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Synthesis and Preclinical Evaluation of Folate-NOTA-AI¹⁸F for PET Imaging of Folate Receptor-Positive Tumors

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Abstract

Folate receptor-targeted PET radiotracers can potentially serve as versatile imaging agents for the diagnosis, staging, and prediction of response to therapy of patients with folate receptor (FR)-expressing cancers. Because current FR-targeted PET reagents can be compromised by complex labeling procedures, low specific activities, poor radiochemical yields or unwanted accumulation in FR negative tissues, we have undertaken to design an improved folate-PET agent that might be more amenable for clinical development. For this purpose, we have synthesized a folate-NOTA-AI¹⁸F radiotracer and examined its properties both *in vitro* and *in vivo*.

Methods—Radiochemical synthesis of folate-NOTA-AI¹⁸F was achieved by incubating ¹⁸F[−] with AlCl₃ for 2 min followed by heating in the presence of folate-NOTA for 15 min at 100 °C. Binding of folate-NOTA-AI¹⁸F to FR was quantitated in homogenates of KB and Cal51 tumor xenografts in the presence and absence of excess folic acid as a competitor. *In vivo* imaging was performed on *nu/nu* mice bearing either FR+ve (KB cell) or FR−ve (A549 cell) tumor xenografts, and specific accumulation of the radiotracer in tumor and other tissues was assessed by high-resolution micro-PET and *ex vivo* biodistribution in the presence and absence of excess folic acid. Image quality of folate-NOTA-AI¹⁸F was compared with that of ^{99m}Tc-EC20, a clinically established folate-targeted SPECT imaging agent.

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SUPPORTING INFORMATION

Supporting information is included with this manuscript consisting of

1. The revised manuscript copy with tracked changes (for review only)
2. The revised supporting information file (for publication)
 - Materials and Methods
 - Detailed Experimental section
 - References
 - Supplemental table

Results—Total radiochemical synthesis and purification of folate-NOTA- Al^{18}F was completed within 37 min, yielding a specific activity of 68.82 ± 18.5 GBq/ μmol , radiochemical yield of $18.6 \pm 4.5\%$, and radiochemical purity of $98.3 \pm 2.9\%$. Analysis of FR binding revealed a K_d of ~ 1.0 nM, and micro-PET imaging together with *ex vivo* biodistribution analyses demonstrated high FR-mediated uptake in an FR+ tumor and the kidneys.

Conclusions—Folate-NOTA- Al^{18}F constitutes an easily prepared FR-targeted PET imaging agent with improved radiopharmaceutical properties and high specificity for folate receptor expressing tumors. Given its improved properties over $^{99\text{m}}\text{Tc}$ -EC20 (i.e. higher resolution, shorter image acquisition time, etc.), we conclude that folate-NOTA- Al^{18}F constitutes a viable alternative to $^{99\text{m}}\text{Tc}$ -EC20 for use in identification, diagnosis, and staging of patients with FR-expressing cancers.

Graphical abstract

Keywords

folate receptor; Al^{18}F -NOTA chelate; ^{18}F -PET imaging; cancer imaging; imaging autoimmune disease

INTRODUCTION

Uptake of physiological folates is mediated in most cells by either the reduced folate carrier ($K_m \sim 1\text{--}5 \times 10^{-6}$ M)¹ or proton coupled folate transporter ($K_m \sim 0.5\text{--}1.0 \times 10^{-6}$ M),² however, a few cells (a subset of epithelial cells and activated pro-inflammatory macrophages) may internalize the vitamin via folate receptor-mediated endocytosis.^{3, 4} Although the reduced folate carrier and protein coupled folate transporter do not facilitate influx of folate conjugates (i.e. folate-linked drugs and imaging agents), the folate receptor does catalyze internalization of folate and folate conjugates with roughly equal affinity. For this reason, folic acid has been frequently exploited to deliver imaging and therapeutic agents selectively to pathologic cells that over-express FR.^{4–7}

