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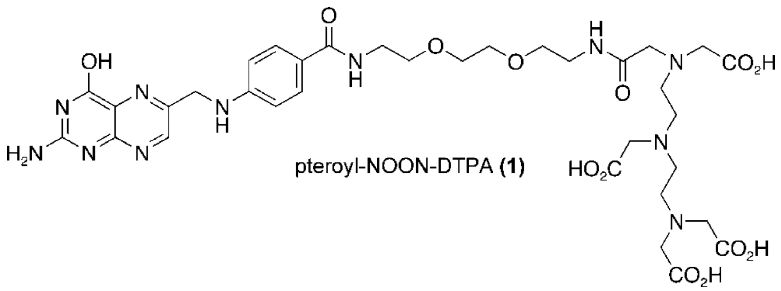

pteroyl-NOON-DTPA (**1**)

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Targeting the Tumor-Associated Folate Receptor with an ^{111}In−DTPA Conjugate of Pteroic Acid

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Abstract: The cell membrane folate receptor is a potential molecular target for tumor-selective drug delivery. To probe structural requirements for folate receptor targeting with low molecular weight radiometal chelates, specifically the role of the amino acid fragment of folic acid (pteroylglutamic acid) in mediating targeting selectivity, the amide-linked conjugate pteroyl-NHCH$_2$CH$_2$OCH$_2$CH$_2$OCH$_2$CH$_2$NH-DTPA was prepared by a three-step procedure from pteroic acid, 2,2′-(ethylenedioxy)-bis(ethylamine), and *t*-Bu-protected DTPA. This conjugate, 1-{2-[2-[(2-(biscarboxymethyl-amino)ethyl)-carboxymethyl-amino]ethyl]-carboxymethyl-amino}-acetylamino-3,6-dioxa-8-pteroylamino-octane (**1**), was employed for synthesis of the corresponding ^{111}In(III) radiopharmaceutical. Following intravenous administration to athymic mice, the ^{111}In complex of **1** was found to selectively localize in folate receptor-positive human KB tumor xenografts and to afford prolonged tumor retention of the ^{111}In radiolabel (5.4 ± 0.8, 5.6 ± 1.1, and 3.6 ± 0.6% of the injected dose per gram of tumor at 1, 4, and 24 h, respectively). The observed tumor localization was effectively blocked by co-administration of folic acid with the ^{111}In−**1** complex, consistent with a folate receptor-mediated targeting process. In control studies, tumor targeting with this pteroic acid conjugate appears as effective as that seen using ^{111}In−DTPA−folate, a radiopharmaceutical that has progressed to clinical trials for detection of folate receptor-expressing gynecological tumors.

Introduction

The cell membrane folate receptor (or folate-binding protein, FBP)[1,2] is a potential molecular target for tumor-selective drug delivery,[3−7] since the folate receptor is found in only a limited range of normal tissues but commonly overexpressed by malignant cells.[8−34] The folate receptor binds, and allows endocytosis of, a range of folate conjugates that are not substrates for the ubiquitous reduced folate carrier.[35−39] Thus,

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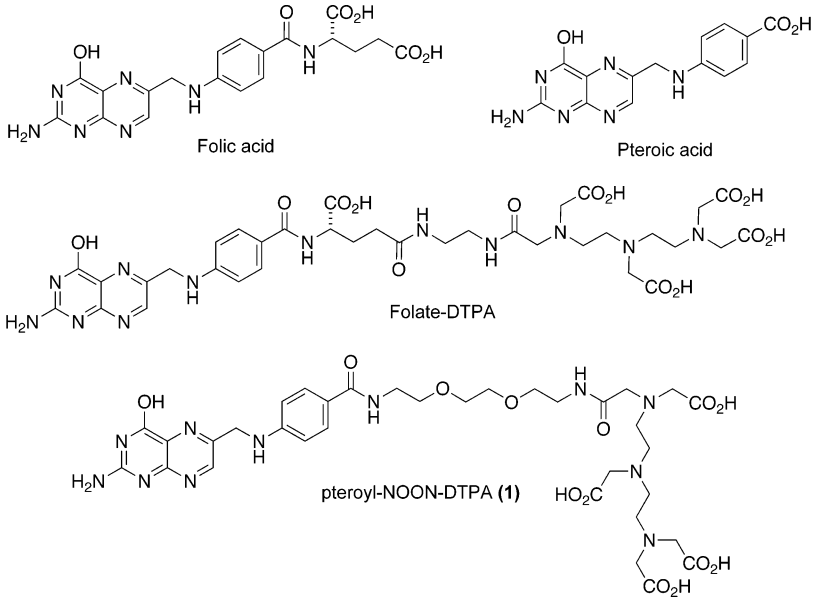
Chart 1. Structural Formulas of Folic Acid, Pteroic Acid, DTPA−Folate, and DTPA-NOON-Pte (**1**)



both in vitro and in vivo, these folate conjugates selectively target folate receptor-expressing cells. Internalization of the folate conjugates appears to occur via a receptor-mediated pathway, since the uptake is specific for the attached folate molecule, saturable at nanomolar concentrations, inhibited at low temperatures, competitively blocked by excess free folate or FBP antibody, and eliminated by removal of FBP from the cell surface.[3,6,40−50]

