

## Development and Pre-clinical Evaluation of New $^{68}\text{Ga}$ -NOTA-folate Conjugates for PET Imaging of Folate Receptor-positive Tumors

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**Abstract.** *In an attempt to develop new folate radiotracers with favorable biochemical properties for detecting folate receptor-positive cancers, we synthesized  $^{68}\text{Ga}$ -NOTA- and  $^{68}\text{Ga}$ -NOTAM-folate conjugates using a straightforward and a one-step simple reaction. Radiochemical yields were greater than 95% (decay-corrected) with total synthesis time of less than 20 min. Radiochemical purities were always greater than 98% without high-performance liquid chromatography (HPLC) purification. These synthetic approaches hold considerable promise as a rapid and simple method for  $^{68}\text{Ga}$ -folate conjugate preparation with high radiochemical yield in a short synthesis time. In vitro tests on the KB cell line showed that significant amounts of the radioconjugates were associated with cell fractions. Biodistribution studies in nude mice bearing human KB xenografts, demonstrated a significant tumor uptake and favorable biodistribution profile for  $^{68}\text{Ga}$ -NOTA-folate over the  $^{68}\text{Ga}$ -NOTAM-folate conjugate. The uptake in the tumors was blocked by excess injection of folic acid, suggesting a receptor-mediated process. These results demonstrate that the  $^{68}\text{Ga}$ -NOTA-folate conjugate may be useful as a molecular probe for detection and staging of folate receptor-positive cancers, such as ovarian cancer and their metastasis, as well as monitoring tumor response to treatment.*

Advancement of scintigraphic imaging is highly determined by the development of more specific radiotracers. Receptor-targeted radiotracers have shown great potential for imaging

tumors and other recurring diseases in nuclear medicine (1). The Membrane-associated-folic acid receptor is a glycosylphosphatidylinositol protein overexpressed in approximately 100% of ovarian adenocarcinomas and various epithelial cancers including cervical, colorectal and renal cancers (2, 3). Meanwhile, this receptor is highly restricted in most normal tissues, which make these tumors excellent candidates for molecular targeting through the folate receptor system. Folate enters into cells by high affinity folate receptors in addition to the classical reduced folate carrier-mediated transport system (4). Folic acid (FA) has been mainly conjugated with chelates covalently *via* its gamma carboxylate of the glutamic acid fragment (5-8). These conjugates have shown considerable success for delivering various single-photon emission computed tomography (SPECT) radionuclides to folate-receptor-positive KB cell tumor xenografts in athymic mouse models. Among these conjugates,  $^{67}\text{Ga}$ -deferoxamine-folate ( $^{67}\text{Ga}$ -DF-folate) (7, 9, 10) and  $^{111}\text{In}$ -diethylenetriamine pentaacetic acid ( $^{111}\text{In}$ -DTPA-folate), which appeared to be feasible for targeting tumor folate receptors *in vivo* (11, 12). However,  $^{111}\text{In}$ -DTPA-folate provided tumor-selective radionuclide delivery *in vivo* that has led to the initial clinical study for imaging of ovarian malignancy and differentiation between malignant and benign ovarian masses (13). Due to its favorable physical properties and wide availability of  $^{99\text{m}}\text{Tc}$ , numerous  $^{99\text{m}}\text{Tc}$ -labeled-folate conjugates have also been synthesized and biologically evaluated (14-17). For instance, the folate conjugates of  $^{99\text{m}}\text{Tc}$ -6-hydrazino-nicotinamido-hydrazido ( $^{99\text{m}}\text{Tc}$ -HYNIC),  $^{99\text{m}}\text{Tc}$ -ethylenedicycysteine ( $^{99\text{m}}\text{Tc}$ -EC),  $^{99\text{m}}\text{Tc}$ -EC20 and  $^{99\text{m}}\text{Tc}$ -DTPA have shown the capability of imaging folate receptor-positive tumors. Recently, our group described the synthesis and evaluation of  $^{99\text{m}}\text{Tc}$ -mercaptoacetyltriglycine-folate ( $^{99\text{m}}\text{Tc}$ -MAG<sub>3</sub>-folate) and  $^{99\text{m}}\text{Tc}$ -mercaptoacetyldiglycine-folate ( $^{99\text{m}}\text{Tc}$ -MAG<sub>2</sub>-folate) conjugates (17). These conjugates, in particular,  $^{99\text{m}}\text{Tc}$ -MAG<sub>3</sub>-folate retained sufficient binding affinity to breast tumor MDA-MB 231

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cells *in vitro* and *in vivo*. With the increased demand of positron-emission tomography (PET), there has been a great interest in the development of positron-emitting radiopharmaceuticals for the early detection and characterization of cancer, molecular assessment of treatment effects and more fundamental understanding of the disease process (18). Therefore, several  $^{18}\text{F}$ -labeled folate derivatives were synthesized in high radiochemical yield and preclinically characterized (19, 20). These derivatives exhibited very high binding affinities to KB cells and demonstrated significant tumor uptake and favorable pharmacokinetics as molecular probes for detecting folate receptor-positive cancers. Lately,  $^{68}\text{Ga}$ -labeled DF and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-folate conjugates were synthesized in high radiochemical purity (21, 22). Imaging of folate-receptor-positive tumor bearing mice with  $^{68}\text{Ga}$ -DF and DOTA-folate were feasible and the characteristic of  $^{68}\text{Ga}$ -DOTA-folate was similar to  $^{111}\text{In}$ -DTPA-folate, which has been used in clinical trials.

Due to the exceptionally high stability of  $\text{Ga}^{\text{III}}$ -NOTA complexes with  $\log K=31$  in comparison with their corresponding DOTA complexes ( $\log K=21$ ) (23, 24) and in an attempt to develop new folate conjugates capable of being labeled with metallic radionuclides that possess favorable properties for detecting PET imaging of folate receptor-positive cancers, we here report the synthesis and preclinical evaluation of new  $^{68}\text{Ga}$ -NOTA-folate conjugates using 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) and 1,4,7-triazacyclononane-1,4,7-triacetamide (NOTAM) as chelating groups.

