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A practical synthesis of [^{18}F]FtRGD: an angiogenesis biomarker for PET†

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Integrins have become increasingly attractive targets for molecular imaging of angiogenesis with positron emission tomography or single-photon emission computed tomography, but the reliable production of radiopharmaceuticals remains challenging. A strategy for chemoselective labeling of the integrin ligand—c(RGDyK) peptide—has been developed on the basis of the Cu(I)-catalyzed conjugation reaction. Recently, we reported a nucleophilic detagging and fluorous solid-phase extraction method providing an easy way to implement an approach for obtaining 2-[^{18}F]fluoroethyl azide. In this work, we report the practical use of this method for the preparation of the 2-[^{18}F]fluoroethyl-triazolyl conjugated c(RGDyK) peptide: [^{18}F]FtRGD. The two-step, two-pot synthesis, HPLC purification, and reformulation could be readily performed with a standard nucleophilic radiofluorination synthesizer (GE TRACERlab FX$_{FN}$), with minimal modifications. [^{18}F]FtRGD was obtained in a solution for injection (>500 MBq/mL) in 10–30% nondecay-corrected radiochemical yield, excellent radiochemical purity (>98%), and 28 ± 13 GBq/μmol specific activity. [^{18}F]FtRGD (K_i = 54 ± 14 nM for $\alpha_V\beta_3$ and 1.7 ± 0.2 nM for $\alpha_V\beta_5$) was evaluated in mice and showed good stability in vivo, good tumor-to-background ratio (1.6 ± 0.3 %ID/g at 1.5 h post-injection in U87-MG tumors), and rapid urinary excretion. Therefore, [^{18}F]FtRGD proved valuable for preclinical positron emission tomography imaging of integrin expression.

Keywords: PET; F-18; angiogenesis; integrins; RGD; fluorous; CuAAC

Introduction

The capability to induce the formation of new blood supply from preexisting vasculature—angiogenesis—is widely accepted as one of the hallmarks of cancer.[1] The development of capillaries is essential for the supply of nutrients and oxygen to growing tumors and metastases. Multiple factors are known to stimulate endothelial cells and induce angiogenesis.[2] All of those growth factors promote endothelial cell adhesion and migration through integrin ligation. Integrins are cell surface receptors that bind to extracellular matrix proteins. Amongst the integrin family, the $\alpha_V\beta_3$-, $\alpha_V\beta_5$-, and $\alpha_5\beta_1$-glycoproteins are overexpressed on activated endothelial cells versus quiescent cells and play a key role in the induction of blood vessel formation. Therefore, the expression of such endothelial integrins has been shown to correlate with the angiogenic activity.[3,4]

Integrins are attractive targets for angiogenesis imaging, and significant effort has been made toward the development of ligands for positron emission tomography (PET) and single-photon emission computed tomography (SPECT) molecular imaging.[5–8] Such biomarkers can prove useful for early detection of integrin-positive tumors and for monitoring the efficacy of antiangiogenic therapies. Those integrins share a common characteristic; the recognition of the amino acid sequence Arg-Gly-Asp (RGD).[3] Therefore, numerous RGD-containing ligands have been developed as radioactive probes for nuclear imaging since the structure–activity relationship study with RGD-containing cyclic pentapeptides in 1995 and the first ^{125}I-radiolabeling of c(RGDyV) in 1999.[9,10] Many studies have led to an increase of the hydrophilicity and the conformational restriction of the RGD sequence so as to optimize of the pharmacokinetics and the binding affinity and selectivity.

Nowadays, most of the radiolabeled ligands are based on the optimized c(RGDyK) and c(RGDfK) sequences.[11] Various radiolabeled derivatives of the cyclic pentapeptides have actually been reported to selectively bind the endothelial integrins in vivo, including the ^{18}F-, ^{68}Ga-, and ^{64}Cu-labeled ones for PET and the ^{99m}Tc- and ^{111}In-labeled ones for SPECT imaging.[12] These radiopharmaceuticals all exhibit excellent tumor-targeting efficacy, good metabolic stability, and a favorable pharmacokinetic profile. Noteworthy multimeric peptides, such as [^{18}F]FPP(RGD)$_2$, have demonstrated a higher binding affinity than monomeric ones.[7,12–14] It is also worth noting that a GE Healthcare (Little Chalfont, UK) proprietary compound—fluciclatide, an ^{18}F-labeled polyethylene glycol conjugate of an RGD-containing cyclic peptide—and a c(RGDfK) derivative, developed by Siemens Healthcare (Erlangen, Germany) (^{18}F-RGD-K5), are gaining interest and have entered clinical trials.[15–17]

As part of our ongoing PET oncology programs with rodents, we expressed some interest for such molecular imaging probes

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that could be used to determine the therapy efficacy of antiangiogenic agents in experimental tumor models. However, we noticed that most of the reported ^{18}F-radiopharmaceuticals rely on very specific and proprietary procedures, reactants, and radiosynthesis systems: for instance, various radiopharmaceuticals production requires custom-made precursors and multistep, multipot synthesis modules with several HPLC purification steps. Hence, the procedures do not allow for the quick implementation of the on-demand preparation radiopharmaceuticals, in a typical setup equipped with a standard nucleophilic radiofluorination synthesizer (e.g., GE TRACERlab FX_{FN}). Such synthesis units are available worldwide and allow for the full automation of the process but are limited to (i) aqueous fluoride processing; (ii) nucleophilic fluorination and additional reaction steps in the same reaction vessel; (iii) HPLC or solid-phase extraction (SPE) purification; (iv) and reformulation. The growing distribution of ^{68}Ge/^{68}Ga generators should allow for the relatively easy preparation of ^{68}Ga-labeled RGD biomarkers, but the shorter half-life of Ga-68 (68 vs. 110 min. for F-18) limits the number of animals to be imaged with a single radiopharmaceutical preparation.