Characterizations of the folate "binding pocket" and structural requirements for folate receptor ligands have been investigated[51−53] to improve the binding affinity and selectivity of ligands to the receptor; however, the detailed structure of the folate receptor has not been crystallographically established. Notably, pteroic acid, a fragment of folic acid lacking the distal glutamyl residue (Chart 1), does not appreciably bind to the high-affinity folate receptor.[54]

We, and others, have shown that the folate receptor can be targeted for medical diagnostic imaging with a variety of radiometal-labeled (^{111}In, $^{66-68}$Ga, ^{99m}Tc) folate−chelate conjugates.[36,55] The present study was undertaken to better probe the structural requirements for folate receptor targeting with low molecular weight radiometal chelates, specifically examining the role of the amino acid fragment of folic acid (pteroylglutamic acid) in mediating folate-receptor affinity.

Results and Discussion

Pteroic acid was conjugated with diethylenetriaminepentaacetic acid (DTPA) via an amide-linked hydrophilic spacer, 2,2′-(ethylenedioxy)-bis(ethylamine), yielding the bifunctional chelate: pteroyl-NHCH$_2$CH$_2$OCH$_2$CH$_2$OCH$_2$CH$_2$NH-DTPA (**1**). The three-step synthesis is outlined in Scheme 1. Briefly, pteroic acid and 2,2′-(ethylenedioxy)-bis(ethylamine) were coupled using benzotriazole-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) and *N*-hydroxybenzotriazole (HOBt) in the presence of *N*-methylmorpholine (NMM) as a base. Due to the poor solubility of pteroic acid and its derivatives, dimethyl sulfoxide (DMSO) was used as a solvent for all the synthetic steps. After removal of DMSO by trituration with methanol and diethyl ether and vacuum evaporation of volatile reagents, pteroyl-ethylenedioxy-bis(ethyldiamine) (intermediate **2**, yellow solid) was produced in 57% yield.

DTPA dianhydride is a commercially available and commonly used starting material for synthesis of DTPA bioconjugates. However, to avoid the undesired cross-linking side reaction that is possible with DTPA dianhydride,[56−59] tri-*tert*-butyl-protected DTPA was instead prepared and utilized.[60] The yellow solid **2** was mixed with DTPA tri-*tert*-butyl ester using the same coupling conditions as the previous step; however, a strong base,

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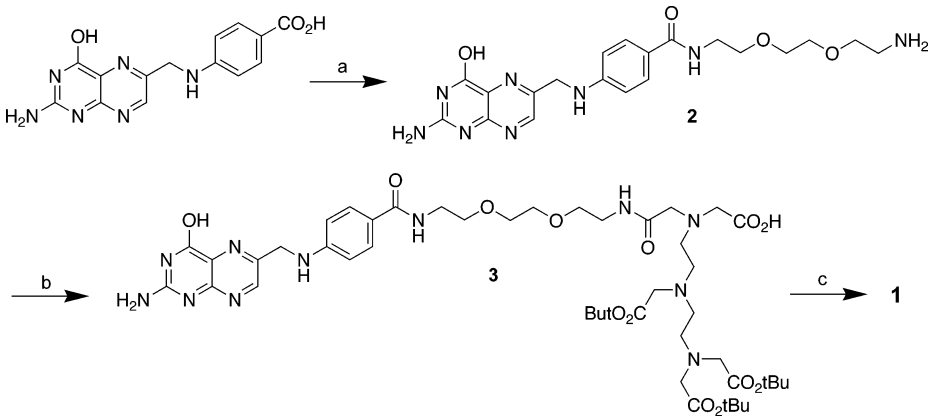
Scheme 1. Synthesis of Compound **1**[a]



[a] Reagents: (a) 2,2′-(ethylenedioxy)bis(ethylamine), PyBOP, HOBt, NMM, DMSO; (b) DTPA tri-*tert*-butyl ester, PyBOP, HOBt, NMM, DMSO, MTBD; (c) 70% TFA/CH_2Cl_2.

