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The synthesis of pteroyl-lys conjugates and its application as Technetium-99m labeled radiotracer for folate receptor-positive tumor targeting

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ABSTRACT

Aiming to develop a new ^{99m}Tc-labeled folate derivative for FR-positive tumor imaging, a simpler method has been established to synthesize the folate-drug conjugates with free α-carboxyl group. In this study, the conjugate pteroyl-lys-HYNIC was synthesized and labeled with ^{99m}Tc using tricine and TPPTS as co-ligands. The radiochemical purity of the final complex ^{99m}Tc(HYNIC-lys-pteroyl)(tricine/TPPTS), **5** was high (>98%), and it remained stable in saline and plasma over 6 h after preparation. The biologic evaluation results showed that the ^{99m}Tc labeled pteroyl-lys conjugate was able to specifically target the FR-positive tumor cells and tissues both in vitro and in vivo, highlighting its potential as an effective folate receptor targeted agent for tumor imaging.

The folate receptor (FR), a glycosylphosphatidylinositol-anchored membrane glycoprotein, has been respected as a very attractive molecular target for tumor selective drug delivery, since it is over-expressed on a wide variety of tumor cells, but highly restricted in most normal human tissues.[1–3] The FR binds the vitamin folic acid with high affinity ($K_d = 10^{-10}$ M), and mediates the transport of folic acid into cell interior via an endocytic process.[4] Thus, the folic acid has been successfully used as a 'Trojan horse' to conjugate with drug molecules, peptides, protein, liposomes and radionuclides for targeting of FR-positive tumors.[5–7]

The folic acid, a combination of the pteroic acid (Pte) and glutamate (Glu), offers two positions for derivatization, the α- and γ-carboxylic acid (Fig. 1). 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.[8–10] The first findings indicated that the glutamate moiety of folic acid is essential for FR binding, because pteroic acid (Fig. 1), a fragment of folic acid lacking the distal glutamic acid residue shows a poor binding affinity to the FR. But the results of subsequent studies showed that the pteroic-conjugates could still selectively bind to the FR-positive tumor cells, which indicate that the Glu residue of folate is not critical for the folate receptor recognition.[10–13] The findings reported by Leamon and Müller et al. suggest that the free α-carboxyl group is not necessary for FR binding either, since both γ-glutamyl- and α-glutamyl-linked conjugates were able to bind to FR-positive cells in vitro.[11,13] However, the further biodistribution results of the radiotracers suggest that the free α-carboxyl group of glutamic acid moiety may have a significant influence on the biologic properties in vivo, since the γ-derivative possessed more favorable pharmacokinetic features than others, and the pteroate derivative showed the lower uptakes in FR-positive tissues than the folate derivatives did.[14] Therefore, the γ-carboxyl group should be the preferred site for derivatization to synthesize the folate conjugates with free α- carboxyl group.

Unfortunately, the traditional coupling reactions with folic acid result in mixtures of α- and γ-derivatives, since both carboxyl groups of the glutamate moiety have similar reactivities. Separation of these mixtures is often difficult and some biologic results have been obtained on mixtures.[15] Many efforts have been done to synthesize the defined α- and γ-derivatives.[10,11,13]

It has been shown that folate-based radiopharmaceutical can be a useful tool for the detection and characterization of FR-positive tumors by means of noninvasive molecular imaging.[15–19] In this study, in order to develop a new ^{99m}Tc-labeled pteroate derivative as a potential radiopharmaceutical for FR-positive tumor imaging, we designed and synthesized pteroyl-lys conjugates (Fig. 1), in which the glutamate residue of folate is replaced by a lysine. It is a new strategy to synthesize pteroyl-lys conjugate with free α-carboxylic group, since the ε-amion group of the lysine moiety can be easily modified and selectively couple with a functional agent.

