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Preparation and biological evaluation of ^{99m}TcN-labeled pteroyl-lys derivative as a potential folate receptor imaging agent

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In order to develop a novel ^{99m}Tc-labeled folate receptor (FR) imaging agent, a dithiocarbamate derivative, pteroyl-lys-DTC, was synthesized and radiolabeled with ^{99m}Tc through the $[^{99m}TcN]^{2+}$ intermediate. The radiochemical purity of the corresponding ^{99m}Tc-complex, ^{99m}TcN-pteroyl-lys-DTC, was over 95% as measured by reversed-phase HPLC. The ^{99m}TcN complex was stable under physiological conditions. ^{99m}TcN-pteroyl-lys-DTC exhibited specific FR binding in FR-positive KB cells *in vitro*. The biodistribution in tumor-bearing mice showed that the ^{99m}TcN-labeled radiotracer had good uptake (3.56 ± 0.09%ID/g at 2 h postinjection) in FR-positive KB tumors, as well as in the kidneys (30.34 ± 3.53%ID/g at 2 h postinjection). After coinjection with excess folic acid, the uptake in tumor and kidneys was significantly blocked. The results indicated that ^{99m}TcN-pteroyl-lys-DTC was able to target the FR-positive tumor cells and tissues specifically both *in vitro* and *in vivo*.

Keywords: ^{99m}Tc-nitrido complex; folate receptor; tumor uptake; pteroyl-lys

Introduction

Folate, vitamin B_9, is required for the survival and proliferation of eukaryotic cells and involved as a coenzyme in many biosynthetic and epigenetic processes.[1] Three folate transporters have been found to contribute to the folates' absorption mechanisms[2,3]: (i) the reduced folate carrier, a high capacity but low affinity membrane transporter; (ii) the proton-coupled folate transporter, which is highly expressed in the duodenum and jejunum and transports preferentially at low pH; and (iii) the folate receptor (FR), which binds folate in high affinity and internalizes folic acid into cells by endocytosis. The FR is over-expressed on ovarian cancers (more than 90%) and various epithelial tumor cells, such as breast, cervical, colorectal, renal, and nasopharyngeal cancers. Meanwhile, the FR is highly restricted in most normal human tissues,[4–6] which makes the FR an important molecular target to develop novel radiolabeled probes for FR-positive tumor detection.[7]

For the past few years, several folate-based radiopharmaceuticals have been investigated for single-photon emission computed tomography and positron emission tomography imaging purpose.[8–10] Among them, ^{111}In-DTPA-folate (γ) has been evaluated in phase I/II clinical studies and appeared to be an effective probe to distinguish benign and malignant ovarian masses.[11–13] Compared with ^{111}In, ^{99m}Tc is the most widely used radionuclide in routine nuclear medicine because of its almost ideal physical decay properties ($T_{1/2}$ = 6.02 h, E_γ = 140 Kev) and the availability through commercial ^{99}Mo/^{99m}Tc generator.[14] The development of new ^{99m}Tc-based FR imaging agents with optimal characteristics has been a subject of interest in the radiopharmaceutical field. Several bifunctional chelating systems, such as the hydrazinonicotinic acid (HYNIC),[9] DTPA-type ligand, [15,16] $^{99m}Tc(CO)_3$ chelating systems,[17,18] and N_3S-type (Dap-Asp-Cys for EC20) ligand[19,20] have been reported for the preparations of these ^{99m}Tc-labeled folate conjugates. In this study, we selected the [^{99m}TcN] chelating system to prepare a novel ^{99m}Tc-based FR imaging agent.

Compared with ^{99m}TcO core, the nitride ligand of $[^{99m}Tc \equiv N]^{2+}$ is a more powerful π-electron donor and has a higher capacity to stabilize the Tc (V) oxidation state.[21] Reported herein are the synthesis of the pteroyl-lys-DTC, radiolabeling, cell binding, and preliminary *in vivo* evaluation of ^{99m}TcN-pteroyl-lys-DTC.

Results and discussion

Chemistry

On the basis of the structure of pteroyl-lys[22], in which the glutamate (Glu) residue of folate is replaced by a lysine, the novel pteroyl-lys-DTC derivative was prepared by treatment with carbon disulfide in basic condition (Scheme 1). The active ε-amino group of the lysine moiety reacted with carbon disulfide in KOH solutions to form the corresponding dithiocarbamate derivative. The pteroyl-lys-DTC was synthesized in a moderate yield of 55%, and the product was characterized by infrared (IR) spectrum, NMR spectrum and electrospray ionization mass spectrometry (ESI-MS). In IR spectra, the stretching band at 998cm^{-1} (characteristic of C = S) indicated the desired dithiocarbamate derivative. IR/cm^{-1}: 1510, 1441 (C—N), 998

