1}:2,964 (CH$_2$), 1,586 (C=O), 1,005 (C=S). ^{1}H-NMR (D$_2$O) δ: 4.70-4.72 (m, 1H), 3.78-3.83 (m, 1H), 3.71-3.76 (m, 1H), 2.19-2.24 (m, 1H), 1.89-1.92 (m, 3H). ^{13}C-NMR (D$_2$O) δ: 205.2, 179.8, 69.1, 55.3, 31.1, 24.3. ESI–MS (m/s): 190 [M-2Na + H]$^-$.
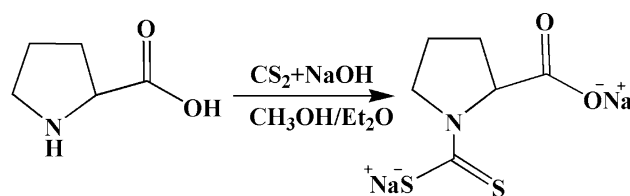
Scheme 1 Synthesis of PRODTC

The preparation of ^{99m}TcN-PRODTC was carried out using the following procedure in Scheme 2.

The method is based on the reaction of $[^{99m}TcO_4]^-$ with succinic dihydrazide (SDH) in the presence of stannous chloride as reducing agent to form a technetium-99m nitrido intermediate. The $[^{99m}TcN]^{2+}$ core is a suitable substrate for the substitution reaction with PRODTC to produce the ^{99m}TcN-PRODTC complex at room temperature.

The radiochemical purity of the complex was routinely checked by TLC and HPLC. By TLC, in saline, $[^{99m}TcO_4]^-$ and ^{99m}TcN-PRODTC remained at the origin while $[^{99m}Tc \equiv N]_{int}^{2+}$ migrated with the front. In acetonitrile, $[^{99m}TcO_4]^-$ migrated at $R_f = 0.3$–0.5, while ^{99m}TcN-PRODTC and $[^{99m}Tc \equiv N]_{int}^{2+}$ remained at the origin. The HPLC pattern of the complex is shown in Fig. 1. It was observed that the retention time of $[^{99m}TcO_4]^-$ was 2.8 min and $[^{99m}Tc \equiv N]_{int}^{2+}$ was 2.5 min, while that of ^{99m}TcN-PRODTC was found to be 10.5 min. The mean radiochemical purity of the product was over 90 % immediately after the preparation. The specific radioactivity of the product was calculated to be 86.95 MBq/μmol.

Based on the previous characterization of ^{99m}Tc-nitrido dithiocarbamate complex [17], it seems reasonable to presume that the structure of ^{99m}TcN-PRODTC should have a square pyramidal geometry with an apical Tc≡N bond and two dithiocarbamate ligands spanning the four positions in the basal plane through the four sulfur atoms. Clearly, further studies should be performed, using macroscopic levels of the long-lived ^{99}Tc, to determine and characterize the structure of ^{99m}TcN-PRODTC.

Stability study

In vitro experiments showed that the complex did not decompose over 6 h at room temperature, suggesting it possesses a great stability in the reaction mixture at room temperature. In serum at 37 °C, the RCP of the complex was over 90 % at 6 h after synthesis, suggesting it was stable over 6 h in human serum conditions.

Human serum albumin binding assay

The percentage of serum protein binding of ^{99m}TcN-PRODTC was relatively high (87.42 ± 0.01 %). The

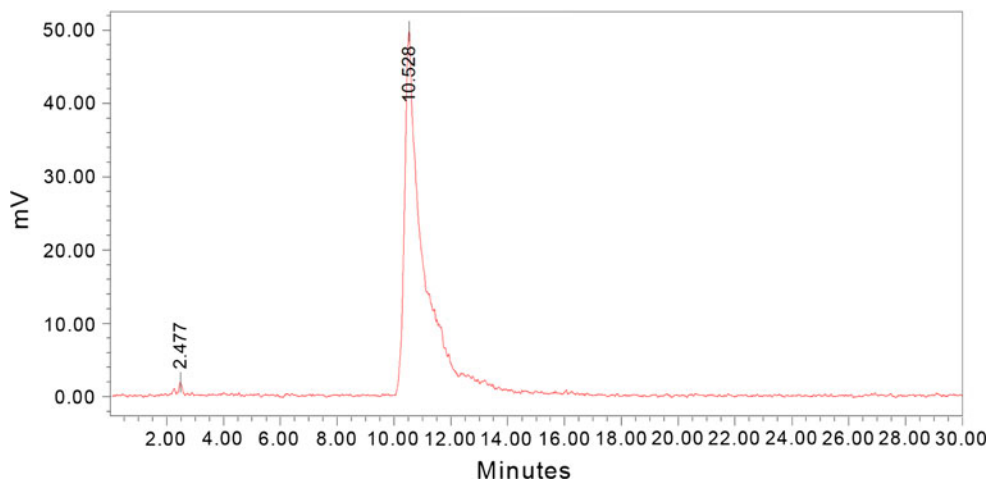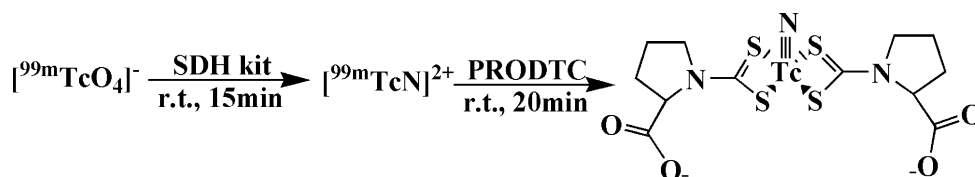**Scheme 2** Preparation procedure and speculative structure of ^{99m}TcN-PRODTC