Scheme 1 shows the synthesis of pteroyl-lys-HYNIC conjugate **2**. The reaction of pteroyl azide **3**[20] with N(ε)-BOC-Lysine in the presence of tetramethylguanidine afforded the pteroyl-lys-BOC conjugate **4**. Treatment the BOC protected compound **4** with TFA

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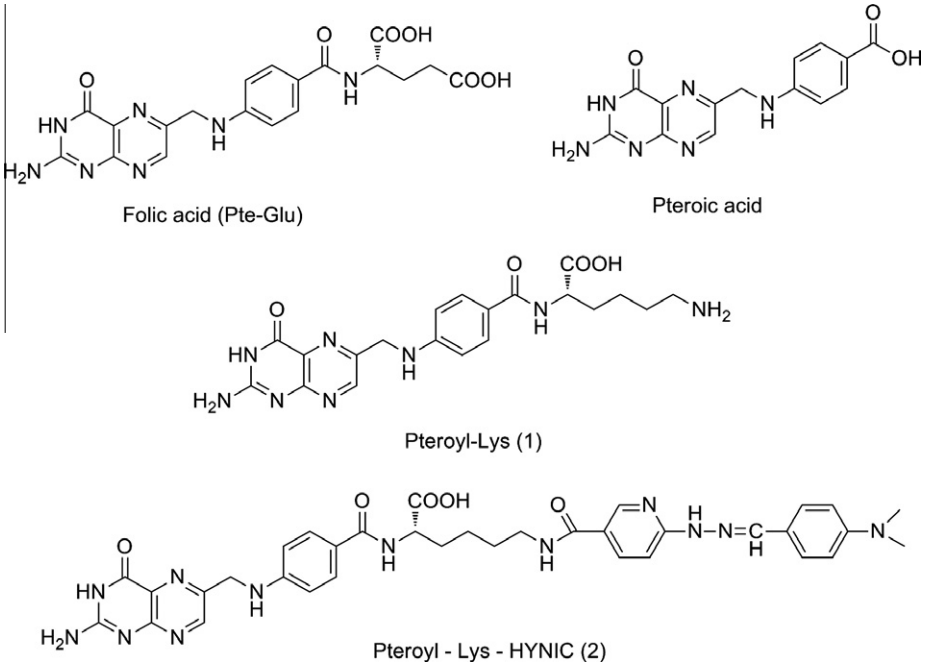
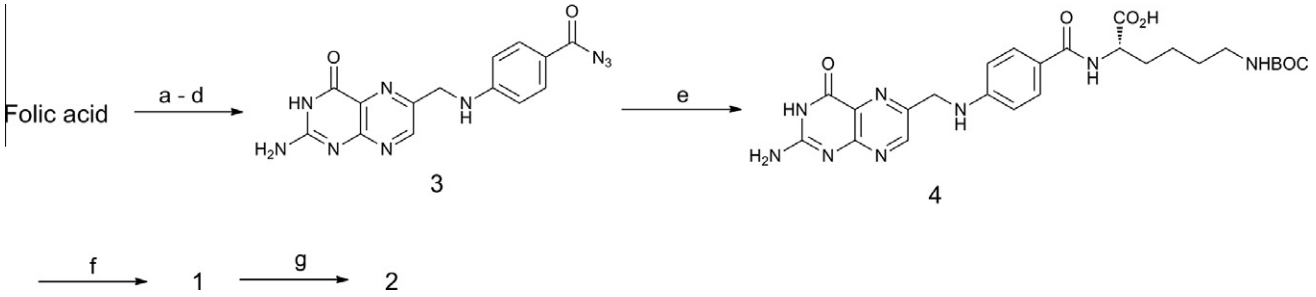


Figure 1. The structures of folic acid, pteroic acid, pteroyl-lys **1** and pteroyl-lys-HYNIC **2**.



Scheme 1. Reagents and conditions: (a) trifluoracetic anhydride, THF, 0 °C–rt, 10 h, yield 40%; (b) ice, THF, rt, 5 h, yield 98%; (c) hydrazine, DMSO, rt, 12 h, yield 72%; (d) potassium thiocyanate, trifluoroacetic, 0 °C to −10 °C; *tert*-butylnitrite, rt, 5 h, yield 67%; (e) N(ε)-BOC-Lysine, tetramethylguanidine, DMSO, rt, 7 h, yield 90%; (f) trifluoroacetic, DMF, pyridine, 0 °C, yield 94%; (g) succinimidyl 6-[2-(4-dimethylamino)benzaldehydehydrazono]nicotinate, DMSO, pyridine, rt, 16 h, yield 65%.