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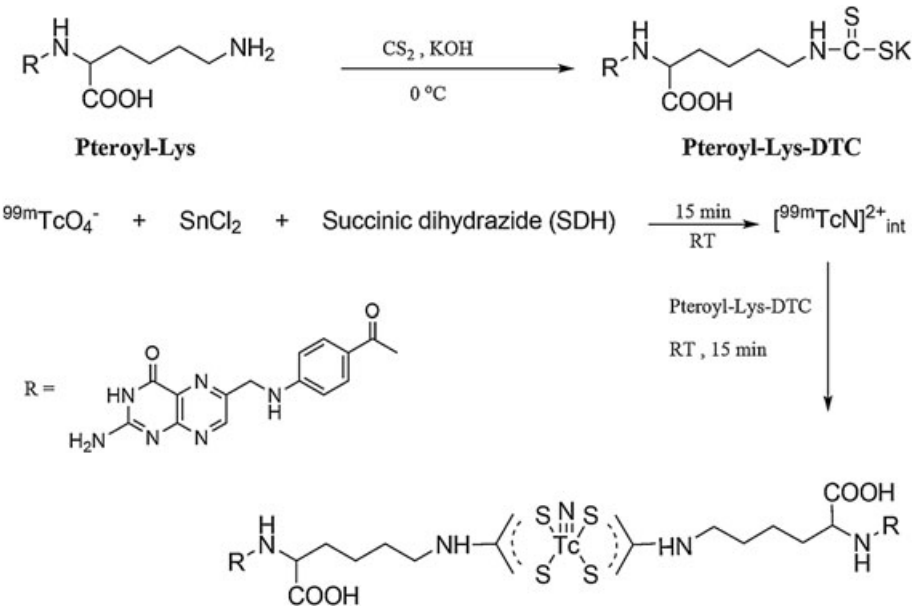

Scheme 1. Synthetic routes of the pteroyl-lys-DTC and the corresponding ^{99m}Tc-nitrido complex.

(C=S). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 1.37 (m, 2H), 1.54 (m, 2H), 1.78 (m, 2H), 2.76 (m, 2H), 4.3 (s, 1H), 4.49 (s, 2H), 6.64 (d, 2H, J = 8.7 Hz), 7.66 (d, 2H, J = 8.7 Hz), 8.64 (s, 1H); ^{13}C-NMR (DMSO-d_6, 400 MHz) δ 23.8, 27.3, 31.3, 45.9, 47.6, 55.5, 112.4, 128.8, 130.8, 147.4, 150.8, 155.3, 163.9, 168.2, 172.9, 175.4, 179.5, 181.3, 209.8; ESI-MS: *m/z* [M^+] calculated for 554.1, found 554.2

Radiochemistry

The complex ^{99m}TcN-pteroyl-lys-DTC was prepared by a two-step procedure, as shown in Scheme 1.

The succinic dihydrazide (SDH) kit vial, containing the following lyophilized formulation: 0.05 mg $SnCl_2 \cdot 2H_2O$, 5.0 mg SDH, and 5.0 mg propylenediamine tetraacetic acid, was used for preparing the $[^{99m}TcN]^{2+}$ intermediate. For $[^{99m}Tc \equiv N]^{2+}$ labeling, SDH plays the role of an efficient donor of nitride atoms (N^{3-}), and $SnCl_2 \cdot 2H_2O$ behaves as reducing agent. The presence of propylenediamine tetraacetic acid is required in order to prevent precipitation of Sn^{2+} in the form of insoluble tin salts. The complex, ^{99m}TcN-pteroyl-lys-DTC, was obtained by adding the pteroyl-lys-DTC into the $[^{99m}TcN]^{2+}$ intermediate at RT for 15 min. The final complex was purified by HPLC to remove the excess unlabeled pteroyl-lys-DTC. After HPLC purification, the ^{99m}TcN-pteroyl-lys-DTC was obtained with overall radiochemical yields of 40~50% (uncorrected for decay) and a specific activity of more than 1.85 GBq/nmol.