## Materials and Methods

The chemicals used in the study were all analytical reagent grade purchased from Aldrich (St. Louis, MO, USA) and were used without further purification unless stated. Acetonitrile (ACN) was kept over molecular sieves. Commercial generator based on a  $\text{SnO}_2$  phase adsorbing was obtained from iThemba Lab (Cape Town, South Africa). Sep-Pak cartridges were purchased from Waters-Millipore (Milford, MA, USA). TLC-SG sheets were purchased from Gelman Sciences Inc (Ann Arbor, MI, USA). High-Performance Liquid Chromatography (HPLC) analysis was carried out on Luna, Phenomenex C-18 reversed-phase column (analytical, 250 mm  $\times$  4.6 mm) (JASCO, Tokyo, Japan). The solvent system used was isocratic (eluant: ACN/ $\text{H}_2\text{O}$ , 95/5 with 0.1% TFA at flow rate of 1.0 ml/min). A Jasco chromatographic system equipped with a variable wavelength ultraviolet monitor and in tandem with a Canberra flow through radioactivity detector was used (Meriden, CT, USA). Ultraviolet absorption was monitored at 254 nm. Chromatograms were acquired and analyzed using the BORWIN software (JASCO, Tokyo, Japan). Melting points were determined on a Thomas-Hoover Unimelt capillary melting point apparatus (Labquip, Ontario, Canada). Mass spectroscopy was run on Quattro electrospray mass spectrometer (ES-MS, Perkin Elmer, Waltham, MA, USA).

### 1,4,7-Triazacyclononane-*N,N',N''*-triacetic acid (NOTA, 2)

Compound 2 in Figure 1 was synthesized utilizing the method reported previously (25) with minor modification and briefly described below. The hydrochloric acid salt of 1,4,7-Triazacyclononane (TACN.HCl, 500 mg, 2.1 mmol) was dissolved in ACN (10 ml) followed by the addition of triethylamine (TEA, 1.16 ml, 8.4 mmol). To the stirred mixture, bromoacetic acid (0.88 g, 6.3 mmol) in ACN (4 ml) was added drop-wise over the course of 5 min. The reaction mixture was then stirred and refluxed for 3 h. The brown solution was concentrated by rotary evaporation to leave a brown solid product which was washed with ACN (10 ml) to leave a creamy precipitate. This was then filtered, washed with ACN (10 ml) and dried *in vacuo* to yield 466 mg of NOTA as an oily material.

### *N*-Succinimidyl-1,4,7-triazacyclononane-*N,N',N''*-triacetic acid (NOTA-NHS, 3)

NOTA (220 mg, 0.72 mmol) was dissolved in ACN (10 ml) followed by the addition of *N*-hydroxysuccinimide (NHS, 78 mg, 0.72 mmol) and dicyclohexylcarbodiimide (DCC, 154 mg, 0.72 mmol). The reaction mixture was stirred at ambient temperature for 3 h. The by-product dicyclohexylurea was then removed by filtration and the filtrate was dried by rotary evaporation to furnish 246 mg of NOTA-NHS as an oily product.

### 1,4,7-Triazacyclononane-*N,N',N''*-triacetic acid-folate (NOTA-folate, 5)

The  $\gamma$ -hydrazide-folate conjugate 4 (folate- $\text{NH}_2\text{-NH}_2$ , 273 mg, 0.6 mmol) was dissolved in dimethylsulfoxide (DMSO, 3 ml) followed by the addition of TEA (83  $\mu\text{l}$ , 0.6 mmol) and NOTA-NHS (240 mg, 0.6 mmol). The reaction mixture was stirred while shielded from light for 3 h at 60°C. The product was precipitated by addition of ACN (2 ml), centrifuged and then washed several times with diethyl ether (ether) before drying under vacuum into a 350 mg of NOTA-folate as a yellow powder.

### 1,4,7-Triazacyclononane-*N,N'*-diacetamide (NOTAM, 6)

Compound 6 in Figure 2 was synthesized utilizing the method described above for compound 2. TACN.HCl salt (500 mg, 2.1 mmol) was dissolved in ACN (10 ml) followed by the addition of TEA (0.87 ml, 6.3 mmol). To the stirred mixture, bromoacetamide (0.57 g, 4.2 mmol) in ACN (4 ml) was added drop-wise over the course of 5 min. The reaction mixture was then stirred and refluxed for 3 h. The brown solution was concentrated by rotary evaporation to leave a brown solid product which was washed with ACN (10 ml) to leave a creamy precipitate. This was then filtered, washed with ACN (10 ml) and dried *in vacuo* to yield 348 mg of NOTAM as an oily material.

### *N*-Succinimidyl-1,4,7-triazacyclononane-*N,N'*-diacetamide (NOTAM-NHS, 8)

NOTAM 6 (348 mg, 1.42 mmol) was dissolved in ACN (10 ml) followed by the addition of TEA (0.4 ml, 1.42 mmol). To the stirred mixture, bromoacetic acid (0.2 g, 1.42 mmol) in ACN (3 ml) was added drop-wise over the course of 5 min. The reaction mixture was then stirred and refluxed for 3 h. The brown solution was concentrated by rotary evaporation to leave a brown solid product which was washed with ACN (8 ml) to leave a creamy precipitate. The creamy precipitate was then filtered, washed with ACN (10 ml) and dried *in vacuo* to yield 432 mg of NOTAM 7 as an oily material. The oily material (120 mg, 0.39 mmol) was dissolved in