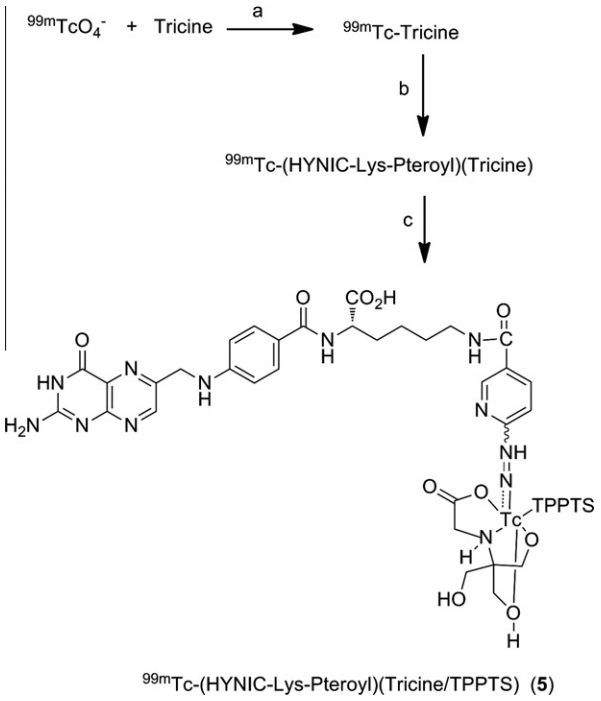
yielded the pteroyl-lys **1**.[21] Compound **1** was then coupled with succinimidyl 6-[2-(4-dimethylamino)benzaldehydehydrazono]nicotinate[22] to give the pteroyl-lys-HYNIC conjugate **2**.[23]

The conjugate **2** was labeled with ^{99m}Tc using tricine and TPPTS as co-ligands in three steps (Scheme 2). The resulting solutions were analyzed by radio-HPLC.[24] The retention times of $^{99m}TcO_4^-$, ^{99m}Tc-tricine, ^{99m}Tc(HYNIC-lys-pteroyl)(tricine) and ^{99m}Tc(HYNIC-lys-pteroyl)(tricine/TPPTS) **5** were 10.0, 12.8, 14.9 and 21.6 min, respectively. The complex **5** was purified by HPLC and its final radiochemical purity was over 98% (Fig. 2). In this study, HYNIC has been chosen as the bifunctional coupling agent due to its high labeling efficiency at low HYNIC-conjugate concentrations. The complex **5** was hydrophilic ($\log P = -2.89 \pm 0.06$, $n = 5$) and remained stable in saline and plasma over 6 h after preparation.

To assess the ability of the complex **5** to target the folate receptor, in vitro experiments were performed with KB cells, a human oral cancer cell line over expressing the FR.[13,14,25,26] As shown in Figure 3, the complex **5** displayed a high cell binding of 28.38 ± 4.15% of the total added radioactivity. The internalized fraction was 20.18 ± 3.43% of total activity. Pre-incubation of the cells with excess folic acid resulted in a significant blockade (<2% of the total activity, $p < 0.05$). These data indicate that the complex **5** can target the FR specifically and be internalized in the KB cells.

The biodistribution and pharmacokinetics of the radiotracer were evaluated in athymic mice bearing KB tumors to further investigate the FR binding properties of ^{99m}Tc-labeled folate conjugate **5** in vivo.[27] The radiolabeled complex was purified by HPLC for the biodistribution studies to remove the excess of cold ligand. A volume of 0.1 ml of the purified radiotracer solution (~150 kBq, ~90 pmol/l) was injected into the mice via the tail vein. Then the mice ($n = 3$) were sacrificed by cervical dislocation at 60, 120 and 240 min post-injection. Blocking experiments were performed by co-injected with excess folic acid (100 μg/mouse), and the mice were sacrificed at 120 min post-injection. The selected tissues and organs were removed, weighed and measured in a well-type NaI(Tl) γ-counter. The results were expressed as the percent uptake of injected dose per gram of tissue (%ID/g).