A variety of folate receptor-targeted imaging agents have been developed for optical,^{8–14} nuclear,^{15–19} and magnetic resonance imaging of cancers (ovarian cancer, non-small lung cancer, kidney cancer, and endometrial cancer etc.) and sites of inflammation (e.g. rheumatoid arthritis, atherosclerosis, and pulmonary fibrosis etc.).^{20–26} For whole body

imaging, positron emission tomography (PET) has attracted the greatest interest because of its higher sensitivity, better spatial resolution, greater signal to noise ratio, and superior tracer quantification.^{27, 28} Most PET imaging protocols also require only low doses of radiotracer, thereby minimizing radiation exposure and allowing for repeated imaging of the same patient.²⁹ When pharmacokinetic and/or pharmacodynamic data on drug candidates are required, PET methodologies also allow for quantitative dynamic imaging, facilitating acquisition of kinetic data on target engagement and retention.

While multiple radionuclides have been successfully employed for PET imaging (e.g. ¹⁸F, ⁸⁹Zr, ⁶⁶Ga, ⁶⁸Ga, ⁶⁴Cu, and ¹²⁴I), ¹⁸F is often preferred because of its commercial availability and optimal decay characteristics, including: i) relatively long half-life (109.8 min; enabling distribution to clinics following centralized syntheses), ii) ideal positron energy ($E_{\text{max}} = 0.635$ MeV; Eaverage energy is 0.25 MeV), and iii) high probability of β^+ emission (97 %), permitting high resolution images and relatively low tissue energy deposition. Although several ¹⁸F-labeled folate-targeted PET agents have already been developed and evaluated in preclinical studies, a need still exists for improved folate-targeted ¹⁸F-PET agents with shorter preparation times, higher radiochemical yields, or increased specific activities.^{30–32} In this investigation, we report the chemical synthesis and one-step radiochemical labeling of a folate-NOTA-Al¹⁸F PET imaging agent and describe its radiopharmaceutical properties both *in vitro* and *in vivo*.

EXPERIMENTAL SECTION

General

Water was distilled and then deionized (18 MΩ/cm²) by passing through a Milli-Q water filtration system (Millipore Corp., Milford, MA). All chemicals and solvents, unless otherwise specified, were purchased from Sigma (St. Louis, MO) and were used without further purification. 2,2'-(7-(2-((2,5-dioxopyrrolidin-1-yl)oxy)-2-oxoethyl)-1,4,7-triazonane-1,4-diyl)diacetic acid (NOTA-NHS) was purchased from CheMatech (France). N¹⁰-TFA-pterioic acid was kindly provided by Endocyte, Inc. The diammonium salt of ³H-folic acid (37 MBq/mL, 1483.7 GBq/mmol) was purchased from Moravек Biochemicals. The folate-NOTA PET precursor and all other intermediates were synthesized as described below. High-performance liquid chromatography (HPLC) analysis and purification of the folate-NOTA precursor were performed on an Agilent 1200. Carrier-free ¹⁸F-fluoride was obtained from PETNET. The scintillation solution, Ultima Gold high-flash-point liquid scintillation cocktail, was purchased from Packard Co. Folic acid deficient RPMI cell culture medium (lacking folic acid, vitamin B12, and phenol red) was obtained from Cell Culture Technologies GmbH. Raw data acquired *in vitro* were analyzed using Prism software (GraphPad Prism 4), and PET imaging files were evaluated using dedicated software PMOD 2.95 (PMOD Technologies, Adliswil, Switzerland).