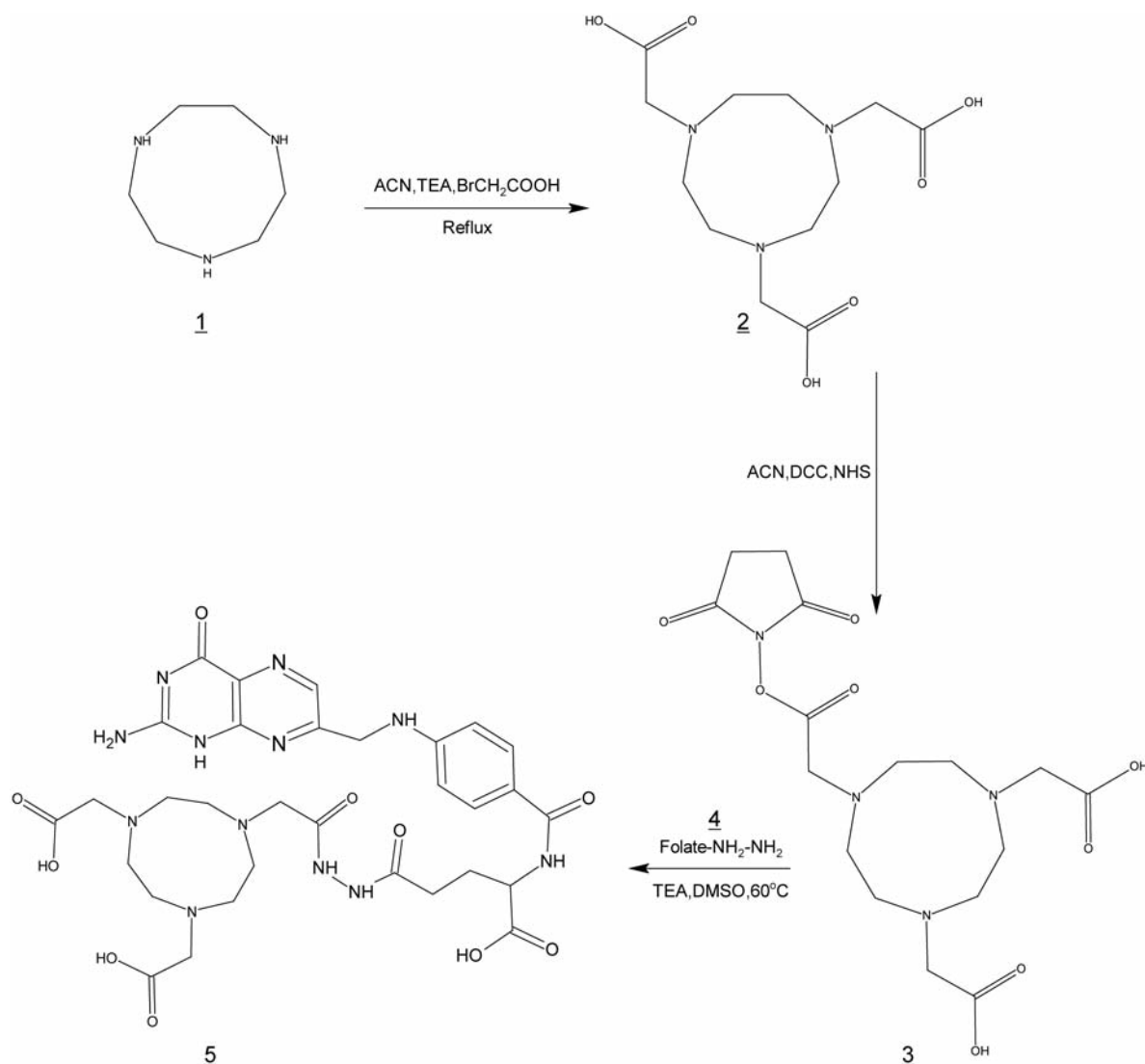


Figure 1. Synthesis of NOTA-folate conjugate precursor 5.

ACN (6 ml) followed by the addition of NHS (43 mg, 0.39 mmol) and DCC (85 mg, 0.39 mmol). The reaction mixture was stirred at ambient temperature for 3 h. The by-product dicyclohexylurea was then removed by filtration and the filtrate was dried by rotary evaporation to furnish 155 mg of NOTAM-NHS as an oily product.

*1,4,7-Triazacyclononane-N,N',N''-triacetamide-folate (NOTAM-folate, 2)*

The folate-NH<sub>2</sub>-NH<sub>2</sub> conjugate 4 (181 mg, 0.39 mmol) was dissolved in dimethylsulfoxide (DMSO, 3 ml) followed by the addition of TEA (54  $\mu$ l, 0.39 mmol) and NOTAM-NHS (155 mg, 0.39 mmol). The reaction mixture was stirred while shielded from light for 3 h at 60°C. The product was precipitated by addition of ACN (2 ml), centrifuged and then washed several times with diethyl ether (ether) before drying under vacuum into a 70 mg of NOTA-folate as a yellow powder.

*Reference Ga-conjugates (Ga-NOTA- and Ga-NOTAM-folates)*

Reference compounds Ga-NOTA- and Ga-NOTAM-folate conjugates were prepared by reacting compounds 5 and 9 (5 mg each, 6.75  $\mu$ mol) with gallium chloride (GaCl<sub>3</sub>, 7.0  $\mu$ mol) in acetic acid (0.1% in EtOH, pH~4.5, 500  $\mu$ l) at 80°C for 30 min. The reference compound conjugates were precipitated by addition of ACN (2 ml), centrifuged and then washed several times with ACN before drying under vacuum into yellow powders.

**Radiochemistry.**  $^{68}\text{Ge}/^{68}\text{Ga}$  generator was eluted with suprapure HCl (0.6 M, 6 mL) in 0.5 ml fractions. The two fractions with the highest  $^{68}\text{GaCl}_3$  37-300 MBq were generally used for labelling purposes (26, 27). The synthetic approaches for the preparation of  $^{68}\text{Ga}$ -NOTA- and  $^{68}\text{Ga}$ -NOTAM-folate conjugates were simple and straightforward. Fractionated solutions of  $^{68}\text{GaCl}_3$  solutions were reacted in sealed vials with NOTA- and NOTAM-folate conjugates