The labeling efficiency and radiochemical purity of the $[^{99m}Tc \equiv N]^{2+}$ intermediate and of ^{99m}TcN-pteroyl-lys-DTC were evaluated with thin layer chromatography (TLC) and HPLC. The thin-layer chromatography analysis was carried out on two different systems. The Rf values of $^{99m}TcO_4^-$, $^{99m}TcO_2 \cdot xH_2O$, $[^{99m}Tc \equiv N]^{2+}$ intermediate, and ^{99m}TcN-pteroyl-lys-DTC are listed in Table 1. In system 1 (a polyamide film with saline as the mobile phase), $^{99m}TcO_4^-$ and $^{99m}TcO_2 \cdot xH_2O$ remained at the point of spotting (R_f = 0.0~0.1), while the $[^{99m}Tc \equiv N]^{2+}$ intermediate moved with the solvent front (R_f = 0.8~1.0). As confirmed by TLC, the $[^{99m}Tc \equiv N]^{2+}$ intermediate can be easily produced in high labeling yield (>95%) with the SDH kit. When the pteroyl-lys-DTC was added, successful ligand exchange was evidenced by the disappearance of the radioactivity at R_f of 0.8~1.0 and formation of new radioactivity accumulation at origin (R_f 0.0~0.1). The system 2 (an instant TLC-silica gel strip with acid-citrate-dextrose buffer as the mobile phase) [26] was used to distinctly separate the $^{99m}TcO_4^-$ and $^{99m}TcO_2 \cdot xH_2O$ from the ^{99m}TcN-pteroyl-lys-DTC, in which, ^{99m}TcN-pteroyl-lys-DTC remained at the origin (R_f = 0.0~0.1), while $^{99m}TcO_4^-$ and $^{99m}TcO_2 \cdot xH_2O$ moved at R_f 0.7~1.0. Figure 1 shows the HPLC chromatograms of $[^{99m}Tc \equiv N]^{2+}$ intermediate and ^{99m}TcN-pteroyl-lys-DTC. The retention time of $[^{99m}Tc \equiv N]^{2+}$ intermediate was 2.3 min, while that of ^{99m}TcN-pteroyl-lys-DTC was found to be 20.5 min. The single peak suggested that only one product was formed. The radiochemical purity of ^{99m}TcN-pteroyl-lys-DTC was more than 95% determined by TLC and HPLC.

^{99m}TcN-pteroyl-lys-DTC exhibited a high stability in saline at room temperature. After 4 h incubation with mouse serum at 37 °C, more than 60% of the radioactivity added remained with the precipitate fraction when 200 μL of methanol was added to precipitate the proteins. However, the radioactivity

Table 1. The Rf values of $^{99m}TcO_4^-$, $^{99m}TcO_2 \cdot xH_2O$, $[^{99m}Tc \equiv N]^{2+}$ intermediate, and ^{99m}TcN-pteroyl-lys-DTC in two thin layer chromatography systems

	System 1 (polyamide film/saline)	System 2 (ITLC-SG/ACD)
$^{99m}TcO_4^-$	0.0~0.1	0.7~1.0
$^{99m}TcO_2 \cdot xH_2O$	0.0	0.7~1.0
$[^{99m}Tc \equiv N]^{2+}$ intermediate	0.8~1.0	0.7~1.0
^{99m}TcN-pteroyl-lys-DTC	0.0~0.1	0.0~0.1

ITLC-SG, instant TLC-silica gel; ACD, acid-citrate-dextrose.