Synthesis of Folate-NOTA Conjugate (1)

A detailed description of the chemical synthesis of the PET precursor, folate-NOTA (**1**), and the reference compound, folate-NOTA-Al¹⁸F, is available in the Supporting Information. Briefly, a protected derivative of folic acid **4** was prepared via PyBOP-mediated coupling of

N¹⁰-TFA-ptericoic acid to H₂N-Glu(OtBu)-OMe, followed by deprotection of the tert-butyl ester with trifluoroacetic acid (TFA). Deprotected **4** was then conjugated with 2,2'-[1,2-ethanediylbis(oxy)] bisethanamine using PyBOP, followed by Boc-removal with TFA to produce **6**. Incubation of **6** with NOTA-NHS ester in DMSO then yielded the protected folate-NOTA **7**, which upon deprotection in 1 M NaOH (aq.) led to the folate-NOTA labeling precursor **1** (Figure 1 and Supporting Information). The crude product was purified by preparative reverse-phase HPLC using a gradient mobile phase of A = 10 mM ammonium acetate buffer and B = acetonitrile; solvent gradient 5% B to 100% B in 30 minutes (XBridge Prep C18, 5 μ m; 19 $\times$ 150 mm). Elution of the conjugate was monitored at λ = 280 nm and the identities of the eluted compounds were analyzed by LC-MS.

Radiochemistry

A cartridge containing ¹⁸F-fluoride was first washed with 1.5 mL of ultrapure water and then ¹⁸F-fluoride was eluted with 1.0 mL of 0.4 M KHCO₃. The eluted ¹⁸F-fluoride solution (100 μ L) was added to a stem vial charged with 10 μ L acetic acid, 25 μ L AlCl₃ (2 mM in 0.1 M NaOAc buffer, pH 4) and 125 μ L 0.1 M NaOAc pH 4. The solution was incubated for 2 min at r.t., mixed with 0.25 mg folate-NOTA precursor (**1**) in 125 μ L of 0.1 M NaOAc, pH 4, and heated immediately to 100 °C for 15 min. After cooling to room temperature, the crude material was mixed with 0.7 Ml 0.1% formic acid and purified by semi-preparative radioactive HPLC equipped with a γ -counter (Wizard, PerkinElmer) by applying a linear gradient starting from 90% A to 15% B over 15 min at a flow rate of 4 mL/min (A = acetonitrile and B = 0.1% formic acid), and the fraction at 11.5 min was collected. Corresponding UV absorption was recorded at 254 nm wavelength. Volatile solvents were removed from the purified product (folate-NOTA-Al¹⁸F (**2**)) by heating under a nitrogen stream, and the resulting aqueous solution was sterile filtered and diluted in isotonic saline to the desired radioactivity.

Cell Culture and IC₅₀ evaluation

Analysis of ³H-folate binding to FR was conducted on KB cells cultured in folate deficient RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (HIFBS), 1% L-glutamine and 1% penicillin at 37°C in a humidified atmosphere containing 5% CO₂. Spent medium in each well was replaced with 10 nM [³H]-folate in the presence of increasing concentrations of either folate-NOTA or free folic acid. After incubating for 1 h at 37 °C, cells were rinsed with PBS (2 $\times$ 0.5 mL) and 1 M trichloroacetic acid (1 $\times$ 0.5 mL) to remove any unbound radioactive material. Cells were dissolved in 1% sodium dodecylsulfate (0.5 mL) and transferred to Ecolume scintillation cocktail for scintillation counting. Experiments were performed in triplicate for each concentration and relative binding affinities (IC₅₀) were calculated using a plot of cell bound radioactivity versus concentration of test compound using GraphPad Prism 4.