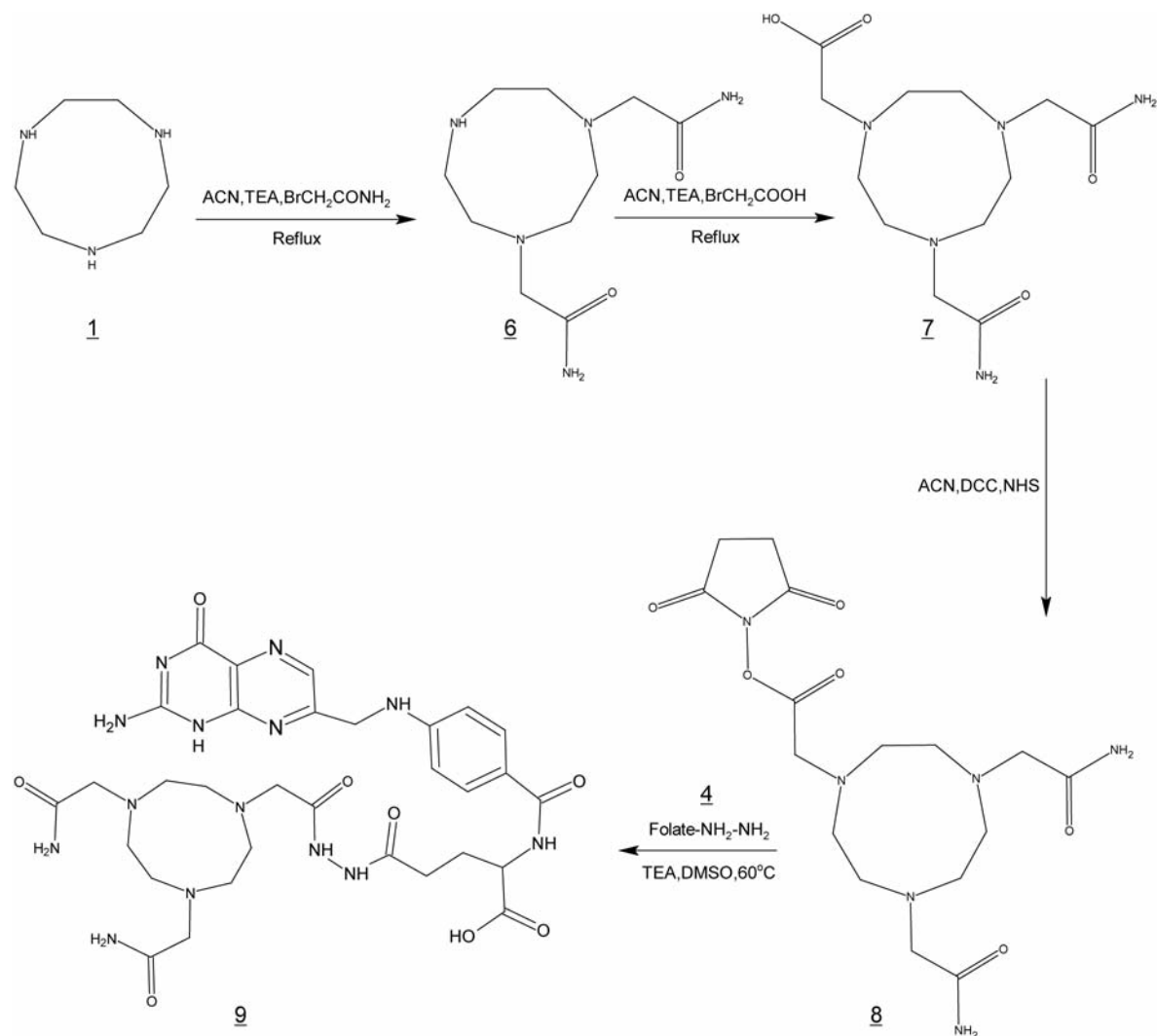


Figure 2. Synthesis of NOTAM-folate conjugate precursor 9.

(50  $\mu\text{g}$  each) in acetic acid (0.1% in EtOH,  $\text{pH}\sim 4.5$ , 200  $\mu\text{l}$ ) at  $80^\circ\text{C}$  for 10 min. The reaction mixtures were dried by rotary evaporation to leave yellowish residues which were then re-solubilized in NaCl (0.9% saline, 1 ml each) before passing through 0.22  $\mu\text{m}$  pore membrane filter for further studies. Similarly,  $^{67}\text{Ga}$ -NOTA- and  $^{67}\text{Ga}$ -NOTAM-folate conjugates were prepared by incubating  $^{67}\text{GaCl}_3$  (185-370 MBq) with NOTA- and NOTAM-folate conjugates (50  $\mu\text{g}$  each) in acetic acid (0.1% in EtOH,  $\text{pH}\sim 4.5$ , 200  $\mu\text{l}$ ) at  $80^\circ\text{C}$  and different time range (5-60 min).  $^{67/68}\text{Ga}$ -NOTA- and  $^{67/68}\text{Ga}$ -NOTAM-folate conjugates were obtained in quantitative radiochemical yields and purities as assessed by HPLC and confirmed by TLC.

**Partition coefficient.** One hundred  $\mu\text{l}$  of  $^{68}\text{Ga}$ -NOTA- and  $^{68}\text{Ga}$ -NOTAM-folate conjugates were added into test tubes containing 1 ml of each n-octanol and buffered  $\text{H}_2\text{O}$  ( $\text{pH}=7.3$ ). The tubes were shaken for 1 min. After partial separation of the phases by gravity, 0.7 ml of each phase was transferred to separate tubes and

centrifuged at 5,000 rpm for 5 min. Duplicate 0.2 ml aliquots of each phase was taken for radioactivity measurement and the partition coefficient was determined by the function:  $\text{Partition coefficient} = \text{Log}_{10} (\text{counts in n-octanol layer}/\text{counts in aqueous layer})$ . The calculation of the electronic charge density distribution was performed using the Extend Hückel MM2 program (Chem-Biodraw Ultra II Office 2008, Cambridge Soft, USA).

**Stability in plasma.** For stability in plasma,  $^{68}\text{Ga}$ -NOTA- and  $^{68}\text{Ga}$ -NOTAM-folate conjugates (100  $\mu\text{l}$ , 20  $\mu\text{Ci}$  each) were incubated with human plasma (500  $\mu\text{l}$ ) in duplicate at  $37^\circ\text{C}$  for 4 h. This was followed by precipitation using a mixture of  $\text{ACN/EtOH}$  (400  $\mu\text{l}$ , 1/1 v/v) and centrifugation at 5,000 rpm for 5 min. The supernatant layer was then analyzed by HPLC under the conditions described above.

**In vitro cell binding.** KB cells, a human oral carcinoma cell line that overexpresses the folate receptor, was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The KB cell