N-methyl-1,5,9-triazabicyclo[4.4.0]decene (MTBD), was added to improve the solubility of pteroyl-ethylenedioxy-bis(ethyldiamine).[61] The pteroyl-ethylenedioxy-bis(ethyldiamine)−DTPA *tert*-butyl ester, **3**, was double-purified using semipreparative reverse-phase HPLC. The three *t*-butyl protecting groups were then removed with 70% trifluoroacetic acid in methylene chloride, and the desired product, **1**, was isolated as a pure yellow solid by trituration with diethyl ether. Both **1** and **3** exhibit the expected molecular ion peaks in their positive ion and negative ion electrospray mass spectra.

The pteroic acid−DTPA conjugate **1** ("DTPA-NOON-Pte") was used to prepare the corresponding indium(III) chelate radiolabeled with ^{111}In (67.3 h half-life; abundant γ photons at 171 keV (90.2%) and 245 keV (94.0%)). The radiopharmaceutical was conveniently obtained from ^{111}In chloride via ligand exchange in acetate buffer. The radiochemical purity of the resulting crude ^{111}In−DTPA-NOON-Pte was evaluated by radio HPLC (Figure 1a); the major radioactive HPLC peak, eluting with a retention time of 14.2 min, was collected. To verify this peak as the desired $^{111}In^{III}$−**1** complex, stable indium(III)[$^{113/115}$In] chloride was added to the crude radiopharmaceutical solution (containing compound **1** in excess) generating carrier-added ^{111}In−DTPA-NOON-Pte at a concentration sufficient for detection by both the UV and radioactivity detectors connected in a series at the HPLC column outlet. As expected, the radiochromatogram of the carrier-added ^{111}In−DTPA-NOON-Pte preparation was identical with that of the no-carrier-added ^{111}In−DTPA-NOON-Pte, but with the appearance of UV intensity for the carrier-added product (Figure 1b). Electrospray mass spectrometry confirmed the chemical identity of the nonradioactive carrier product, [$^{113/115}$In−DTPA-NOON-Pte]$^{1-}$, eluting at 14 min.

The HPLC-purified ^{111}In−DTPA-NOON-Pte complex was isolated from the HPLC solvent system by solid-phase extraction and then repeatedly analyzed by HPLC to assess complex stability. This HPLC analysis showed the isolated [^{111}In−DTPA-NOON-Pte]$^{1-}$ radiopharmaceutical to remain stable for at least 7 days at room temperature (Figure 2). Radio TLC of the HPLC-purified ^{111}In−DTPA-NOON-Pte product (Figure 3) confirms the absence of ^{111}In chloride and ^{111}In acetate, both of which irreversibly adsorb to C_{18} ($R_f = 0$).

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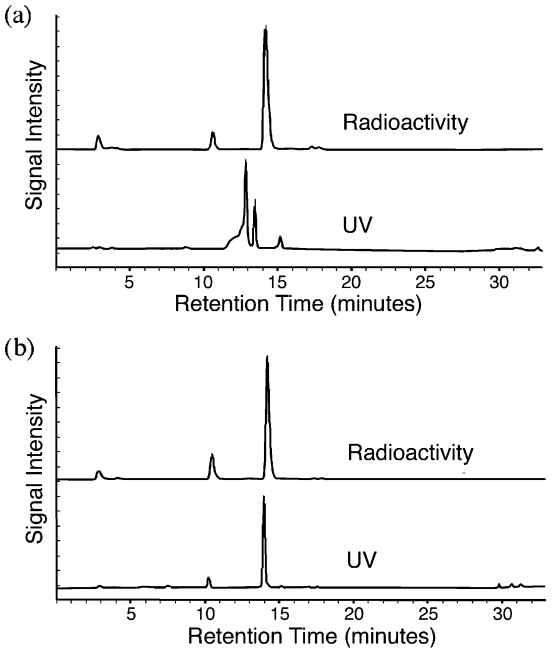

Figure 1. (a) HPLC of the crude no carrier-added ^{111}In−DTPA-NOON-Pte product. The ^{111}In−DTPA-NOON-Pte product, eluting at 14.2 min, corresponds to 82% of the total radioactivity. The identities of the radiochemical impurities, eluting as 2.8 and 10.6 min, are unknown. The UV signals arise from the excess of **1** in the formulation, which may be present as a mixture of the free ligand and its complexes with trace metal contaminants. (b) HPLC of the carrier-added ^{111}In−DTPA-NOON-Pte product, obtained by addition of carrier indium(III) chloride to an aliquot of the crude reaction mixture that produced (a), showing the appearance of carrier [In−DTPA-NOON-Pte]$^{1-}$ in the output from the UV detector. In both cases, the eluate passes through the UV−vis detector ~15 s before the radioactivity detector.