$$[^{99m}TcO_4]^- \xrightarrow[\text{r.t., 15min}]{\text{SDH kit}} [^{99m}TcN]^{2+} \xrightarrow[\text{r.t., 20min}]{\text{PRODTC}}$$

Fig. 1 HPLC pattern of ^{99m}TcN-PRODTC

similar results are also found in $[^{99m}Tc(Tyrosine)(H_2O)(CO)_3]$ and $[^{99m}Tc(Lysine)(H_2O)(CO)_3]$ complexes [18].

Partition coefficient (log P)

The partition coefficient (log P) value of ^{99m}TcN-PRODTC was calculated as -2.80 ± 0.11, indicating it was hydrophilic.

Paper electrophoresis

The electrophoresis results showed that the complex moved to the anode (percentage of radioactivity >98 %), suggesting it was negative charged.

In vitro cell study

The result of cell uptake of ^{99m}TcN-PRODTC is given in Fig. 2. From Fig. 2, ^{99m}TcN-PRODTC had optimal uptake between 2 h and 3 h post-incubation and reached a high cell uptake. However, at 4 h post-incubation, a decreased uptake of ^{99m}TcN-PRODTC in murine sarcoma S180 cells was observed.

Biodistribution study

The results of biodistribution of ^{99m}TcN-PRODTC in mice bearing S180 tumor are shown in Table 1. Results of biodistribution of recently reported ^{99m}Tc complexes as tumor imaging agents are shown in Table 2 for comparison.
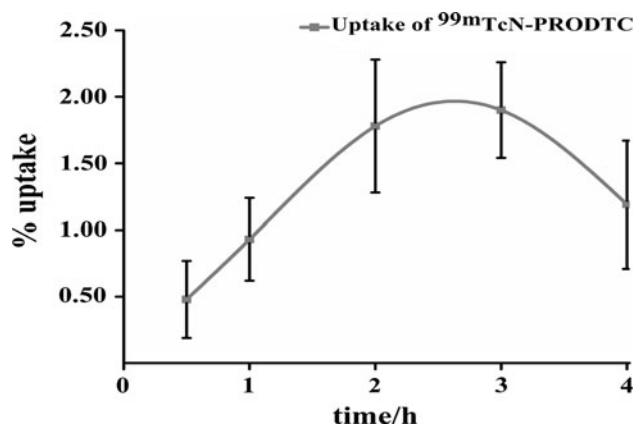
Fig. 2 In vitro cell uptake of ^{99m}TcN-PRODTC

As described in Table 1, ^{99m}TcN-PRODTC has a certain tumor uptake (1.10 % ID/g at 0.5 h post-injection). The initial blood uptake is high, but its clearance is faster than that of the tumor so that the tumor/blood ratio is higher. The muscle uptake is low so the T/N ratio is better. The tumor uptake decreases at 4 h post-injection. The results are in accordance with the cell uptake results. Therefore, the optimal imaging time may be conducted between 2 and 3 h post-injection. The initial uptakes of the liver and kidneys are high, suggesting the main routes of excretion are via the urinary tract and through the hepatobiliary. The clearance of the liver and kidneys is rapid, thus making it possible to acquire good imaging results. Further imaging studies in more extensive animal models are in due course.

Table 1 Biodistribution of ^{99m}TcN-PRODTC in mice bearing S 180 tumor (%ID/g)

Tissue	0.5 h	2 h	4
Heart	1.15 ± 0.43	0.28 ± 0.17	0.06 ± 0.03
Liver	10.52 ± 3.80	1.23 ± 0.14	0.41 ± 0.08
Lung	1.76 ± 0.48	0.31 ± 0.12	0.19 ± 0.07
Kidney	3.59 ± 0.86	1.42 ± 0.30	0.79 ± 0.14
Spleen	0.81 ± 0.30	0.12 ± 0.04	0.05 ± 0.01
Brain	0.14 ± 0.07	0.03 ± 0.02	0.01 ± 0.00
Muscle	0.32 ± 0.03	0.13 ± 0.06	0.09 ± 0.05
Blood	3.20 ± 1.21	0.27 ± 0.15	0.21 ± 0.09
Tumor	1.10 ± 0.24	0.59 ± 0.09	0.32 ± 0.09
T/N ratio	3.44	4.54	3.56
T/B ratio	0.34	2.19	1.52