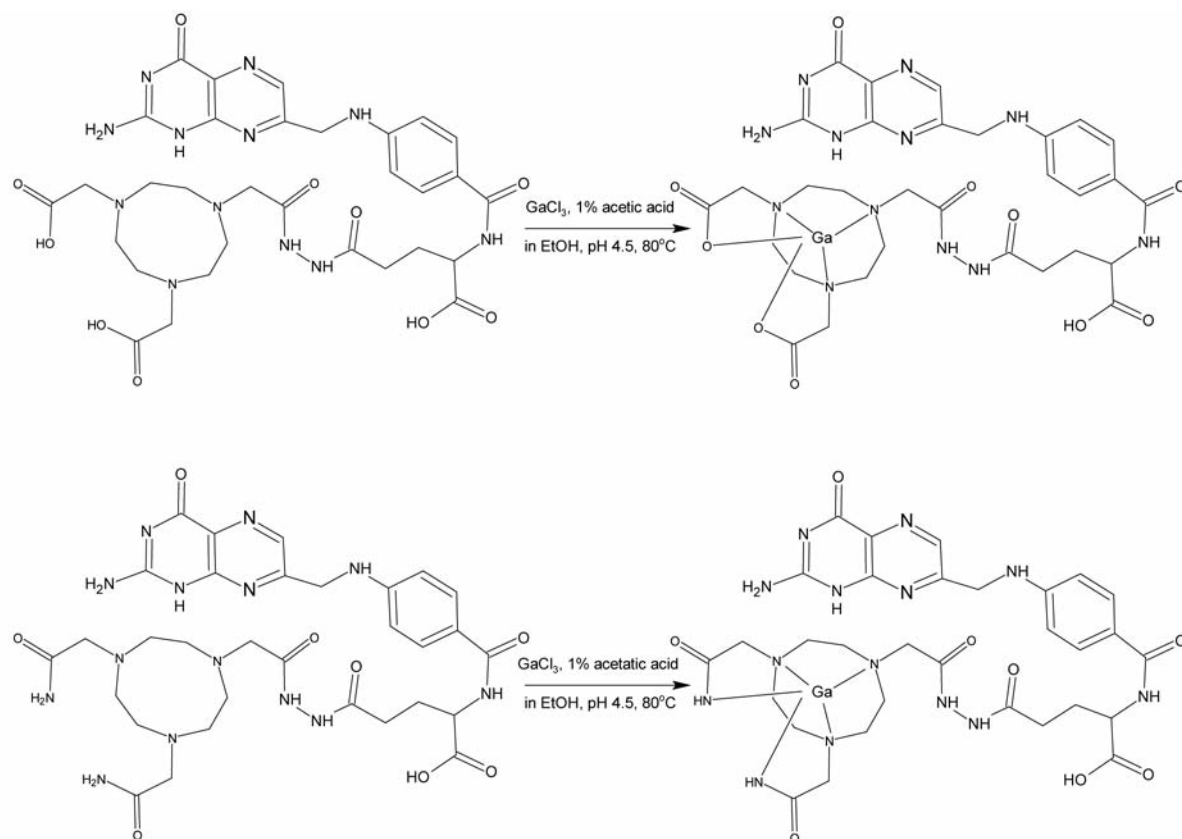


Figure 3. Synthesis of the Ga-NOTA- and Ga-NOTAM-folate conjugates ( $\text{Ga} = ^{67/68}\text{natGa}$ ).

line was grown in folate-deficient RPMI-1640 culture media with 10% fetal bovine serum (FBS) in tissue culture flasks (Fisher Scientific, Waltham, MA, USA). Twenty-four h prior conducting the cell-binding assay, media was replaced with RPMI-1640 without further addition of FBS. Confluent cultures were harvested by trypsinization, and  $6 \times 10^6$  cells were suspended in 1.8 ml of sterile saline for binding assay. Approximately 300,000 cells (in 0.3 ml of sterile saline) were incubated with various amounts of  $^{68}\text{Ga}$ -NOTA- and  $^{68}\text{Ga}$ -NOTAM-folate conjugates ranging from 0.3-18 nM in duplicate for 60 min at 37°C. Incubation was terminated by dilution with cold saline (0.3 ml) and cells were pelleted by centrifugation. The cell pellets were then washed with cold saline to remove unbound radioactivity and centrifuged to collect supernatants. Radioactivity in the cell pellets (total bound) and washings (unbound) were measured in a  $\gamma$ -well counter (Perkin Elmer, Waltham, MA, USA). Non-specific binding, was determined in the presence of approximately 100 fold excess of unlabeled FA. Specific binding was calculated by subtracting the non-specific bound radioactivity from that of the total binding. The data were analyzed by a non-linear regression analysis program (Graph-Pad Software Inc., San Diego, CA, USA) using one-site binding equation. All binding data were corrected for non-specific binding and presented as the mean  $\pm$  standard deviation (S.D.). For internalization measurement, the cell-pellets were incubated again with 300  $\mu\text{l}$  of acidic buffer (0.02 M sodium acetate in saline, pH 4.0) (2) at 37°C

for 20 min. Then, cells were separated by centrifugation and washed with cold acidic buffer. The amount of cell surface-bound (acidic-wash) and intracellular radioactivity (acid-resistant) were determined by measuring the radioactivity of the supernatant and cell pellet, respectively, in a  $\gamma$ -well counter.

***In vivo biodistribution and tumor targeting.*** Approval for the animal protocol used in this study was obtained from the Institutional Animal Care and Use Committee. Animal biodistribution experiments were performed according to the international regulations governing the safe and humane use of laboratory animals in research (28). Human KB xenografts mouse models were used for *in vivo* tumor targeting experiments. For the implantation of tumor xenografts, approximately  $3 \times 10^6$  KB cells in suspension of 100  $\mu\text{l}$  sterile saline were injected subcutaneously into the right thigh of each mouse. Tumors were allowed to grow for 2-3 weeks by which tumors had reached weights of  $\sim 500$  mg. Mice were injected *via* the lateral tail vein with 100  $\mu\text{l}$  of the radiotracers formulated in saline. Each dose contained  $\sim 20$   $\mu\text{Ci}$  (740 kBq) of the  $^{68}\text{Ga}$ -NOTA- and  $^{68}\text{Ga}$ -NOTAM-folate radiotracers. For the blocking studies, each animal was intravenously injected with excess cold FA ( $\sim 100$   $\mu\text{g}$ ) 10 min prior to the radiotracers injection. Animals were sacrificed at different time intervals and the tissues of interest were dissected, weighed and assayed for radioactivity. The percentage of the injected dose per gram (% ID/g) was then



calculated by counting all tissues in a  $\gamma$ -well counter using a stored sample of the injection solution as a standard to estimate the total dose injected per mouse.

**Statistical analysis.** Data are expressed as mean $\pm$ S.D., where appropriate. For data comparisons, a Student's *t*-test was performed of the mean values using the Graph-Pad Software (Graph-Pad Software Inc., San Diego, CA, USA). A probability value of  $p < 0.05$  was considered statistically significant.

## Results and Discussion

**Organic chemistry.** As there exists debate on the affinity of the  $\alpha$  regioisomer to have high affinity for the FR (11, 29), derivatization of the folate at the  $\gamma$ -carboxyl moiety revealed that its affinity for the folate receptors remains unaltered and comparable to folic acid (5). Therefore,  $\gamma$ -hydrazide-folate conjugate **4** was synthesized to serve as a linkage between folic acid and chelating moieties and characterized as reported in detail previously (29). Due to the exceptionally high stability of Ga<sup>III</sup>-NOTA complexes with log K being 31 in comparison with their corresponding DOTA complexes (log K=21) (23, 24) and in an attempt to develop new folate radiotracers with favorable biochemical properties for detecting folate receptor-positive cancers, we have synthesized NOTA- and NOTAM-folate conjugates and chemical purities of the  $\gamma$ -isomers were found to be greater than 98%, as assessed by HPLC and MS analysis. The synthetic approaches for the preparation of NOTA and NOTAM-folates **5** and **9** are outlined in Figures 1 and 2. Starting from compound TACN **1**, NOTA **2** was obtained in 74% yield. NOTA **2** was converted to its corresponding ester (NOTA-NHS, **3**) in 85% yield, which was then conjugated to folate-NH<sub>2</sub>-NH<sub>2</sub> **4** to furnish NOTA-folate conjugate precursor **5** in 56%. The calculated molecular masses for compounds **2**, **3**, and **5** were 303, 400 and 740, respectively, which were in agreement with the found ES-MS [M+1]<sup>+</sup>=304, 401 and 741, respectively. Similarly, starting from compound TACN **1**, NOTAM **6** was obtained in 68% yield. NOTAM **6** was converted to its corresponding ester (NOTAM-NHS, **8**) in 85% yield, which was then conjugated to folate-NH<sub>2</sub>-NH<sub>2</sub> **4** to furnish NOTAM-folate conjugate precursor **9** in 23%. The calculated molecular masses for compounds **6**, **8**, and **9** were 243, 398 and 738, respectively, which were in agreement with the found ES-MS [M+1]<sup>+</sup>=244, 399 and 739, respectively. Both conjugate precursors **5** and **9** appear to decompose beyond 185°C and the base peak  $m/z$ =373 and 371 correspond to the  $\gamma$  moiety of the conjugated folate precursors -(CH<sub>2</sub>-CH<sub>2</sub>-CO-NH-NH-NOTA) and -(CH<sub>2</sub>-CH<sub>2</sub>-CO-NH-NH-NOTAM), respectively.