The results of biodistribution are shown in Table 1. As noted from Table 1, the tumor accumulation of radioactivity was high already at 1 h after injection (5.39 ± 0.40%ID/g). The maximal tumor accumulation was found after 2 h (9.60 ± 1.46%ID/g) and was almost completely retained over the time of investigation (7.85 ± 1.37%ID/g, 4 h after injection). Clearance from the blood was fast and the nonspecific retentions of radioactivity in nontargeted tissues (lung, spleen, liver, muscle) were low, leading to increasing tumor-to-blood and tumor-to-muscle ratios over time



Scheme 2. Reagents and conditions: (a) tricine 40 mg, $SnCl_2{\cdot}2H_2O$ 20 μg, rt, 15 min; (b) conjugate **2** 20 μg, pH 3–5, 100 °C, 40 min; (c) trisodium triphenylphosphine-3,3′,3″-trisulfonate (TPPTS) 1 mg, pH 4.5, 100 °C, 20 min.

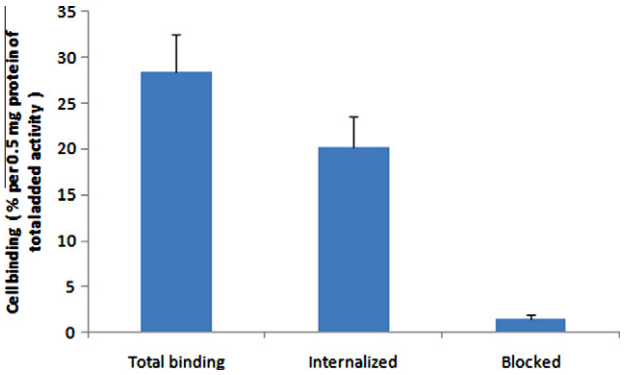


Figure 3. In vitro experiment of the ^{99m}Tc-radiotracer **5** with KB cells; cell total binding, internalization and blocked by excess folic acid (p <0.05). The cell binding were calculated per 0.5 mg protein and expressed as percentage of total added radioactivity.

(from 5.04 and 3.05 at 1 h p.i. to 25.32 and 8.01 at 4 h p.i., respectively). A significant accumulation and retention of radioactivity were also found in the kidneys (63.26 ± 3.40%ID/g at 1 h p.i. and 88.61 ± 9.17%ID/g at 4 h p.i.), since FRs are abundant in the renal proximal tubules.[28,29] Co-injection of excess folic acid almost completely blocked the tumor uptake (0.36 ± 0.10%ID/g at 2 h p.i. p <0.05), as well as the accumulation of radioactivity in the kidneys (3.79 ± 0.71%ID /g at 2 h p.i. p <0.05), indicating the FR-specific uptakes of radiofolate in these FR-positive tissues. Compared to