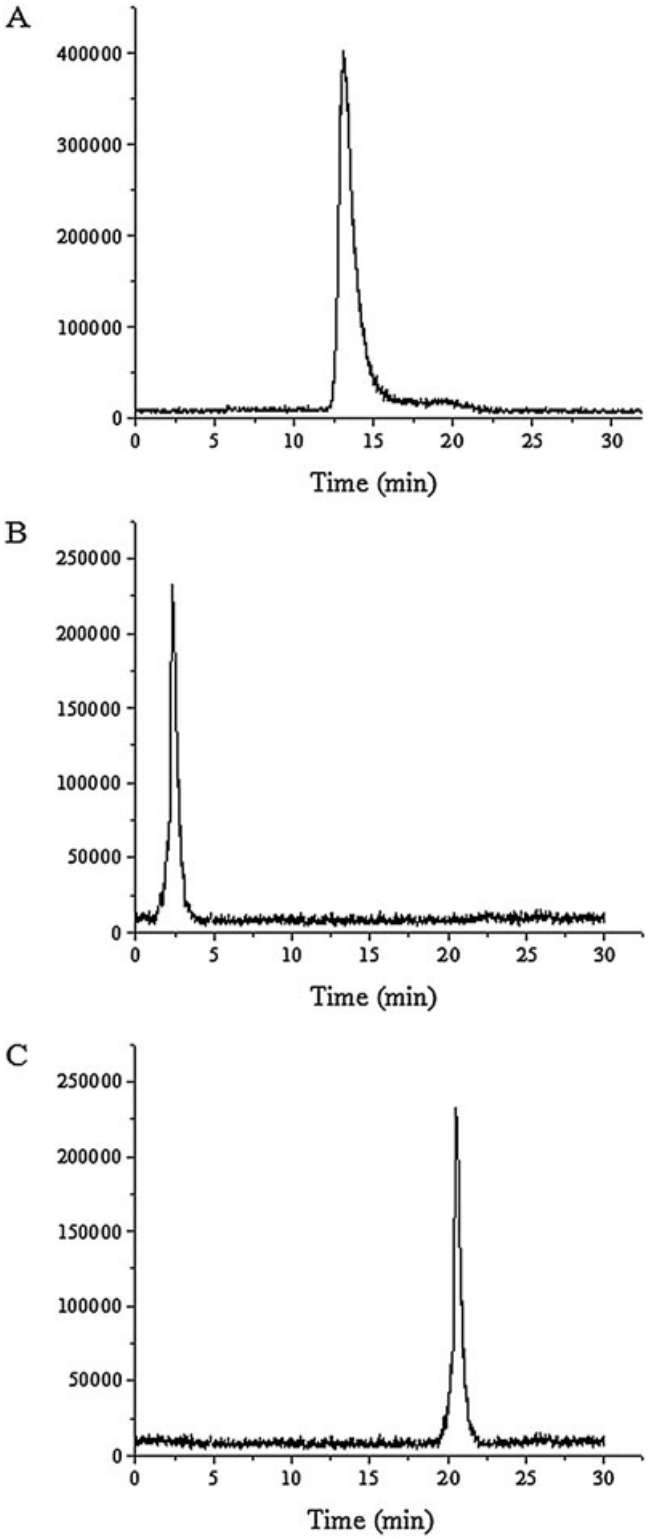

Figure 1. Radioactive HPLC profiles of $^{99m}TcO_4^-$ (A), $[^{99m}TcN]^{2+}$ intermediate (B), and ^{99m}TcN-pteroyl-lys-DTC (C). The retention time of $^{99m}TcO_4^-$, $[^{99m}TcN]^{2+}$ intermediate, and ^{99m}TcN-pteroyl-lys-DTC were 12.9, 2.3, and 20.5 min, respectively.

could disassociate when more methanol was added to wash the proteins, indicating significant unspecific serum protein binding. The analytical results of the supernatant displayed that no new radioimpurities were detected by reversed-phase (RP)-HPLC, and the radiochemical purity was more than 90% after 4 h of incubation in serum. The partition coefficient (log*P*) of the complex ^{99m}TcN-pteroyl-lys-DTC was -1.40 ± 0.01 at pH 7.0.

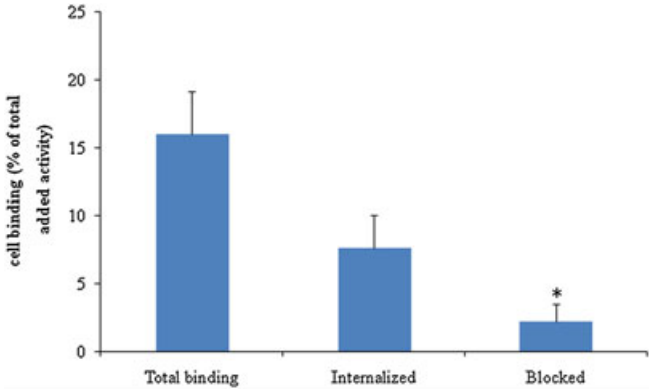

Figure 2. Binding of ^{99m}TcN-pteroyl-lys-DTC in KB cells; cell total binding, internalization, and blocked by excess folic acid (*$p < 0.05$).

Cell experiment

To evaluate the ability of ^{99m}TcN-pteroyl-lys-DTC to target FRs, the specificity of cell uptake and internalization studies were performed with the FR-positive KB tumor cells. The results are shown in Figure 2. The complex ^{99m}TcN-pteroyl-lys-DTC displayed a high cell binding of $16.03 \pm 3.15\%$ of the total added radioactivity. The internalized fraction was $7.63 \pm 2.43\%$ of total added radioactivity. The cellular uptake of ^{99m}TcN-pteroyl-lys-DTC was significantly blocked by incubation of excess folic acid (<2% of the total activity, $p < 0.05$). These data demonstrated that the cellular uptake of ^{99m}TcN-pteroyl-lys-DTC was FR specific, and more than 40% of total cellular uptake could be internalized in the cells.

Biodistribution

To further evaluate the ability of ^{99m}TcN-pteroyl-lys-DTC to target FR *in vivo*, the biodistribution and pharmacokinetics were performed in athymic BALB/C nude mice bearing KB tumor. The results are shown in Table 2. The complex ^{99m}TcN-pteroyl-lys-DTC exhibited significant tumor uptake of $3.56 \pm 0.09\%$ID/g at 2 h postinjection and a good retention ($1.71 \pm 0.53\%$ID/g at 4 h postinjection). Clearance from the blood was fast, leading to an increasing tumor-to-blood ratio over time (from 1.26 at 1 h post injection to 4.14 at 2 h postinjection). A significant accumulation and retention of radioactivity were observed in the kidneys ($30.34 \pm 3.53\%$ID/g at 2 h postinjection and $18.12 \pm 1.08\%$ID/g at 4 h postinjection), because the kidney proximal tubule cells express a high concentration of FR on their apical membranes. Coinjection of excess folic acid significantly blocked the tumor uptake ($1.67 \pm 0.22\%$ID/g at 2 h postinjection, $p < 0.05$), as well as the accumulation of radioactivity in the kidney ($18.78 \pm 1.62\%$ID/g at 2 h postinjection, $p < 0.05$), indicating the FR-specific uptake of the radiotracer in these FR-positive tissues.