K_d evaluation

For analysis of folate-NOTA-Al¹⁸F binding, KB tumors were collected, weighed, added to 9 volumes of lysis buffer (50 mM Tris-HCL containing 2 mM EDTA, pH 7.4), and homogenized at 4 °C using a Potter-Elvehjem homogenizer attached to a variable-speed drill

and a tissuemizer. The homogenized tissue was centrifuged for 5 minutes in a microfuge at 1000 rpm, and the cell pellet was resuspended in folate deficient 1640 RPMI medium, seeded in 24-well (100,000 cells/well) Falcon plates and allowed to form monolayers over a period of 24 h. To each well were added increasing concentrations of folate-NOTA-Al¹⁸F (**2**) in fresh medium (0.5 mL). After incubating for 1 h at 37 °C, cells were rinsed with PBS (2 × 0.5 mL) and 1 M trichloroacetic acid (1 × 0.5 mL) to remove any unbound radioactive materials. 1% sodium dodecylsulfate in PBS (0.5 mL) was then added and solubilized cells were transferred into individual test tubes and bound folate-NOTA-Al¹⁸F was measured in a γ -counter (Wizard; PerkinElmer) using an energy window of 300–700 keV. Experiments were performed in triplicate at each concentration. The B_{max} and K_d values were calculated using GraphPad Prism 4.

Tumor implantation in mice

All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Merck, West Point, PA. KB (high FR expression) or Cal-51 (moderate FR expression) cells were cultured in complete growth medium containing folate free RPMI 1640 medium with 5% fetal bovine serum, while A549 cells (low FR expression) were cultured in complete growth medium containing F-12K medium with 10% fetal bovine serum at 37°C with 5% CO₂. The growth media was changed 2 or 3 times per week and the cells were subcultured at a ratio of 1:10 when needed.

Female CD1 nu/nu (KB) and nu/nu (Cal51 or A549), 6–8 week old, were ordered from Charles River Laboratories (Stone Ridge, NY, USA) and housed in a temperature and humidity controlled room and maintained on a folate-deficient rodent diet. After 7 to 14 days of acclimatization, tumors were implanted at the right shoulder with subcutaneously injection of 1×10⁶ KB cells in 100 μ L folate free RPMI, 5×10⁶ Cal51 or A549 cells in 100 μ L PBS + Growth Factor Reduced Matrigel (1:1). Animal experiments were then performed 2–3 weeks after tumor cell injection, when the tumors had grown to a mass of 200 to 400 mg.

MicroPET Imaging

In order to evaluate the *in vivo* imaging performance of folate-NOTA-Al¹⁸F (**2**) to FR+ tumors, microPET imaging was performed on nude mice bearing KB tumor xenografts both in the presence and absence of excess free folate to block all accessible FR. PET experiments were performed with a dedicated small-animal PET system (Focus220, Siemens Medical Solution, Hoffman Estate, IL). Anesthesia was induced with 4–5% isoflurane and maintained with 1–3% isoflurane delivered with a mixed air/oxygen through a closed nose cone throughout the PET imaging session. Mice bearing KB tumor xenografts on their left shoulders were injected with 8.5–12.9 MBq (0.12–0.24 nmol) of folate-NOTA-Al¹⁸F (**2**). The competition group (n = 3) received an intravenous injection of 100 μ g folic acid (FA) 2 min before radiotracer administration, and the baseline control (n = 4 per tumor type) was injected with the same radiotracer plus the corresponding volume of isotonic saline.

PET data were collected for 90 minutes following radiotracer administration, and reconstructed using maximum a posteriori reconstruction (MAP, β = 0.2, 256 × 256 × 95

voxels, $0.9 \times 0.9 \times 0.80 \text{ mm}^3$, ^{57}Co transmission-based photon attenuation and scatter correction). PET images were converted to percent injected dose per gram (%ID/g tissue) for evaluation of tumor specificity, Image analysis was performed using the software program MATLAB (MathWorks, Natick, Massachusetts, USA).