To assess the ability of the ^{111}In−DTPA-NOON-Pte complex to target the folate receptor in vivo, the biodistribution and pharmacokinetics of this radiopharmaceutical were evaluated in athymic mice bearing subcutaneous folate receptor positive human KB cell tumor xenografts.[6,7] The ^{111}In−DTPA-NOON-Pte agent rapidly cleared from the blood (Table 1) and was found to selectively localize in the folate receptor positive tumors (5.4 ± 0.8 and 5.6 ± 1.1% of the injected ^{111}In dose per gram of tumor at 1 h and 4 h postinjection, respectively). Prolonged tumor retention of the radiolabel was observed, with 3.6 ± 0.6% of the injected ^{111}In dose per gram of tumor still remaining at 24 h postinjection. Tumor localization of the ^{111}In radiolabel

Table 1. Biodistribution of ^{111}In−DTPA-NOON-Pte in KB Tumor-Bearing Athymic Mice at Various Times Following Intravenous Administration

	percentage of injected ^{111}In dose per gram (tissue wet mass)[a]			
	1 h	4 h	4 h – blocked[b]	24 h
blood	0.18 ± 0.01	0.050 ± 0.009	0.016 ± 0.010	0.035 ± 0.002
heart	3.5 ± 0.4	2.9 ± 0.9	0.015 ± 0.010	1.2 ± 0.5
lungs	1.5 ± 0.2	1.4 ± 0.3	0.041 ± 0.027	0.72 ± 0.21
liver and gall bladder	4.3 ± 0.7	1.9 ± 0.6	0.051 ± 0.029	1.2 ± 0.6
spleen	0.31 ± 0.09	0.29 ± 0.07	0.028 ± 0.017	0.27 ± 0.08
kidney (one)	61 ± 5	81 ± 7	0.90 ± 0.59	105 ± 7
stomach, intestines, and contents	1.7 ± 0.2	1.5 ± 0.9	1.7 ± 1.1	1.2 ± 0.2
muscle	3.5 ± 0.5	2.8 ± 0.8	0.080 ± 0.057	1.9 ± 0.6
tumor	5.4 ± 0.8	5.6 ± 1.1	0.12 ± 0.07	3.6 ± 0.6
tumor/blood	*30 ± 5*	*111 ± 11*	*7.5 ± 2.4*	*105 ± 20*
tumor/kidney	*0.088 ± 0.013*	*0.069 ± 0.013*	*0.12 ± 0.03*	*0.035 ± 0.008*
tumor/liver	*1.3 ± 0.4*	*3.0 ± 0.5*	*2.2 ± 0.6*	*3.7 ± 1.4*
tumor/muscle	*1.6 ± 0.4*	*2.1 ± 0.2*	*1.5 ± 0.7*	*2.0 ± 0.6*
tumor mass (g)	*0.15 ± 0.10*	*0.080± 0.031*	*0.077 ± 0.010*	*0.104 ± 0.097*
animal mass (g)	*29.5 ± 1.2*	*28.6 ± 1.6*	*28.2 ± 1.1*	*28.6 ± 0.8*
animal quantity and gender	*3M*	*4M*	*4M*	*4M*

[a] Values shown represent the mean ± standard deviation. [b] Folate receptors blocked by co-injection of folic acid dihydrate at a dose of 4.1 ± 0.4 mg/kg.

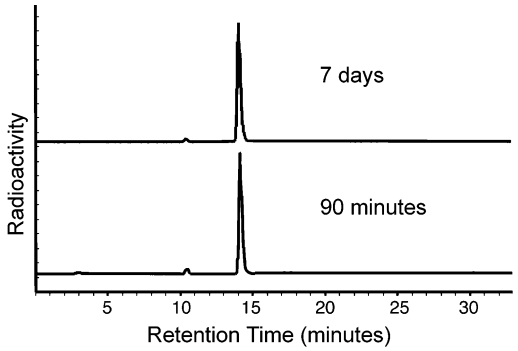

Figure 2. Repeat HPLC analyses of the HPLC-purified no carrier-added [^{111}In−DTPA-NOON-Pte]$^{1-}$ (t_R = 14.2 min, Figure 1a). Bottom: Chromatogram ca. 90 min following radiopharmaceutical isolation. Top: Chromatogram 7 days later, confirming the radiochemical stability of this product.