The reference Ga-NOTA- and Ga-NOTAM-folate conjugates were obtained as yellow powder with overall chemical yields of 75% and 80%, respectively, which appear to decompose beyond 185°C. Chemical purities of Ga-

NOTA- and Ga-NOTAM-folate conjugates were greater than 97% as determined by HPLC with retention times of 13.3 and 13.6 min, respectively (Figure 4). Elemental analysis calculated for Ga-NOTA- and Ga-NOTAM-folate conjugates were: C,45.98; H,4.94; N,20.77 and C,46.10; H,5.20; N,24.29, found: C,46.31; H,4.52; N, 20.23. and C,45.65; H, 4.96; N,23.89, respectively.

**Radiochemistry.** Folate receptor can be used a tumor-associated molecular target since it is over-expressed in ovarian and endometrial carcinomas. Thus, we designed and developed <sup>68</sup>Ga-folate radiotracers utilizing <sup>68</sup>Ga generator to facilitate production of some PET radiopharmaceuticals in PET centers lacking cyclotrons. The synthetic approaches for preparation of <sup>68</sup>Ga-NOTA- and <sup>68</sup>Ga-NOTAM-folate conjugates entailed a straightforward and a simple one-step reaction (Figure 3). The eluted <sup>68</sup>GaCl<sub>3</sub> was reacted with NOTA- and NOTAM-folate conjugates in acetic acid (0.1% in EtOH, pH~4.5) at 80°C for 10 min to provide, after EtOH evaporation and re-constitution in saline, <sup>68</sup>Ga-NOTA- and <sup>68</sup>Ga-NOTAM-folate radiotracers in quantitative radiochemical yields (decay corrected) in less than 20 min. Radiochemical purities of <sup>68</sup>Ga-NOTA- and <sup>68</sup>Ga-NOTAM-folate conjugates were always greater than 98% as determined by HPLC and confirmed by TLC with retention times of 13.6 and 13.9 min, respectively (Figure 4). On the other hand, NOTA- and NOTAM-folate conjugates were labeled with <sup>67</sup>Ga ( $T_{1/2}$ ~78 h) to give the possibility of investigations at a later time point. The <sup>67</sup>GaCl<sub>3</sub> was reacted with NOTA- and NOTAM-folate conjugates using similar conditions and the optimum reaction time was found to be 30 min. These radiotracers were obtained in less than 40 min and the overall radiochemical yields (decay-corrected) and purities were quantitative as assessed by HPLC and TLC. In comparison to the generator produced <sup>68</sup>GaCl<sub>3</sub>, longer reaction time was required for <sup>67</sup>GaCl<sub>3</sub>, which might be attributed to the impurities (Zn, Cu, etc.) present in the final <sup>67</sup>GaCl<sub>3</sub> solution obtained using the cyclotron <sup>68</sup>Zn(p,n)<sup>67</sup>Ga nuclear process. The optimized synthetic approach appears to be faster and advantageous over other synthetic strategies reported previously for the preparation of folate analogs (22, 31) and holds considerable promise in the synthesis of <sup>67/68</sup>Ga-DOTA-folate conjugate analogs, where the overall radiochemical yield and purity were 10% and 92%, respectively.

In addition, the calculated partition coefficient for <sup>68</sup>Ga-NOTA- and <sup>68</sup>Ga-NOTAM-folate conjugates were found to be -1.92 $\pm$ 0.13 and -1.26 $\pm$ 0.09, respectively, representing at least 30% lower lipophilic characteristic for the former, in comparison with the latter conjugate. Moreover, the calculated charged densities for <sup>68</sup>Ga-NOTA- and <sup>68</sup>Ga-NOTAM-folate conjugates were found to be around 0.70 and 0.23, respectively, which correlate well with partition coefficient measurements.

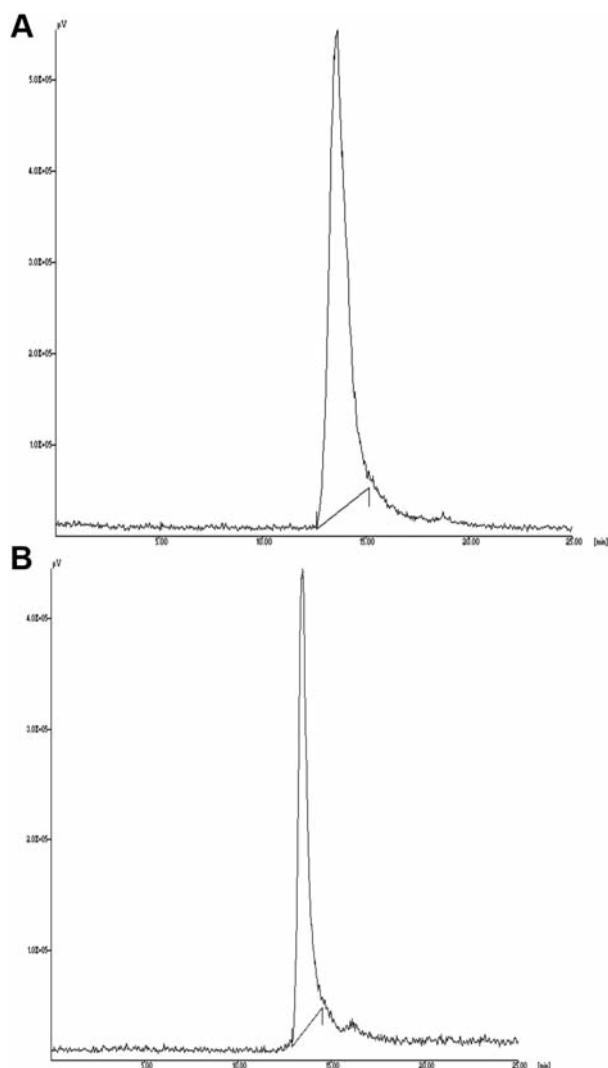


Figure 4. HPLC chromatograms of  $^{68}\text{Ga}$ -NOTA- (A) and  $^{68}\text{Ga}$ -NOTAM-folate (B) conjugates.