Although ^{99m}TcN-pteroyl-lys-DTC was stable in mouse serum *in vitro*, the urine analysis showed that about 53% of ^{99m}TcN-pteroyl-lys-DTC was metabolized into pertechnetate formation at 1 h after injection (Figure 3), which might be responsible for the high radioactivity accumulation in the stomach ($7.66 \pm 2.52\%$ID/g at 1 h after injection).

Table 2. Biodistribution of ^{99m}TcN-pteroyl-lys-DTC in athymic nude mice bearing KB tumor xenografts[a]

	%ID/g (mean ± standard deviation)			
	1 h	2 h	2 h (blocking)[b]	4 h
Tissue				
Heart	1.85 ± 0.05	1.72 ± 0.30	2.46 ± 0.29	1.96 ± 0.08
Liver	9.84 ± 0.43	9.57 ± 0.94	13.71 ± 4.76	7.37 ± 0.61
Lung	4.52 ± 0.59	5.58 ± 1.33	6.76 ± 1.79	6.45 ± 1.13
Kidney	18.93 ± 0.64	30.34 ± 3.53	18.78 ± 1.62[c]	18.12 ± 1.08
Spleen	2.55 ± 0.43	2.85 ± 0.03	3.92 ± 1.40	2.85 ± 0.33
Stomach	7.66 ± 2.52	2.89 ± 0.38	12.31 ± 8.6	11.51 ± 1.15
Bone	1.06 ± 0.10	2.14 ± 0.85	3.04 ± 0.42	2.20 ± 0.58
Muscle	1.21 ± 0.12	1.14 ± 0.33	2.35 ± 1.10	1.09 ± 0.36
Intestines	2.66 ± 0.63	3.32 ± 0.87	4.72 ± 2.34	2.43 ± 1.05
Blood	1.84 ± 0.39	0.86 ± 0.75	1.75 ± 0.62	0.89 ± 0.03
Thyroid	4.77 ± 0.76[d]	-	-	-
Tumor	2.31 ± 0.21	3.56 ± 0.09	1.67 ± 0.22 [c]	1.71 ± 0.53
Ratios				
Tumor/Blood	1.26	4.14	-	1.92
Tumor/Muscle	1.90	3.12	-	1.56

[a]All data are the mean percentage ($n = 5$) of the injected dose of ^{99m}TcN-pteroyl-lys-DTC per gram of tissue (%ID/g), ± the standard deviation of the mean.
[b]Coinjection of excess folic acid.
[c]$p < 0.05$, significance comparisons on tumor and kidney uptakes between the radiotracers with or without folate blockade at 2 h postinjection.
[d]The thyroid uptake of ^{99m}TcN-pteroyl-lys-DTC in normal nude mice ($n = 3$) at 1 h postinjection.

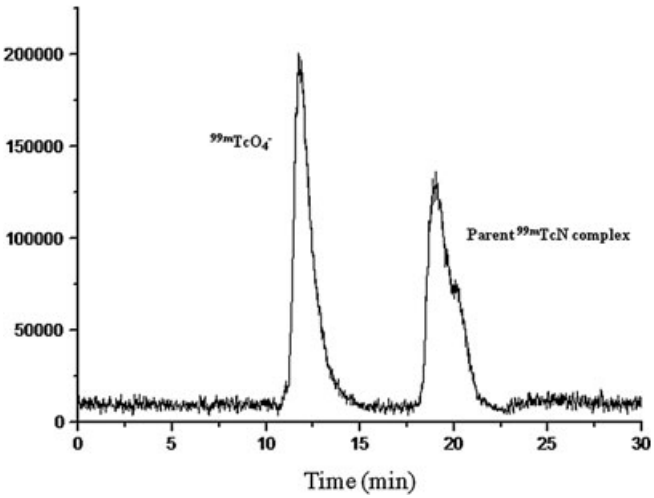

Figure 3. Radioactive HPLC profile of the urine sample of a nude mouse at 1 h postinjection of ^{99m}TcN-pteroyl-lys-DTC.