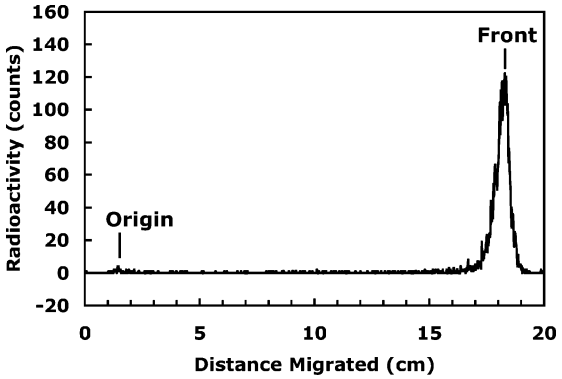

Figure 3. Radio TLC of the HPLC-purified no carrier-added [^{111}In−DTPA-NOON-Pte]$^{1-}$ (^{111}In−**1**) (C18 plate eluted with 25% NH_4OAc:75% CH_3CN).

clearly appears to be mediated by the cellular folate receptor, since the tumor uptake of radiotracer drops dramatically (0.12 ± 0.07% of the injected ^{111}In dose per gram at 4 h, Table 1) when ^{111}In−DTPA-NOON-Pte was co-injected with an excess of folic acid, which will compete for available folate receptor sites. Urinary excretion appears to be the primary whole body clearance pathway for the ^{111}In−DTPA-NOON-Pte, since the radiotracer was never appreciably found in the intestinal contents (Table 1). This is similar to the current (Table 2) and prior[6,7] findings for ^{111}In−DTPA−folate and contrasts with the combination of fecal and urinary clearance observed for ^{67}Ga-deferoxamine−folate.[3−5]

The substantial retention of ^{111}In in the kidneys (Table 1) is fully consistent with binding of ^{111}In−DTPA-NOON-Pte to tissue folate receptors, since folate receptors are abundant in the renal proximal tubules.[7,13−16] This interpretation is supported by the expected, and observed, marked reduction in renal ^{111}In levels when ^{111}In−DTPA-NOON-Pte was co-administered with excess folic acid. Prior studies of ^{111}In−DTPA−folate and ^{67}Ga-deferoxamine−folate in this mouse tumor model have shown that tumor/nontarget contrast can be substantially manipulated by co-administering modest doses of folic acid with the radiopharmaceutical;[4−5,7] however, for the purposes of the present study, no effort was made to employ such a strategy to further optimize ^{111}In−DTPA-NOON-Pte targeting selectivity in vivo.

The behavior of the ^{111}In−DTPA-NOON-Pte radiopharmaceutical in this mouse tumor model (Table 1) is virtually identical to that observed for ^{111}In−DTPA−folate (Table 2), an agent that has met with success in clinical trials for imaging folate receptor-expressing gynecologic cancers.[62] Thus, although pteroic acid itself is a poor ligand for the folate receptor, pteroic acid conjugates appear viable as candidates for folate receptor-directed drug targeting. The present results indicate that the glutamate residue of folate (pteroylglutamate) need not be viewed as an essential component of drug conjugates designed for tumor targeting via the folate receptor. This finding is consistent with the reported retention of folate receptor affinity by conjugates based structurally upon substitution of glycine for the glutamate of folate.[52] The slight decline in the tumor concentration of ^{111}In 24 h following ^{111}In−DTPA-NOON-Pte administration is similar to the findings previously reported for ^{111}In−DTPA−folate at 24−48 h postinjection.[7]

The affinity of the In−DTPA-NOON-Pte conjugate for the folate receptor has not been directly measured; however, the

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Table 2. Biodistribution of ^{111}In−DTPA−Folate (^{111}In−DTPA-en-γGlu-Pte) in Athymic Mice with Subcutaneous KB Cell Tumor Xenografts

	percentage of injected ^{111}In dose per gram (tissue wet mass)[a]		
	1 h postinjection	4 h postinjection	4 h postinjection blocked[b]
blood	0.14 ± 0.03	0.064 ± 0.007	0.029 ± 0.011
heart	2.3 ± 0.4	2.0 ± 0.3	0.022 ± 0.010
lungs	1.3 ± 0.1	1.1 ± 0.3	0.065 ± 0.021
liver and gall bladder	4.0 ± 1.5	2.2 ± 0.4	0.12 ± 0.03
spleen	0.36 ± 0.03	0.35 ± 0.11	0.060 ± 0.021
kidney	90 ± 9	85 ± 12	5.0 ± 5.5
muscle	3.5 ± 0.8	2.9 ± 0.7	0.023 ± 0.013
stomach, intestines, and contents	1.0 ± 0.2	1.0 ± 0.2	0.49 ± 0.20
tumor	5.3 ± 0.4	6.8 ± 1.2	0.16 ± 0.07
tumor/blood	*38 ± 7*	*106 ± 15*	*5.5 ± 0.8*
tumor/kidney	*0.060 ± 0.011*	*0.080 ± 0.012*	*0.050 ± 0.023*
tumor/liver	*1.5 ± 0.5*	*3.3 ± 1.1*	*1.4 ± 0.4*
tumor/muscle	*1.6 ± 0.3*	*2.5 ± 0.9*	*8.4 ± 3.8*
tumor mass (g)	*0.138 ± 0.051*	*0.202 ± 0.083*	*0.193 ± 0.078*
animal mass (g)	*24 ± 2*	*25 ± 1*	*24 ± 1*