Biodistribution Study

The binding specificity of folate-NOTA- Al^{18}F (**2**) to FR was further evaluated through *ex vivo* biodistribution studies in mice in comparison with $^{99\text{m}}\text{Tc}$ -EC20 under both baseline and competition conditions. Immediately after PET imaging, mice were euthanized via CO_2 inhalation. Tissues (blood, plasma, heart, lung, liver, spleen, kidney, muscle and tumor) were collected, cleaned and weighed, then counted in a gamma counter (Wizard 3, PerkinElmer). All mice that underwent PET imaging with folate-NOTA- Al^{18}F (**2**) including mice with A549 tumors, were included in the biodistribution study. For $^{99\text{m}}\text{Tc}$ -EC20 studies, 0.37–0.74 MBq (0.036 – 0.084 nmol) was administered, and mice were sacrificed by decapitation and dissected 120 min post injection (p.i.) of radiotracer. Competition with free folic acid was achieved using the same methodology as in folate-NOTA- Al^{18}F (**2**) studies. Radiotracer accumulation for all biodistribution studies was calculated as %ID/g.

Radioactive Saturation Binding Assay

KB and Cal51 xenografts were harvested and stored at -80°C freezer before use. Crude homogenates of the xenografts were prepared by homogenizing them separately in ice cold buffer (50 mM Tris pH 7.5, 120 mM NaCl, 2mM KCl, 1 mM MgCl_2 , 1 mM CaCl_2) for 30 sec. at 4°C on setting 5 of Polytron. Crude homogenates of KB and Cal51 xenografts (1 mg/mL of wet tissue weight) were used in the assay. Tissue homogenates were first pre-incubated at room temperature for 2 hours in assay buffer (50 mM Tris-HCl pH 7.5, 120 mM NaCl, 2 mM KCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 1:1000 Protease Inhibitor (P-8340) with 2% DMSO or 5 μM self-block using Skatron tube strips (SK15776) covered with aluminum foil. Non-displaceable binding of folate-NOTA- Al^{18}F was defined using 5 μM self-block. Following pre-incubation, twelve concentrations of folate-NOTA- Al^{18}F from 0.1 nM to 30 nM were added to the assay tubes in duplicates with final assay volume 0.25 mL per tube. The assay tubes were mixed by brief vortex, and then incubated at room temperature for 60 minutes. After completion of incubation, each assay tube mixtures were transferred onto Skatron GF/C filters (SK11731), which was pre-soaked in 0.2% PEI for 30 minutes at RT before use, using a Skatron Combi cell 12-well harvester. The filters were promptly washed 3 times on setting 3-3-3 with ice cold wash buffer (50 mM Tris, pH 7.5, 120 mM NaCl, 2 mM KCl, 1 mM MgCl_2 , 1 mM CaCl_2). The filters were punched into Pico Pro vials, and then counted in Gamma counter (Wizard 3, PerkinElmer). The data were analyzed using nonlinear fit (one site binding) model with Prism software (GraphPad, CA)

RESULTS

Radiochemistry

Radiochemical synthesis of folate-NOTA- Al^{18}F (**2**) was performed by reacting the folate-NOTA precursor **1** with Al^{18}F followed by HPLC purification ($R_t=11.5 \text{ min}$) to afford compound **2** (Figure 2A). Starting with $99.3 \pm 25.4 \text{ mCi}$ ($n=3$) of ^{18}F]fluoride, compound **2**

was isolated in a decay corrected radiochemical yield of $18.6 \pm 4.5\%$ (17.7 ± 0.1 mCi) with a radiochemical purity of $98.3 \pm 2.9\%$ (Figure 2B) and a specific activity of 68.82 ± 18.5 GBq/ μmol in a total synthesis time of 37 min.

***In Vitro* Characterization**

Relative Binding Affinity—To investigate the potential influence of conjugation of NOTA to folate on the binding affinity of the folate conjugate to FR, a relative binding affinity assay was performed by evaluating the concentration of conjugate required to block binding of ^3H -folic acid. As shown in figure 3, folate-NOTA conjugate (**1**) displayed high affinity for FR with a mean IC_{50} of 18.7 nM at the concentration of ^3H -folic acid used, which is only slightly weaker than the affinity of free folic acid (4.6 nM), suggesting that attachment of the NOTA chelating moiety has only a minor effect on FR binding affinity.