The folic acid, a combination of the pteroic acid and Glu, offers two positions for derivatization, the α-carboxylic and γ-carboxylic acid. The role of the Glu residue of folate and the necessity of a free α-carboxyl group to retain binding affinity to FR are still debated in the literature.[23,24] The results of recent studies showed both γ-glutamyl-linked and α-glutamyl-linked conjugates were able to bind to FR-positive cells *in vitro*.[17,25] However, the γ-derivative possessed more favorable pharmacokinetic features than the α-derivative, which indicated that the γ-carboxyl group should be a better site for derivatization to synthesize the folate conjugates. Unfortunately, the preparation of the defined γ-derivatives is often difficult, because both carboxyl groups of the Glu moiety have similar reactivities for the traditional coupling reactions. Recently, our group has reported a new strategy to synthesize derivatives with free α-carboxylic group based on the structure of pteroyl-lys, in which the Glu residue of folate is replaced by a lysine.[22] In this study, a dithiocarbamate derivative of pteroyl-lys was obtained by the reaction of the active ε-amine group of the lysine moiety with carbon disulfide in KOH solutions. As we anticipated, the corresponding ^{99m}TcN-labeled complex still retained the FR binding potential and could target the receptor specifically both *in vitro* and *in vivo*. These results further confirmed that the Glu moiety of folate is not critical for the FR recognition, highlighting the potential of this class of ^{99m}TcN-labeled pteroyl-lys conjugates for the FR imaging.^{99m}TcN-pteroyl-lys-DTC exhibited good tumor uptake (3.56 ± 0.09%ID/g at 2 h postinjection). The tumor-to-muscle and tumor-to-blood ratios were 3.12 and 4.14 at 2 h postinjection, respectively. These results indicated the potential of this class of ^{99m}TcN-pteroyl-lys-DTC conjugates for FR-positive tumor imaging.

Several ^{99m}Tc labeling bifunctional chelating systems, such as the ^{99m}Tc(CO)$_3$ chelating systems [17,18] and HYNIC-conjugated ligands,[9,22] have been used for the preparations of FR targeting agents. Table 3 illustrates the comparison of the partition coefficient values (log*P*) and biodistribution data between ^{99m}TcN-pteroyl-lys-DTC, ^{99m}Tc(CO)$_3$-his-folate,[17] and ^{99m}Tc(HYNIC-lys-pteroyl)(Tricine/TPPTS)[22] in the same KB tumor-bearing mice model at 1 h postinjection. The data for ^{99m}Tc(HYNIC-lys-pteroyl)(Tricine/TPPTS) were obtained from previous studies of our group. The tumor uptake of ^{99m}TcN-pteroyl-lys-DTC (2.31 ± 0.21%ID/g at

Table 3. Comparison of the partition coefficient value (log*P*) and biodistribution data between ^{99m}TcN-pteroyl-lys-DTC, ^{99m}Tc(CO)$_3$-his-folate, and ^{99m}Tc(HYNIC-lys-pteroyl)(Tricine/TPPTS) in the same KB tumor-bearing mice model at 1 h postinjection

Complex	^{99m}TcN-pteroyl-lys-DTC	^{99m}Tc(CO)$_3$-his-folate	^{99m}Tc(HYNIC-lys-pteroyl)(Tricine/TPPTS)
log*P*	−1.40 ± 0.10	-	−2.89 ± 0.06
Tumor (%ID/g)	2.31 ± 0.21	2.63 ± 0.48	5.39 ± 0.40
Liver (%ID/g)	9.84 ± 0.43	9.73 ± 1.32	3.19 ± 0.09
Intestines (%ID/g)	2.66 ± 0.63	3.84 ± 2.23	1.60 ± 0.58
Reference	Present study	[17]	[22]

1 h postinjection) is comparable with that of ^{99m}Tc(CO)$_3$-his-folate (2.63 ± 0.48%ID/g at 1 h postinjection) but is 50% less than that of ^{99m}Tc(HYNIC-lys-pteroyl)(Tricine/TPPTS) (5.39 ± 0.40% ID/g at 1 h postinjection). ^{99m}TcN-pteroyl-lys-DTC displayed a significant accumulation of radioactivity in liver (9.84 ± 0.43%ID/g at 1 h postinjection), as well as in the intestines (2.66 ± 0.63%ID/g at 1 h postinjection), which were similar to those of ^{99m}Tc(CO)$_3$-his-folate (9.73 ± 1.32%ID/g and 3.84 ± 2.23%ID/g at 1 h postinjection, respectively). The results suggested these radiotracers were partially excreted via the hepatobiliary and gastrointestinal extraction. Generally, hepatobiliary elimination often correlates with the lipophilicity of a drug. ^{99m}TcN-pteroyl-lys-DTC (log*P* = −1.40 ± 0.10) exhibited higher lipophlicity than ^{99m}Tc(HYNIC-lys-pteroyl)(Tricine/TPPTS) (log*P* = −2.89 ± 0.06). The liver uptake of ^{99m}TcN-pteroyl-lys-DTC was three times higher than that of ^{99m}Tc(HYNIC-lys-pteroyl)(Tricine/TPPTS). The hepatobiliary extraction of ^{99m}TcN-pteroyl-lys-DTC is undesirable, because the high abdominal accumulation will hamper the tumor visualization in this region. Introduction of one more hydrophilic linker between the ^{99m}TcN chelating moiety and targeting molecule might decrease the gastrointestinal tract accumulation of this class of ^{99m}TcN-radiolabeled radiotracers in future studies.