[a] Values shown represent the mean ± standard deviation; $n = 4$. Each mouse received ~3 μCi In-111 in a formulation containing 0.2 μg of DTPA−folate. [b] Folate receptors blocked by co-injection of folic acid dihydrate at a dose of 0.50 ± 0.08 mg/kg.

literature has shown that folate conjugates, such as DTPA−folate, can exhibit folate receptor affinities very similar to that of folic acid itself.[63] In screening candidate folate receptor-targeted radiopharmaceuticals, we have instead focused on assessment of targeting efficiency and selectivity in vivo, where success implicitly depends not only on receptor affinity, but also on factors such as the effectiveness of drug delivery to receptor-rich tissue, levels of nonspecific binding, the rates and routes of agent metabolism, and the rates and routes of radionuclide clearance and excretion. The reported data indicate that, in vivo, ^{111}In−DTPA-NOON-Pte targets the folate receptor as efficiently, and selectively, as the clinically studied ^{111}In−DTPA−folate radiopharmaceutical.[62]

Conclusion

Tumor-selective drug targeting via the folate receptor is feasible with pteroic acid conjugates lacking amino acid fragments, such as the glutamic acid moiety of folic acid. This finding is expected to somewhat simplify folate receptor-based drug targeting strategies, obviating the need to selectively control conjugation via the α- or γ-carboxylates of folic acid, while also avoiding amino acid chirality and/or racemization issues that can exist in preparation of receptor-targeted conjugates based on folic acid itself.

Experimental Section

Materials and General Methods. Unless otherwise specified, all reagents were purchased and used without further purification. Distilled, deionized water (18 MΩ) was used throughout. DMSO was distilled from CaH_2 under reduced pressure and stored over activated 4 Å molecular sieves. ^{111}In chloride was obtained from Mallinckrodt Medical, Inc., St. Louis, MO. The tri-*tert*-butyl-protected DTPA was synthesized as described by S. Achilefu et al.[60] C18 Sep-Pak Light solid-phase extraction cartridges were purchased from Millipore, Inc. A Capintec CRC-127R radioisotope calibrator was used for assays of ^{111}In radioactivity in the microcurie to millicurie range, whereas low-level (<0.01 μCi) samples of ^{111}In were counted in a Packard A5530 automatic γ scintillation counter with a 3-in. large-bore NaI(Tl) crystal. ^{111}In−DTPA−folate was prepared as described previously for use in control biodistribution studies.[7,64] The resulting ^{111}In−DTPA−folate, obtained by reaction of 342 μCi ^{111}In with 15 μg of DTPA−folate, had a radiochemical purity >98% by C_{18} TLC.[6,64] High-field NMR spectra were recorded in DMSO-d_6 on a Bruker ARX 300 (^{1}H at 300 MHz) spectrometer. ^{1}H chemical shifts are reported in δ ppm relative to residual DMSO (2.49 ppm) as internal standard. Multiplicities in ^{1}H are reported as (s) singlet, (d) doublet, (t) triplet, (q) quartet, and (m) multiplet. The HPLC methods employed a Rainin Rabbit-HP system with a Knauer UV−vis detector and a NaI(Tl) radioactivity detector (Bioscan). The HPLC flow rate was 0.5 mL/min for 4.6 × 250 mm column, and 2.35 mL/min for 10 × 250 mm column. Program 1: 5% solvent B at 0 min, ramped to 25% B at 25 min, then to 60% B at 27 min, and reached 100% B at 30 min (solvent A is 56 mM NH_4OAC (99.99+%) in water; solvent B is CH_3CN). Program 2: 5% solvent B at 0 min, to 70% B at 30 min, then ramped to 100% B at 32 min, and remained 100% B to 40 min (solvent A is 5% CH_3CN in 0.1% aqueous TFA; solvent B is 10% water in CH_3CN with 0.1% TFA). Program 3: (using the same solvents as program 2) 5% solvent B at 0 min, ramped to 40% B at 30 min, then to 100% B at 32 min, and remained 100% B to 40 min.