**Stability in plasma.** The proteolytic degradation of the  $^{68}\text{Ga}$ -NOTA- and  $^{68}\text{Ga}$ -NOTAM-folate conjugates was determined in human plasma *in vitro*. HPLC analysis of the human plasma revealed that  $^{68}\text{Ga}$ -NOTA- and  $^{68}\text{Ga}$ -NOTAM-folate conjugates remained sufficiently stable (>99%) during incubation at 37°C for at least 4 h demonstrating a high *in vitro* stability of these radioconjugates.

**In vitro cell binding.** The binding affinities ( $K_d$ ) of  $^{68}\text{Ga}$ -NOTA- and  $^{68}\text{Ga}$ -NOTAM-folate conjugates for positive-folate receptor were evaluated using the KB human oral carcinoma cell line. The  $K_d$  values of these conjugates were determined by saturation assays (Figure 5). The results demonstrate that binding affinities of  $^{68}\text{Ga}$ -NOTA- and  $^{68}\text{Ga}$ -

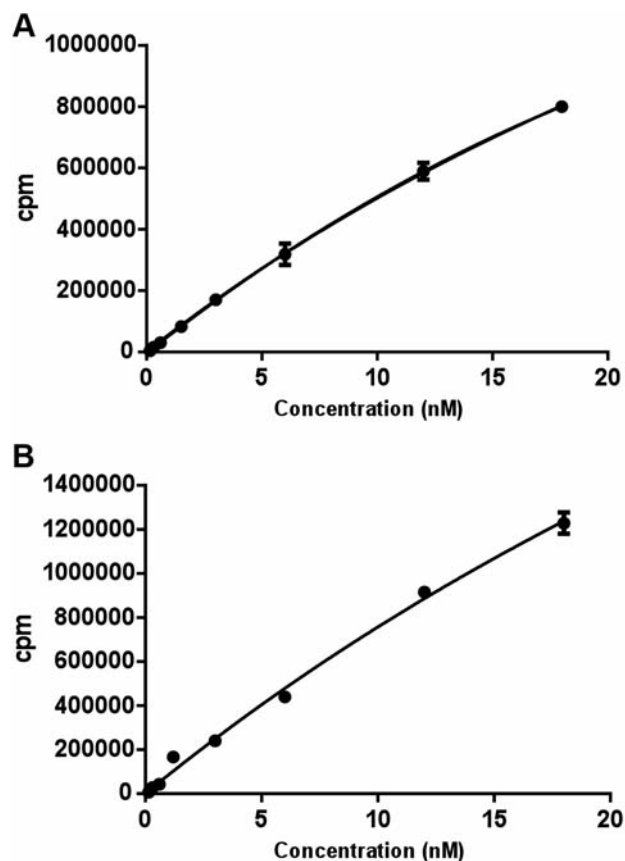


Figure 5. Determination of binding affinity ( $K_d$ ) values of  $^{68}\text{Ga}$ -NOTA- (A) and  $^{68}\text{Ga}$ -NOTAM-folate (B) conjugates for positive-folate receptor using KB human oral carcinoma cell line.

NOTAM-folate conjugates are  $54.36 \pm 6.31$  nM and  $78.01 \pm 13.0$  nM, respectively. These results appeared to be well at least ten-fold lower than binding affinities for their corresponding  $^{68}\text{Ga}$ -DOTA conjugates. Blocking experiments performed with excess amount of native FA showed negligible binding on cell surface, which indicate the high specificity of  $^{68}\text{Ga}$ -NOTA- and  $^{68}\text{Ga}$ -NOTAM-folate conjugates for the over-expressed folate receptor *in vitro*.

It is important for therapeutic radiopharmaceuticals that radiolabeled molecules be internalized into the tumor cells after binding to the cell surface receptors. *In vitro* cellular internalization studies were performed to determine the extent of internalization of  $^{68}\text{Ga}$ -NOTA- and  $^{68}\text{Ga}$ -NOTAM-folate conjugates by human cancer KB cells using acidic buffer. A rapid and significant internalization was observed for both conjugates with  $35.22 \pm 0.50\%$  and  $45.0 \pm 1.13\%$  of the cell surface bound conjugates internalized after incubation in acidic buffer for 20 min at 37°C, respectively. These results appear to be at least two-fold higher than their corresponding  $^{68}\text{Ga}$ -DOTA conjugates ( $\sim 16.5\%$ ). These findings of cell-

binding and internalization into tumor cells demonstrate that, despite the modifications in the folate for  $^{68}\text{Ga}$  chelation, these conjugates maintained their integrity and high affinity and specificity towards positive folate receptor.

*In vivo biodistribution and tumor uptake.* The biodistribution data in nude mice bearing human KB cell line xenografts for  $^{68}\text{Ga}$ -NOTA- and  $^{68}\text{Ga}$ -NOTAM-folate conjugates at 1, 2, 4 and 24 h post injection (*p.i.*) are shown in Tables I and II. Both  $^{68}\text{Ga}$ -NOTA- and  $^{68}\text{Ga}$ -NOTAM-folate conjugates displayed moderate clearance from the blood and most of the other organs. A low to moderate uptake of both  $^{68}\text{Ga}$ -NOTA- and  $^{68}\text{Ga}$ -NOTAM-folate conjugates was exhibited in the kidney ( $2.97\pm 1.12$  and  $5.56\pm 0.78\%$  ID/g, respectively, 4 h *p.i.*), which are known to express folate receptor. Surprisingly, kidney uptake was found to be much lower than the  $^{67/68}\text{Ga}$ -DOTA-folate conjugate analogs (85-103% ID/g, 4h *p.i.*) reported previously (22, 31). The exact reason for the low uptake is unclear, but we assumed that this behavior may be attributed to the nature of  $^{68}\text{Ga}$ -NOTA and  $^{68}\text{Ga}$ -NOTAM as chelating groups and overall charge on  $^{68}\text{Ga}$ -NOTA- and  $^{68}\text{Ga}$ -NOTAM-folate complexes (32). More studies are required to validate this assumption. High tumor uptake was observed for both  $^{68}\text{Ga}$ -NOTA- and  $^{68}\text{Ga}$ -NOTAM-folate conjugates at all time-point intervals, however, the maximum uptake was observed at 4 h *p.i.* ( $17.75\pm 4.27$  for the former and  $8.65\pm 0.56\%$  ID/g for the latter, respectively). The high tumor uptake for both conjugates at 24 h *p.i.* ( $6.08\pm 0.65$  and  $7.33\pm 0.31\%$  ID/g) indicates a slow wash-out from the tumor, which is comparable to  $^{67/68}\text{Ga}$ -DOTA-folate conjugate analogs previously reported (22, 31). Tumor-to-blood, tumor-to-muscle and tumor-to-intestine ratios for  $^{68}\text{Ga}$ -NOTA- and  $^{68}\text{Ga}$ -NOTAM-folate conjugates at 4 h *p.i.* were 6.65, 31.14, 5.04 and 1.92, 5.62, 2.19, respectively, rendering the former conjugate more suitable as a tumor imaging agent than the latter. It is worth mentioning that the tumor uptake of  $^{68}\text{Ga}$ -NOTA-folate conjugate is superior to uptake profile reported for  $^{67/68}\text{Ga}$ -DOTA-folate conjugates (22, 31), while radioactivity accumulation in the liver and intestine was below 10% ID/g at 4 h *p.i.* In addition, receptor blocking studies in which 100  $\mu\text{g}$  dose of FA was injected 10 min prior the administration of both conjugates, significantly reduced the uptake in the target organs and tumors.  $^{68}\text{Ga}$ -NOTA-  $^{68}\text{Ga}$ -NOTAM-folate conjugates uptake was reduced in tumor ( $17.75\pm 4.27$  vs.  $2.01\pm 0.56\%$  and  $8.65\pm 0.56$  vs.  $1.12\pm 0.25\%$  ID/g, respectively,  $p<0.05$ ) by approximately 85%, indicating receptor-specificity of these radioconjugates. Uptake by the kidneys of both radioconjugates was dramatically reduced by at least 70%.