Radioactive Saturation Binding Assay

In saturation binding assay, folate-NOTA- Al^{18}F (**2**) was found to bind to crude homogenates of KB and Cal51 xenografts (1 mg/mL wet weight) in a monophasic manner, consistent with a single class of binding sites (Figure 4). The K_d values estimated for folate-NOTA- Al^{18}F were 0.7 nM (KB) and 1.1 nM (Cal51) respectively. Non-displaceable binding (assessed in the presence of 5 μM self-block) was low, approximately < 1% (KB) and 11% (Cal51) of total binding at concentrations near the K_d value. B_{max} values of folate-NOTA- Al^{18}F were found to vary 14-fold between KB (511 nM) and Cal51 (36 nM) xenografts. Folate-NOTA- Al^{18}F binding potentials (B_{max}/K_d) in xenografts were 730 (KB) and 33 (Cal51) respectively, resulting in 22-fold greater binding potential of folate-NOTA- Al^{18}F in KB xenografts than in Cal51 xenografts. The lower expression of FR in Cal51 xenografts could account for the reduced retention of folate-NOTA- Al^{18}F in the Cal51 samples.

***In Vivo* microPET imaging**

As shown in figure 5, tumors were readily visible under baseline conditions (injection of radiotracer with no competing folic acid), whereas the uptake of folate-NOTA- Al^{18}F (**2**) was completely blocked with excess free folic acid, supporting a high specificity of folate-NOTA- Al^{18}F binding to FR *in vivo*. The high radioactivity found in kidneys was due both to FR-mediated uptake by folate receptors expressed on the proximal tubule cells of the kidneys and the presence of excreted folate-NOTA- Al^{18}F flowing through the urine concentrating ducts and tubules of the kidneys. No significant uptake in other organs was found except limited accumulation in liver.

***Ex Vivo* Biodistribution Studies**

As shown in table 1 and figure 6, uptake of folate-NOTA- Al^{18}F (**2**) in KB tumors was 10.9 ± 2.7 %ID/g in baseline animals, whereas the radioactive accumulation in the animals subjected to competition with excess free folic acid was substantially reduced to 1.3 ± 0.1 %ID/g, indicating a highly specific uptake mediated by FR. In contrast, the uptake of folate-NOTA- Al^{18}F (**2**) in the very low FR expressing A549 tumors was 2.8 ± 0.7 %ID/g, i.e., substantially lower than that in KB tumors (Figure 6). The high accumulation of folate-NOTA- Al^{18}F (**2**) in kidneys, along with much lower uptake in liver shown in figure 7

suggests that renal elimination is the predominant route for excretion. Moreover, a measurable blocking effect of excess folic acid was also found in heart, lung, muscle and spleen, suggesting low levels of FR present in these organs. Overall, the biodistribution of folate-NOTA-Al¹⁸F (**2**) is comparable but perhaps slightly better than that of ^{99m}Tc-EC20, the clinically established folate-based SPECT tracer.

DISCUSSION

In this study, we have shown that a rationally-designed folate-NOTA-Al¹⁸F (**2**) can be used as a PET imaging agent for targeting FR+ tumor xenografts in mice. The highly specific binding of **2** suggests that attachment of a chelating group like NOTA to folic acid has little effect on the conjugate's affinity for FR. Radiochemical synthesis of folate-NOTA-Al¹⁸F was achieved in one step by reaction with Al¹⁸F₃, yielding folate-NOTA-Al¹⁸F in good radiochemical yield (18.6±4.5%), high radiochemical purity (98.3±2.9%, after HPLC purification), and a total synthesis time of 37 min, making the entire procedure compatible with a standard automated production system.