Experimental

Methods and materials

Pteroyl-lys[22] was prepared according to literature methods. SDH kit was obtained from Beijing Shihong Pharmaceutical Center, Beijing Normal University, China. All other chemicals were of reagent grade and used without further purification. Technetium-99m as sodium pertechnetate ($Na^{99m}TcO_4$) was obtained from commercial ^{99}Mo/^{99m}Tc generator (China Institute of Atom Energy, China) just before use. IR spectrum was obtained with an AVATAR 360 FT-IR spectrometer using KBr pellets. NMR spectra were recorded on Bruker Avance-400 (400 MHz) spectrometers. ESI-MS spectrum was recorded on a LC-MS Shimadzu 2010 series. TLC were performed on polyamide films with saline as the mobile phase (system 1) and instant TLC-silica gel strips with acid-citrate-dextrose buffer (0.068 mol/L citrate, 0.074 mol/L glucose, pH 5.0) as the mobile phase (system 2). RP-HPLC experiments were performed on a SHIMAZU SCL-10AVP HPLC pump system (SHIMAZU Corporation, Japan) and BIOSCAN flow-counter, using a Venusil XBP C18 reversed-phase column (250 × 10 mm, 5 μm), working at a flow rate of 1.0 mL/min. 10 mM NH_4HCO_3 (A) and CH_3OH (B) mixtures were used as mobile phase (0–10 min, B: 5–50%; 10–20 min, B: 50–50%; 20–25 min, B: 50–5%). All biodistribution studies were carried out in compliance with the national laws related to the conduct of animal experimentation

Synthesis of pteroyl-lys-DTC

In the dark, pteroyl-lys[22] (65 mg, 0.148 mmol) was dissolved in a solution of KOH (10 mL, 0.3 mmol). A 0.3-mL portion of carbon disulfide (4.96 mmol) was added dropwise to the solution stirred in an ice bath at 0 °C. The solution was then stirred for 3 h in the ice bath. The solvent was removed under reduced pressure and dried in vacuum. The crude product was recrystallized from ethanol/water, then washed with Et_2O (1 × 3 mL) and dried under vacuum to afford 45 mg of pteroyl-lys-DTC, yield 55%. The pteroyl-lys-DTC was characterized by IR, ^{1}H-NMR, ^{13}C-NMR, and ESI-MS spectroscopy.

Radiolabeling of ^{99m}TcN-pteroyl-lys-DTC

^{99m}TcN-pteroyl-lys-DTC was prepared by a two-step procedure. First, 1.0 mL of saline containing $^{99m}TcO_4^-$ (277 MBq) was added into a SDH kit and kept at room temperature for 15 min to obtain the $[^{99m}TcN]^{2+}$ intermediate. Then, 1 mg of the pteroyl-lys-DTC ligand was added to the $[^{99m}TcN]^{2+}$ intermediate. The mixture was kept at room temperature for 15 min. The radiolabeled ^{99m}TcN-pteroyl-lys-DTC was purified by RP-HPLC to completely separate from its excess unlabeled pteroyl-lys-DTC. The fraction (19.5–21.0 min, Rt = 20.5 min) was collected, and volatiles in HPLC mobile phase were removed under vacuum. The residues were dissolved in saline for further stability, specific binding, and animal studies. The labeling efficiency and radiochemical purity of the $[^{99m}Tc \equiv N]^{2+}$ intermediate and of ^{99m}TcN-pteroyl-lys-DTC were evaluated with TLC and HPLC.

In vitro stability

The *in vitro* stability of this ^{99m}TcN-pteroyl-lys-DTC complex was evaluated by monitoring the RCP at different time points. Briefly, HPLC purified ^{99m}Tc complex was diluted with 4–5 mL saline and kept at room temperature for 4 h. Samples of the resulting solutions were analyzed by radio-HPLC at 0, 2, and 4 h.

To 900 μL of fresh mouse serum, 100 μL of the purified ^{99m}TcN-pteroyl-lys-DTC complex solution was added and incubated at 37 °C. At 0, 2, and 4 h, 100 μL aliquots were withdrawn and treated with 200 μL methanol to precipitate the proteins. Sample was centrifuged at 3000 rpm. The supernatant was purged with N_2 gas to remove the ethanol. The resulting sample was mixed with 100 μL of water and analyzed by radio-HPLC.