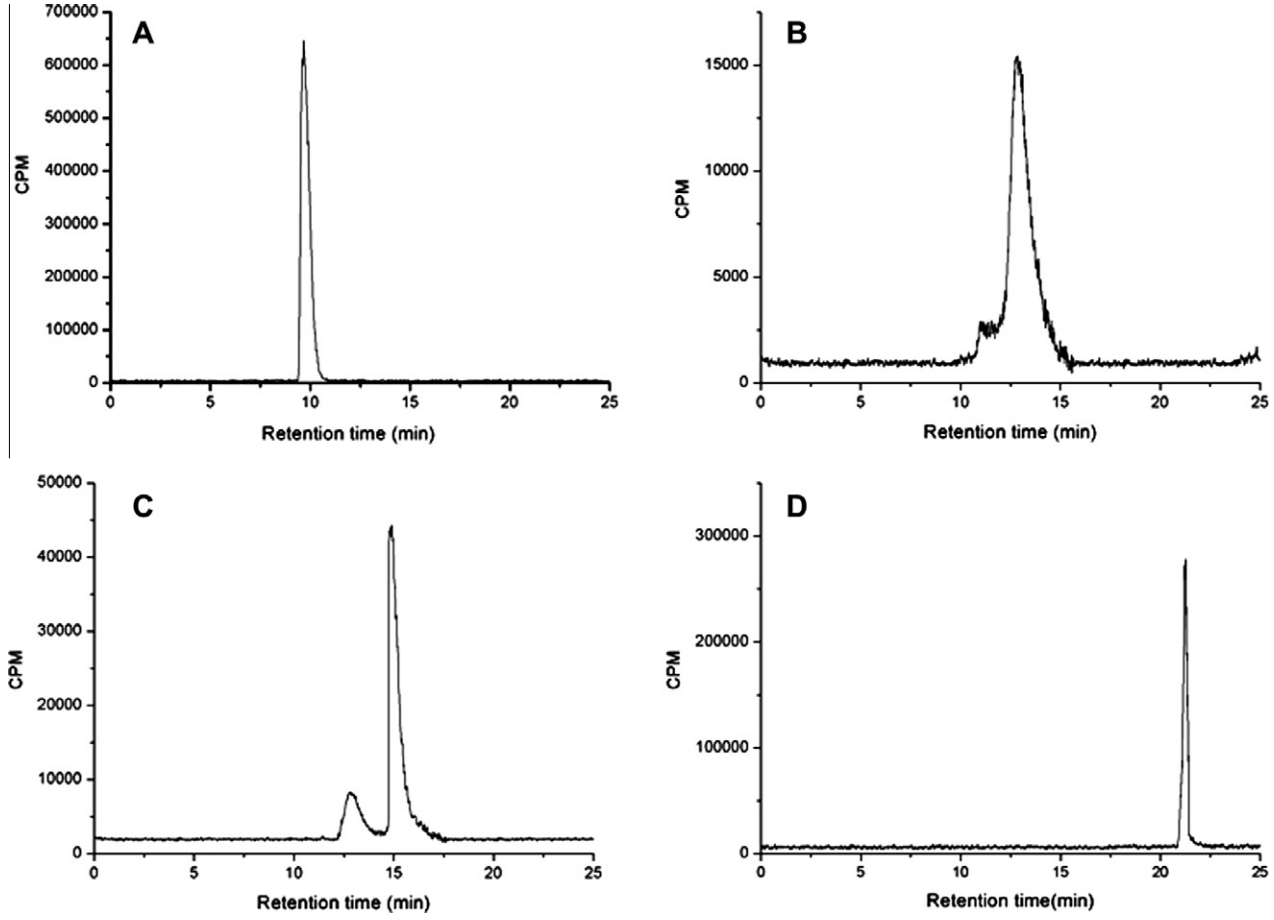


Figure 2. The HPLC profiles of $^{99m}TcO_4^-$ (A, R_t = 10.0 min), ^{99m}Tc-tricine (B, R_t = 12.8 min), ^{99m}Tc-(HYNIC-lys-pteroyl)(tricine) (C, R_t = 14.9 min) and ^{99m}Tc(HYNIC-lys-pteroyl)(tricine/TPPTS) **5** (D, R_t = 21.6 min).

Table 1
Biodistribution of ^{99m}Tc(HYNIC-lys-pteroyl)(tricine/TPPTS) in athynic nude mice bearing KB tumor xenografts[a]

	1 h	2 h	2 h blockade [b]	4 h
Tissues				
Heart	2.48 ± 0.65	2.71 ± 0.18	0.47 ± 0.06	2.03 ± 0.18
Liver	3.19 ± 0.09	2.47 ± 0.06	1.83 ± 0.33	2.08 ± 0.18
Lungs	2.30 ± 0.18	2.01 ± 0.64	0.76 ± 0.11	1.73 ± 0.13
Kidney	63.26 ± 3.40	89.60 ± 5.06	3.79 ± 0.71[c]	88.61 ± 9.17
Spleen	0.96 ± 0.33	1.01 ± 0.16	0.76 ± 0.10	0.60 ± 0.12
Stomach	2.17 ± 0.59	2.13 ± 0.74	1.62 ± 0.90	1.72 ± 0.72
Bone	1.16 ± 0.27	1.64 ± 0.60	0.66 ± 0.10	1.18 ± 0.35
Muscle	1.77 ± 0.17	1.78 ± 0.48	0.30 ± 0.12	0.98 ± 0.17
Intestines	1.60 ± 0.58	1.58 ± 0.95	0.63 ± 0.26	0.62 ± 0.17
Blood	1.07 ± 0.03	0.73 ± 0.06	0.65 ± 0.10	0.31 ± 0.07
Tumor	5.39 ± 0.40	9.60 ± 1.46	0.36 ± 0.10[c]	7.85 ± 1.37
Ratios				
Tumor/Muscle	3.05	5.39	--	8.01
Tumor/Blood	5.04	13.15	--	25.32
Tumor/Liver	1.69	3.89	--	3.77
Tumor/Kidney	0.09	0.11	--	0.09