The favorable biodistribution profile of  $^{68}\text{Ga}$ -NOTA-folate conjugate warrant further evaluation and may tempt one to infer that this PET radiotracer may be useful as a molecular probe for detecting and staging of folate receptor-positive cancers, such as ovarian cancer and their metastasis as well

Table I. Biodistribution of  $^{68}\text{Ga}$ -NOTA-folate conjugate in tumor-bearing nude mice.

	1 h	2 h	4 h	24 h**
Blood	$8.33\pm 0.96$	$7.13\pm 0.43$	$2.67\pm 0.40$	$0.99\pm 0.16$
Liver	$5.53\pm 0.87$	$5.56\pm 0.80$	$6.26\pm 0.92$	$3.29\pm 0.49$
Lung	$3.30\pm 0.13$	$2.80\pm 0.55$	$1.95\pm 0.38$	$1.17\pm 0.32$
Kidney	$4.18\pm 0.56$	$3.04\pm 0.47$	$2.97\pm 1.12$ ( $0.48\pm 0.05$ )*	$3.44\pm 0.79$
Intestine***	$3.37\pm 0.28$	$4.40\pm 0.03$	$3.52\pm 0.35$	$2.10\pm 0.42$
Heart	$2.57\pm 0.31$	$1.75\pm 0.75$	$1.46\pm 0.36$	$0.55\pm 0.05$
Muscle	$1.42\pm 0.26$	$0.72\pm 0.02$	$0.57\pm 0.15$	$0.63\pm 0.12$
Spleen	$3.56\pm 0.59$	$3.10\pm 1.27$	$2.24\pm 0.55$	$2.23\pm 0.57$
Tumor	$6.29\pm 0.33$	$8.94\pm 0.27$	$17.75\pm 4.27$ ( $2.01\pm 0.57$ )*	$6.08\pm 0.65$

The values are average of % injected dose/gram $\pm$ SD for n=4. \*Animals were injected with blocking dose of 100  $\mu\text{g}$  folic acid per mouse 10 min prior to the administration of radiotracers. \*\*Animals were injected with  $^{67}\text{Ga}$ -NOTA-folate conjugate. \*\*\*With content.

Table II. Biodistribution of  $^{68}\text{Ga}$ -NOTAM-folate conjugate in tumor-bearing nude mice.

	1 h	2 h	4 h	24 h**
Blood	$11.55\pm 2.35$	$9.03\pm 1.03$	$4.50\pm 0.56$	$1.71\pm 0.20$
Liver	$5.53\pm 1.61$	$3.64\pm 0.76$	$4.78\pm 0.47$	$6.79\pm 0.82$
Lung	$3.72\pm 1.18$	$3.96\pm 0.28$	$3.71\pm 0.60$	$2.10\pm 0.15$
Kidney	$4.17\pm 1.04$	$4.10\pm 0.26$	$5.56\pm 0.78$ ( $1.50\pm 0.06$ )*	$4.52\pm 0.53$
Intestine***	$3.59\pm 1.32$	$4.10\pm 0.64$	$3.97\pm 0.43$	$3.51\pm 0.69$
Heart	$3.72\pm 0.16$	$3.83\pm 0.43$	$3.34\pm 0.20$	$0.98\pm 0.11$
Muscle	$0.81\pm 0.08$	$1.15\pm 0.07$	$1.54\pm 0.57$	$0.65\pm 0.08$
Spleen	$4.09\pm 0.45$	$2.70\pm 0.39$	$2.93\pm 0.37$	$3.55\pm 0.22$
Tumor	$4.57\pm 0.60$	$7.44\pm 1.44$	$8.65\pm 0.56$ ( $1.12\pm 0.25$ )*	$7.33\pm 0.31$

The values are average of % injected dose/gram $\pm$ SD for n=4. \*Animals were injected with blocking dose of 100  $\mu\text{g}$  folic acid per mouse 10 min prior to the administration of radiotracers. \*\*Animals were injected with  $^{67}\text{Ga}$ -NOTAM-folate conjugate. \*\*\*With content.

as monitoring tumor response to treatment. Low kidney uptake may also, offer this radioconjugate as potential candidate for radionuclide therapy when labeled with therapeutic radionuclides, such as  $^{90}\text{Y}$  or  $^{177}\text{Lu}$ , which is the subject of another study.

## Conclusion

The synthesis of  $^{68}\text{Ga}$ -NOTA-  $^{68}\text{Ga}$ -NOTAM-folate conjugates was performed using a simple one-step reaction. The overall radiochemical yields and purities for these



radioconjugates were quantitative with total synthesis time of less than 20 min. *In vitro* binding studies on the KB cell line showed superior affinity of  $^{68}\text{Ga}$ -NOTA-folate over  $^{68}\text{Ga}$ -NOTAM-folate conjugates for folate receptor. In a nude mice model bearing human KB cell line xenografts,  $^{68}\text{Ga}$ -NOTA-folate demonstrated significant tumor uptake and favorable pharmacokinetics over the  $^{68}\text{Ga}$ -NOTAM-folate conjugate. These results demonstrate that the  $^{68}\text{Ga}$ -NOTA-folate conjugate may be useful as molecular probe for detecting folate receptor-positive cancers, such as ovarian cancer, using positron emission tomography, however, further evaluation is warranted.

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