Octanol/water partition coefficient

The octanol/water partition coefficient was measured by the following procedure: mixing 100 μL of the purified radiotracer solution with 1.9 mL of phosphate buffer (0.05 mol/L, pH 7.0) and 2 mL of octanol in centrifuge tube. The tube was vortexed for 2 min and then centrifuged at 4000 rpm for 5 min. the counts in 100 μL aliquots of both organic and inorganic layers were determined by use of a NaI well-type γ-counter. All experiments were performed in triplicate. The partition coefficient (*P*) was calculated using the following equation: *P* = (cpm in octanol − cpm in background)/(cpm in aqueous layer − cpm in background). The final partition coefficient value was expressed as log*P*.

Cell experiment

The specific FR binding of ^{99m}TcN-pteroyl-lys-DTC was determined in KB carcinoma cell according to our published procedure.[22] Briefly, the KB

carcinoma cell line is cultured as monolayers at 37 °C in a humidified atmosphere containing 5% CO_2, The cells were cultured in FFRPMI medium (modified RPMI 1640, without folic acid) supplemented with 10% fetal calf serum and antibiotics (penicillin 100 IU/mL, streptomycin 100 μg/mL, and fungizone 0.25 μg/mL). Twenty-four hours prior to the experiment, the KB cells were seeded in 24 well plates (2×10^5 cells/well) and incubated at 37 °C to form confluent monolayer. All experiments were performed in triplicate. After being washed once with FFRPMI medium, the cells were incubated at 37 °C for 1 h with approximately 7.4 KBq of HPLC purified complex in 1 mL of FFRPMI medium. The blocking studies were performed by coincubation of free folic acid solution (100 μM) into the incubation medium. After incubation, the reaction media were aspirated, and the cells were rinsed with 2×1 mL of cold PBS (pH 7.4). Cellular internalization of the ^{99m}Tc complex was assessed by washing the cells with 1 mL of acidic buffer (aqueous solution of 0.1 M acetic acid and 0.15 M NaCl, pH 3). Finally, the cells were lysed by treatment with 1 mL of 1 N NaOH for 5 min and transferred to tubes. All the samples were counted for radioactivity using a γ-counter. The cell binding fractions and cell internalized fractions were calculated and expressed in relation to the total added activity (% of total added activity).

Biodistribution studies

In vivo biodistribution study of ^{99m}TcN-pteroyl-lys-DTC was carried out in the athymic BALB/C nude mice bearing KB tumor. The KB tumor model was established by injection 5×10^6 cells of FR-positive KB cells in the athymic BALB/C nude mice (4- to 5-week-old female). During the 10 days after inoculation, the animals were fed with a folate-free diet, and the animals with tumors in the range of 0.5–1.0 cm^3 were used for biodistribution studies. The ^{99m}TcN-pteroyl-lys-DTC (100 μL, 0.185 MBq) was injected into the mice via the tail vein. Then the mice ($n = 5$) were sacrificed by cervical dislocation at 60, 120, and 240 min postinjection. To further determine the receptor-specific uptake of the complex, the blocking experiments were performed by coinjection with excess folic acid (100 μg/mouse). The mice ($n = 5$) were sacrificed at 120 min postinjection. The organs or tissues of interest were removed, weighted, and measured in a well-type NaI (Tl) γ-counter. The percentage of injected dose per gram (%ID/g) for each sample was calculated by comparing its activity with appropriate standard of ID. The values were expressed as mean ± standard deviation.

Metabolite study

The ^{99m}TcN-pteroyl-lys-DTC (300 μL, 18.5 MBq) was injected into a nude mouse via the tail vein. The urine sample was collected at 1 h postinjection by manual void and then directly centrifuged at 13,000 g for 5 min before the radio-HPLC analysis. The HPLC gradient already described was used for the analyses of metabolite.

Statistical Methods

Statistical analysis was performed using the Student's *t*-test for unpaired date to determine the significance of differences between the cell binding and tumor uptake of ^{99m}TcN-pteroyl-lys-DTC with or without folic acid blockade. Difference at the 95% confidence level ($p < 0.05$) was considered significant.

Conclusion

A novel pteroyl-lys conjugate, pteroyl-lys-DTC, was synthesized and labeled with ^{99m}Tc by a two-step method. The radiochemical purity of the corresponding ^{99m}Tc-complex, ^{99m}TcN-pteroyl-lys-DTC was over 95%. Both the binding in FR-positive KB cells and the uptake in KB tumors could be significantly blocked by excess folic acid, indicated that the binding of ^{99m}TcN-pteroyl-lys-DTC was FR specific. In biodistrubution studies, ^{99m}TcN-pteroyl-lys-DTC exhibited a good tumor uptake, suggesting the potential of this class of pteroyl-lys conjugates for the FR-positive tumor imaging; however, further investigation toward novel ^{99m}TcN-labeled pteroyl-lys derivatives with reduced lipophilicity are warranted.

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Conflict of Interest

The authors did not report any conflict of interest.

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