All the data are the mean percentage (n = 5) of the injected dose of ^{99m}TcN-PRODTC per gram of tissue, ±the standard deviation of the mean

T/N Tumor to muscle, *T/B* Tumor to blood

Table 2 Comparison of biodistribution of ^{99m}TcN-PRODTC with some reported tumor imaging agents

Complex	^{99m}TcN-PRODTC	$[^{99m}Tc(CO)_3(PA-TZ-CHC)]^+$	$[^{99m}Tc(CO)_3(IDA-PEG3-CB)]^-$
Time p.i. (h)	2	2	2
Tumor uptake (%ID/g)	0.59 ± 0.09	0.33 ± 0.08	0.85 ± 0.13
T/N ratio	4.54	3.81	2.61
T/B ratio	2.19	1.43	0.50
Reference	Present study	[19]	[20]

As seen from Table 2, among the three complexes, ^{99m}TcN-PRODTC exhibits the highest T/N ratio and T/B ratio. As for tumor uptake, a decrease in the order is observed: $[^{99m}Tc(CO)_3(IDA-PEG3-CB)]^- > {}^{99m}TcN$-PRODTC $> [^{99m}Tc(CO)_3(PA\text{-}TZ\text{-}CHC)]^+$.

Conclusion

In summary, PRODTC ligand was successfully synthesized and its ^{99m}Tc nitrido complex was achieved in high yield. The moderate tumor localization, high tumor/blood and tumor/muscle ratios of the complex in mice exhibited favorable properties, suggesting the possibility of the complex as a novel tumor imaging agent.

References

1. Jager PL, Vaalburg W, Prium J, De Vries EGE, Langen KJ, Piers DA (2001) J Nucl Med 42:432
2. Leskinene-Kallio S, Ruotsalainen U, Någren K, Teräs M, Joensuu H (1991) J Nucl Med 32:1211
3. Nettelbladt OS, Sundin AE, Valind SO, Gustafsson GR, Lamberg K, Långstrom B, Björnsson EH (1998) J Nucl Med 39:640
4. Inoue T, Tomiyoshi K, Higuichi T, Ahmed K, Sarwar M, Aoyagi K, Amano S, Alyafei S, Zhang H, Endo K (1998) J Nucl Med 39:663
5. Inoue T, Shibasaki T, Oriuchi N, Aoyagi K, Tomiyoshi K, Amano S, Mikuni M, Ida I, Aoki J, Endo K (1999) J Nucl Med 40:399
6. Wester HJ, Herz M, Weber W, Heiss P, Senekowitsch-Schmidtke R, Schwaiger M, Stöcklin G (1999) J Nucl Med 40:205
7. Wang MW, Yin DZ, Cheng DF, Li GC, Wang YX (2006) J Radioanal Nucl Chem 270:439
8. Pauleit D, Stoffels G, Bachofner A, Floeth FW, Sabel M, Herzog H, Tellmann L, Jansen P, Reifenberger G, Hamacher K, Coenen HH, Langen KJ (2009) Nucl Med Biol 36:779
9. Langen KJ, Jarosch M, Hamacher K, Muhlensiepen H, Weber F, Floeth F, Pauleit D, Herzog H, Coenen HH (2004) Nucl Med Biol 31:67
10. Kong FL, Zhang YH, Ali MS, Oh C, Mendez R, Kohanim S, Tsao N, Chanda M, Huang WC, Yang DJ (2010) Nucl Med Commun 31:699
11. Sinha D, Shukla G, Tiwari AK, Chaturvedi S, Chuttani K, Chandra H, Mishra AK (2009) Chem Biol Drug Des 74:159
12. Hazari PP, Shukla G, Goel V, Chuttani K, Kumar N, Sharma R, Mishra AK (2010) Bioconjugate Chem 21:229
13. Zhang JB, Guo HX, Zhang SJ, Lin Y, Wang XB (2008) Bioorg Med Chem Lett 18:5168
14. Zhang JB, Ren JL, Lin X, Wang XB (2009) Bioorg Med Chem Lett 19:2752
15. Zhang SJ, Zhang WF, Wang Y, Jin ZH, Wang XB, Zhang JB, Zhang YY (2011) Bioconjugate Chem 22:369
16. Baird IR, Cameron BR, Skerlj RT (2003) Inorg Chim Acta 353:107
17. Baldas J, Bonnyman J, Pojer PM, Williams GA, Mackay MF (1981) J Chem Soc Dalton Trans 9:1798
18. Djokić D, Janković D (2007) J Label Compd Radiopharm 50:155
19. Wang JJ, Duan XJ, Mao HN, Yang J, Tan CM, Tian Y, Wu WS (2013) J Radioanal Nucl Chem 295:227
20. Wang JJ, Yang J, Yan ZY, Duan XJ, Tan CM, Shen YL, Wu WS (2011) J Radioanal Nucl Chem 287:465