Biodistribution data and PET imaging experiments demonstrated that the uptake of folate-NOTA-Al¹⁸F (**2**) was concentrated primarily in FR+ KB tumors and kidneys. As displayed in table 1 and figure 6, uptake of radiotracer **2** in FR+ KB tumors was ~10.9 %ID/g at 90 minutes after injection. Accumulation of this radiotracer was also shown to be FR-mediated, as the administration of excess free folic acid blocked its retention in tumor. As expected, a high uptake of radioactivity was found in the kidneys (78.6 ± 5.1 %ID/g, 90 min p.i.), because of elevated expression of FR in proximal tubule cells. Ongoing renal excretion of this radiotracer may also contribute to the high accumulation in kidneys, especially at early time points. The more limited radioactivity in the liver (5.3 ± 0.5 %ID/g, 90 min p.i.) suggests that only a minor fraction of the conjugate undergoes hepatobiliary excretion. In contrast, the biodistribution study in A549 xenografts revealed a very limited accumulation of **2** in FR- tumor, which further confirmed that the highly specific uptake of **2** is mediated by FR.

Folate-NOTA-Al¹⁸F and ^{99m}Tc-EC20 showed largely similar distribution in mice, with the folate targeted PET agent displaying somewhat lower retention in normal tissues than the ^{99m}Tc- EC20. In a clinical setting, PET may show approximately thirty-fold better sensitivity and eightfold better volumetric resolution than SPECT. Quantitation of tracer concentration is inherent in all clinical PET and PET/CT scanners, whereas for SPECT and SPECT/CT it is still an area of development. These advantages for PET translate into potentially shorter whole-body acquisition times, improved sensitivity of tracer uptake in lesions smaller than ~20 mm, and the ability to quantify the signal in individual lesions with the possibility of establishing a correlation tracer uptake and lesion response to therapy.

It should be emphasized that the folate-NOTA-Al¹⁸F conjugate described here is not the first folate-targeted PET agent to be described in the literature. Indeed, a variety of folate-conjugated ⁶⁸Ga and ¹⁸F PET agents have already been shown to produce highly defined images of FR positive tumors in animal tumor models.^{33–56} Our motivation for seeking yet another folate-targeted PET agent was prompted by the need to create an imaging agent that

could meet the economic requirements for translation of a PET agent into the clinic. ^{18}F was selected ahead of ^{68}Ga not only because of the higher resolution images that it yields,^{28, 57–59} but also because of the extensive production and distribution networks that already exists for ^{18}F .⁵⁸ Folate-NOTA- Al^{18}F was preferred over earlier folate- ^{18}F conjugates for reasons that differ for each previous conjugate, including its higher specific activity,^{39, 45, 47, 55} comparable or in some cases better radiochemical yield,^{39, 40, 47, 49} and faster or simpler synthesis.^{39, 40, 47, 49, 51} As a consequence, we propose that folate-NOTA- Al^{18}F meets most of the requirements for eventual translation into human clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIGURE 1.

Synthesis of folate-NOTA precursor (**1**) and radiochemical synthesis of folate-NOTA-Al¹⁸F PET radiotracer (**2**). All reactions were conducted under N₂ or Ar at room temperature. Reagents and conditions of reactions (a–e) are: a) HCl·H₂N-Glu(OtBu)-OMe, PyBOP, DIPEA, DMSO, 23 °C; b) TFA/DCM, 1/3, 23 °C, 83% for 2 steps; c) PyBOP, BocNH-PEG-NH₂, DIPEA, DMSO, 23 °C; d) TFA/DCM, 1/3, 23 °C, 90% for 2 steps; e) DIPEA, DMSO, 23 °C, 59%; f) 1M NaOH, 23 °C, 5 min, 60% after HPLC; g) AlCl₃-K¹⁸F, NaOAc buffer, pH 4, 100 °C

A. **B.****FIGURE 2.****A.** HPLC Chromatogram Purification of folate- Al^{18}F (**2**)**B.** Representative analytical HPLC chromatogram of purified folate- Al^{18}F (**2**).