Synthesis of 1-{2-[2-[(2-(Biscarboxymethyl-amino)ethyl)-carboxymethyl-amino]ethyl]-carboxymethyl-amino}-acetylamino-3,6-dioxa-8-pteroylamino-octane (1). To a suspension of pteroic acid (0.025 g, 0.080 mmol), PyBOP (0.125 g, 0.240 mmol), and HOBt (0.037 g, 0.240 mmol) in DMSO (0.8 mL), NMM (0.049 g, 0.48 mmol) was added under N_2. After being stirred at room temperature for 10 min, 2,2′-(ethylenedioxy)bis(ethylamine) (0.237 g, 1.600 mmol) was added. The suspension gradually turned clear after the addition of this amine, with the reagents completely dissolved by 1 h. The reaction was stirred at room temperature for 22.5 h, then concentrated under vacuum overnight. The resulting brown oil was triturated with MeOH, and the resultant suspension was centrifuged to produce a light yellow solid. The solid was washed with MeOH and Et_2O and dried in a vacuum desiccator to produce 20 mg of product **2**. Electrospray MS calcd for $C_{20}H_{26}N_8O_4$: *m*/*z* 442, positive mode ($M + H^+$) found 443, negative mode ($M - H^+$) found 441. ^{1}H NMR (DMSO-d_6, 300 MHz): δ ppm 2.73 (2H, m), 3.20−3.70 (10H, m), 4.46 (2H, s, broad), 6.61 (2H, d, $J = 8.3$ Hz), 6.88 (1H, m), 7.02 (2H, m), 7.59 (2H, d, $J = 8.3$ Hz), 8.05 (1H, m), 8.61 (1H, s). For identification purposes, analytical HPLC was performed with a 4.6 × 250 mm C18 reverse-phase column with flow rate 0.5 mL/min using program 3. The desired product has a retention time of 17.3 min detected with UV detector @ 254 nm.

To a solution of tri-*tert*-butyl DTPA (0.075 g, 0.134 mmol), PyBOP (0.084 g, 0.163 mmol), and HOBt (0.025 g, 0.163 mmol) in DMSO (0.4 mL), NMM (0.0247 g, 0.244 mmol) was added under N_2 at room temperature. This premixed solution was then added under N_2 to the

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suspension of the yellow solid **2** (0.0120 g, 0.0271 mmol) in DMSO (0.2 mL). MTBD (0.0532 g, 0.347 mmol) was added after the reaction mixture was stirred at room temperature overnight. The reaction mixture was stirred at room temperature for another day and then concentrated under vacuum. The resulting brown residue was triturated first with Et_2O and then with MeOH to produce crude product as a yellow solid. This yellow solid was purified using semipreparative HPLC twice on a C18 column (10 × 250 mm) with program 2. The peak with retention time 30.5 min, detected by a UV detector at 254 nm, was collected and concentrated to produce the desired pure product **3**. Electrospray MS calcd for $C_{46}H_{71}N_{11}O_{13}$: *m*/*z* 985, positive mode ($M + H^+$) found 986, negative mode ($M - H^+$) found 984. ^{1}H NMR (DMSO-d_6, 500 MHz): δ ppm 1.40 (18H, s), 1.45 (9H, s), 3.02 (4H, m), 3.22 (2H, m), 3.31 (6H, m), 3.39−3.55 (18H, m), 4.46 (2H, s, broad), 6.52 (1H, s, broad), 6.61 (2H, d, $J = 8.3$ Hz), 6.88 (2H, s, broad), 7.59 (2H, d, $J = 8.3$ Hz), 8.04 (1H, d, $J = 5.5$ Hz), 8.09 (1H, s, broad), 8.63 (1H, s).

Removal of the *tert*-Butyl Ester Protecting Groups. To the HPLC-purified coupling product **3** (2 mg) in a 5-mL round-bottomed flask was added 70% TFA/CH_2Cl_2. (The flask was twice rinsed with ultrapure concentrated HCl and several times with water, then dried in a 110 °C oven overnight before use.) The reaction mixture was stirred at 0 °C for 30 min. After being stirred at room temperature for an additional 5 h, the reaction mixture was repeatedly concentrated after two CH_2Cl_2 additions, using rotary evaporator, producing **1** as a yellow residue. Electrospray MS calcd for $C_{34}H_{47}N_{11}O_{13}$: *m*/*z* 817, positive mode ($M + H^+$) found 818, negative mode ($M - H^+$) found 816. ^{1}H NMR (DMSO-d_6, 500 MHz): δ ppm 3.05 (4H, m), 3.22 (2H, m), 3.28−3.60 (24H, m), 4.48 (2H, s), 6.61 (2H, d, $J = 8.6$ Hz), 7.36 (2H, s, very broad), 7.59 (2H, d, $J = 8.6$ Hz), 8.05 (2H, t, $J = 5.4$ Hz), 8.66 (1H, s). This product was used for labeling with ^{111}In chloride without further purification.