[a] All data are the mean percentage ($n = 3$) of the injected dose of ^{99m}Tc(HYNIC-lys-pteroyl)(tricine)(TPPTS) per gram of tissue (%ID/g), ± the standard deviation of the mean.
[b] Co-injection 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 post-injection.

^{99m}Tc-HYNIC-folate, a ^{99m}Tc-labeled folate-hydrazide-HYNIC conjugate reported by Guo et al.,[19] complex **5** had a higher tumor uptake but lower no-target tissues uptakes in liver, spleen and blood (see Table 2). These results suggest that the Glu moiety of folate could be replaced by a Lys residue, and the new pteroyl-lys conjugate still retained the targeting potential to FR. The Lys residue may be acting as an efficient spacer between the Pte moiety and ^{99m}Tc-HYNIC chelating moiety, resulting in more favorable biologic characteristics as a FR imaging agent.

Table 2
The comparison of the bidistribution data between ^{99m}Tc(HYNIC-lys-pteroyl)(tricine/TPPTS) and ^{99m}Tc-HYNIC-folate at 4 h p.i.

Complex	^{99m}Tc(HYNIC-lys-pteroyl)(tricine/TPPTS)	^{99m}Tc-HYNIC-folate*
Tumor/%ID/g	7.85 ± 1.37	5.62 ± 0.75
Liver/%ID/g	2.08 ± 0.18	4.03 ± 0.41
Spleen/%ID/g	0.60 ± 0.12	2.55 ± 0.39
Blood/%ID/g	0.31 ± 0.07	0.48 ± 0.04
Tumor to liver	3.77	1.39
Tumor to spleen	13.08	2.20
Tumor to blood	25.32	11.71

* The data for ^{99m}Tc-HYNIC-folate are obtained from the studies of Liu et al. in the same KB tumor-bearing mice model.[30]

In order to visualize the distribution of the radiotracer in a living animal, planar imaging studies of complex **5** were performed in the athymic nude mice bearing KB tumors using a SPECT device. The mice were injected with approximately 7.4 MBq of complex **5**, and blocking experiment was performed by co-injected with excess folic acid (100 μg/mouse). The mice were anesthetized with 1.5% isoflurane for imaging. Figure 4 illustrates the planar images of the KB tumor-bearing mice administered with complex **5** (A) and complex **5** with excess folic acid (B) at 2 h p.i. The KB tumor could be clearly visualized and the uptakes of radiotracer in FR-positive tumor and kidney were significantly blocked by co-injection of excess folic acid. The images clearly confirmed that the conjugate ^{99m}Tc(HYNIC-lys-pteroyl)(tricine/TPPTS) could