FIGURE 3. Relative binding of folate-NOTA to cultured KB cells: Inhibition of ^3H -folate with folic acid and folate-NOTA (1)

FIGURE 4. Specific binding affinity assays of folate-NOTA-Al¹⁸F (2) to FR in KB (upper graphs) and Cal51 (lower graphs) tumor crude homogenate (1 mg/mL)

FIGURE 5.

PET images with folate-NOTA-Al¹⁸F (**2**) are shown in mice bearing KB tumors on left shoulders (arrows) under baseline and blocking conditions. A blocking dose of 100 μ g Folic acid was administered i.v. immediately before the radiotracer. PET images are corresponding coronal slices through the tumor and kidneys, with PET data summed for 90 min post-folate-NOTA-Al¹⁸F (**2**) administration.

FIGURE 6.

Ex Vivo tumor accumulation of folate-NOTA-Al¹⁸F (**2**) at 90 min post injection in nude mice bearing KB tumor xenografts or A549 tumor xenografts in comparison to the SPECT tracer ^{99m}Tc-EC20 at 120 min. Horizontal lines indicate arithmetic mean, and error bars represent 95% confidence intervals.

FIGURE 7.

Biodistribution of folate-NOTA-AI¹⁸F and ^{99m}Tc-EC20 in athymic tumor bearing mice at 90 min and at 120 min post injection, respectively. Folate receptor specific binding is evaluated by competition with 100 µg excess folic acid. Data on tumor retention for the same animals were shown in Figure 6. Horizontal lines indicate arithmetic mean, and error bars represent 95% confidence interval.

Ex Vivo biodistribution study of folate-NOTA-Al¹⁸F (2) at 90 minutes p.i. in nude mice bearing KB tumor xenografts in comparison to the SPECT Tracer ^{99m}Tc-EC20 evaluated at 120 minutes post-injection. All values are in units of %ID/g ± SEM (except ratios).

TABLE 1

Organ or tissue	Control (N=8) KB Tumor (N = 4)	FA Block (N=6) KB Tumor (N = 3)	Ratio control:block	^{99m} Tc-EC20 Control (N=26) KB Tumor (N = 20)	^{99m} Tc-EC20 FA Block (N=6) KB Tumor (N = 3)
Blood	0.2 ± 0.02	0.3 ± 0.1	0.6	0.3 ± 0.01	0.3 ± 0.1
Heart	0.8 ± 0.03	0.1 ± 0.03	11.5	1.9 ± 0.1	0.1 ± 0.02
Kidney	78.6 ± 5.1	16.6 ± 12.8	4.7	113.0 ± 3.6	6.7 ± 1.6
Liver	5.3 ± 0.5	3.5 ± 0.5	1.5	4.1 ± 0.3	2.5 ± 0.5
Lung	0.9 ± 0.1	0.6 ± 0.4	1.5	2.1 ± 0.1	0.8 ± 0.2
Muscle	1.0 ± 0.1	0.1 ± 0.1	8.4	1.2 ± 0.04	0.1 ± 0.02
Plasma	0.3 ± 0.04	0.5 ± 0.2	0.5	0.5 ± 0.03	0.5 ± 0.1
Spleen	0.6 ± 0.1	0.3 ± 0.1	2.0	0.8 ± 0.1	0.5 ± 0.2
KB Tumor	10.9 ± 2.7	1.3 ± 0.1	8.3	10.9 ± 0.6	0.9 ± 0.1
KB Tumor/Liver	2.0 ± 0.4	0.4 ± 0.2	4.5	3.1 ± 0.3	0.4 ± 0.1
KB Tumor/Kidney	0.1 ± 0.03	0.4 ± 0.3	0.3	0.1 ± 0.01	0.1 ± 0.02
KB Tumor/Blood	55.8 ± 11.5	14.0 ± 12.1	4.0	41.5 ± 2.2	2.9 ± 0.4

In the competition group, each animal received 100 µg of folic acid 10 min before radiotracer injection.