Synthesis of ^{111}In Complex of (1). Briefly, ca. 1.2 mCi no carrier-added ^{111}In chloride in 0.05 mL of 0.05 N HCl was transferred to a test tube, and 0.05 mL of 0.1 N ammonium acetate (pH 5.5), followed by 0.02 mL of 0.5 N ammonium acetate (pH 7.4) were added, producing a solution with pH 7. A sample of ligand **1** was weighed and diluted in water adjusted with 1 N NaOH to pH 9−10. A quantity of 10 μL of this solution, containing 95 μg of **1**, was added to the ^{111}In acetate solution (120 μL) and allowed to stand overnight at room temperature, protected from light.[65] The crude ^{111}In−**1** complex was purified by radio HPLC using a 4.6 × 250 mm Dynamax C18 reverse-phase column (Varian/Rainin) eluting with aqueous NH_4OAC/CH_3CN using program 1 and a flow rate of 1 mL/min. The fraction with retention time 14.2 min was collected and diluted with water to reduce the percentage of CH_3CN to less than 5%. A C18 Sep-Pak light solid-phase extraction cartridge was conditioned by washing with EtOH followed by water. The conditioned C18 Sep-Pak was loaded with the diluted HPLC fraction and washed with water (3 mL), and then the ^{111}In−**1** complex was eluted with EtOH. The ethanol fractions containing radioactivity were combined and evaporated to dryness under a N_2 stream at room temperature to produce the desired ^{111}In−**1** complex for further study. The complex was reconstituted in water for the reported stability study (Figure 2) or in saline for evaluation of radiopharmaceutical biodistribution following intravenous administration to tumor-bearing athymic mice.

The stable In(III) complex of **1**, [$^{113/115}$In−DTPA-NOON-Pte]$^{1-}$, was prepared in a manner analogous to the radiolabeled chelate, but at a 1:1 In:**1** stoichiometry. Electrospray MS calcd for [^{115}In−DTPA-NOON-Pte]$^{1-}$ ($InC_{34}H_{43}N_{11}O_{13}$): *m*/*z* 928.21; negative mode (M^-) found 928.46; positive mode found ($M^- + 2H^+$) 930.14, ($M^- + H^+ + Na^+$) 952.18, and ($M^- + 2Na^+$) 974.16. High-resolution positive ion electrospray mass spectrum for [$InC_{34}H_{43}N_{11}O_{13}^{1-} + 2H^+$]: calcd 930.2237, found 930.2244.

Biodistribution Studies with ^{111}In Radiotracers. All animal studies were conducted in accordance with procedures approved by the Purdue Animal Care and Use Committee. The general procedure for the animal biodistribution studies has been described previously.[4,5,7] The athymic mice used in these studies were maintained for 3 weeks on a folate-free diet (to reduce their serum folate to a level near that of human serum) and had been implanted subcutaneously in the interscapular region with human KB tumor cells, as described elsewhere.[3,4,7] The diethyl ether anesthetized mice were injected with radiotracer via the exposed femoral vein and allowed to recover. Syringes used for radiotracer injections were weighed on an analytical balance before and after injection to quantify the dose received by each animal. Following animal sacrifice and dissection, tissue samples were weighed and counted to quantify ^{111}In. The uptake of radiotracer in each tissue sample was calculated as both a percentage of the injected dose (%ID) per organ (Supporting Information) and as a %ID/g of tissue wet weight (Tables 1 and 2), using reference counts from a weighed and appropriately diluted sample of the original injectate. Blood was assumed to account for 5.5% of total body mass. Muscle was assumed to account for 42% of the total body mass. To competitively block tissue folate receptors, a subset of the mice were co-injected with ^{111}In radiopharmaceutical premixed with folic acid dihydrate at the doses specified (Tables 1 and 2).

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Supporting Information Available: Data tables expressing the biodistribution of ^{111}In−DTPA-NOON-Pte and ^{111}In−DTPA−folate as a percentage of injected ^{111}In dose per organ or tissue (athymic mouse/KB tumor xenograft model). This material is available free of charge via the Internet at http://pubs.acs.org.

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