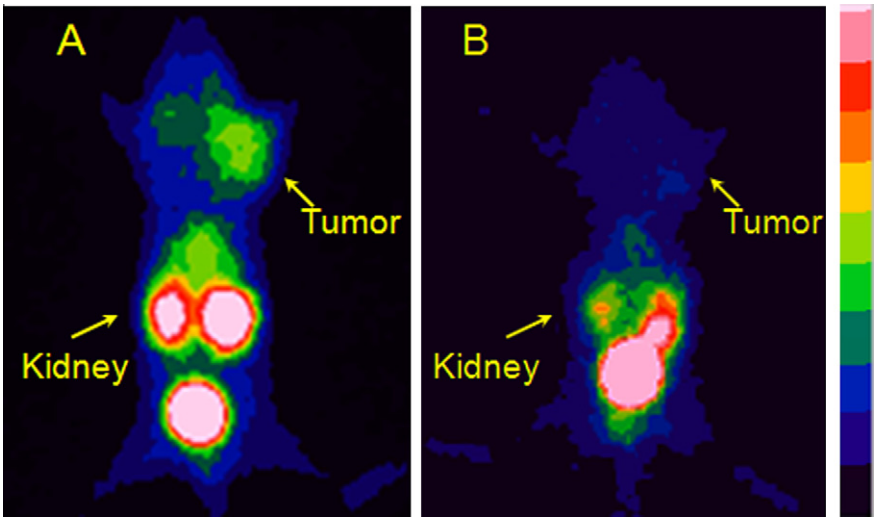


Figure 4. The planar images of the athymic nude mice bearing KB tumors (male, 18–20 g) at 2 h p.i. (A) Control: injection of 7.4 MBq complex **5**; (B) blocked: co-injection of 7.4 MBq complex **5** with excess folic acid (100 μg).

target the folate receptor efficiently and selectively in vivo, highlighting its potential as an effective imaging probe for FR-positive tumor detection.

In summary, we established a simpler method to synthesize folate-drug conjugates with free α- carboxyl group. In this study, the conjugate pteroyl-lys-HYNIC, in which the Glu residue of folic acid was replaced by a lysine as the linker between the pteroic acid and HYNIC, was synthesized and labeled with ^{99m}Tc. As we anticipated, the ^{99m}Tc labeled pteroyl-lys conjugate **5** was able to specifically target FR-positive tumor cells and tissues both in vitro and in vivo. The promising biologic evaluation results suggest that this complex could be a potential folate receptor targeted agent for tumor imaging.

Acknowledgments

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21. Pteroyl-lys,^{1}H NMR (400 MHz, DMSO-d6): δ 8.64 (s,1H), 7.66 (d, J = 8.7 Hz, 2H, Ar), 6.64 (d, J = 8.7 Hz, 2H, Ar), 4.49 (s, 2H), 4.31 (s, 1H), 2.76 (m, 2H), 1.78 (m, 2H), 1.54 (m, 2H), 1.37 (m,2H). ^{13}C NMR (400 MHz, DMSO-d6):174.1, 167.3, 166.3, 153.9, 151.9, 150.7, 149.5, 148.4, 131.0, 127.9, 111.2, 54.0, 53.5, 46.5, 40.1, 39.8, 39.2. ESI-MS: m/z $[M+H]^+$ calcd for $C_{20}H_{25}N_8O_4$: 441.2, found 441.3.
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23. Pteroyl-lys-HYNIC, ^{1}H NMR (400 MHz, DMSO-d6): δ 8.62 (s, 1H), 7.95–8.05 (m, 2H), 7.63 (m, 4H), 7.15 (s, 1H), 6.94 (m, 3H), 6.62 (m, 4H), 4.46 (s, 2H), 4.26 (m, 1H), 3.10 (overlap, 6H), 2.76 (m,2H), 1.35–1.76 (m, 6H). ESI-MS: m/z $[M+H]^+$,707. HRMS $[M+H]^+$ calcd for $C_{35}H_{39}N_{12}O_5$ 707.3166; found 707.3187.
24. HPLC was carried out with a Venusil XBP C18 reversed-phase column (250 × 10 mm, 5 μm), Shimadzu SCL-10AVP series, working at a flow rate of 1.0 ml/min. 10 mM NH_4HCO_3(A) and CH_3OH(B) mixtures were used as the mobile phase (0–10 min, B: 5–50%; 10–20 min, B: 50–50%; 20–25 min, B: 50–5%).
25. The human nasopharyngeal 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, Fungizone 0.25 μg/ml).Twenty-four hours prior to the experiment, the KB cells were seeded in 12-well plates (5×10^5 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 **5** in 1 ml of FFRPMI medium. The blocking studies were performed by addition of free folic acid solution (10 μl, 1 mg/ml) 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. All the samples were counted for radioactivity using a γ-counter. Cellular protein was determined by using BCA protein assay reagent. The cell binding fractions and cell internalized fractions were calculated per 0.5 mg protein and expressed in relation to the total added activity (% per 0.5 mg protein of total added activity).
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