^{18}F-radiolabeling of peptides relies heavily on the radiosynthesis of a prosthetic group and then its conjugation to the functionalized peptide. Various conjugation methods have been employed for the synthesis of radiolabeled RGD derivatives. For instance, the chemoselective oxime ligation can be employed between an ^{18}F-labeled aldehyde (e.g., 4-[^{18}F]fluorobenzaldehyde, [^{18}F]FDG) and the aminooxy-functionalized peptide.[18,19] *N*-succinimidyl-4-[^{18}F]fluorobenzoate can be coupled with a free amino group of the peptide to provide the 4-[^{18}F]fluorobenzoyl-functionalized radiopharmaceutical.[20] The Cu(I)-catalyzed (CuAAC) or strain-promoted Huisgen 1,3-dipolar cycloaddition between an ^{18}F-labeled alkyne and the azide-functionalized peptide or between an ^{18}F-labeled azide and the alkyne-functionalized peptide provides an alternative way to access ^{18}F-labeled RGD peptides.[21,22] Those radiolabeled prosthetic groups all require an HPLC purification step before the conjugation takes place, resulting in incompatibilities with the general nucleophilic fluorination units and lengthy preparation procedures (and lower nondecay-corrected yields).

Hence, we developed a new ^{18}F-labeled radiopharmaceutical for angiogenesis imaging, satisfying the following criteria:

- The radiosynthesis, purification, and reformulation of the radiopharmaceutical shall be performed with a standard nucleophilic radiofluorination synthesizer, with minimal and reversible modifications.
- The radiosynthesis shall rely on commercially available or easy to synthesize reactants.
- The formulation for in vivo injection shall be compatible with rodents imaging, that is, >500 MBq/mL (<10% ethanol).
- The radiopharmaceutical shall target angiogenesis in vivo.
- The radiopharmaceutical shall display a favorable metabolic profile in rodents: fast clearance and optimal tumor contrast 1–2 h post-injection (p.i.).

This manuscript describes the procedure for the reliable and reproducible preparation of ^{18}F-labeled RGD-containing peptide for angiogenesis imaging in rodents. This procedure relies on the synthesis and purification of a radiolabeled synthon 2-[^{18}F]fluoroethyl azide, its subsequent conjugation with the pentynoic derivative of c (RGDyK), and finally the purification and reformulation of the radiopharmaceutical. The conjugation reaction was based on the 1,3-dipolar cycloaddition providing the 1,2,3-triazole linker, an amide bond isostere, not susceptible to cleavage. The radiolabeled derivative of the cyclic pentapeptide c(RGDyK)—[^{18}F]FtRGD (Scheme 1)—was further evaluated in vivo: its tumor targeting efficacy and pharmacokinetics were assessed using PET.

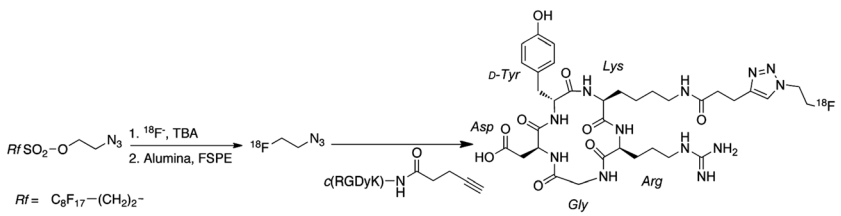
Materials and methods

General

All chemicals obtained commercially were of analytical grade and used without further purification. 2-Azidoethyl 1*H*,1*H*,2*H*,2*H*-perfluorodecane-1-sulfonate and 2-fluoroethyl azide were prepared as described previously.[23] Solvents and chemicals were purchased from Sigma-Aldrich (Singapore) unless stated otherwise. c(RGDyK), pentynoic c(RGDyK), and c(RGDyK)-Bn-NOTA-Ga were purchased from FutureChem Co Ltd (Seoul, S. Korea). Axitinib was purchased from Selleck Chemicals (Houston, TX, USA). The solution tetrabutylammonium bicarbonate solution (TBA·HCO_3, 1 M in MeOH) was prepared by purging CO_2 into a solution of tetrabutylammonium hydroxide (1 M in MeOH, Sigma Aldrich) for 3 h. The solution could be stored for several months at room temperature without noticeable decrease of efficiency.

Nonradioactive analytical and semi-preparative reversed-phase HPLC was performed with a Shimadzu Prominence HPLC system (Kyoto, Japan) equipped with a diode array detector. Plasma metabolites HPLC analysis was performed with the same system equipped with two high-pressure flow-line selection valves (two-positions/six-ports valves). Radioactive analytical HPLC was performed with a PerkinElmer Series 200 HPLC (Shelton, CT, USA) system equipped single-wavelength ultraviolet (UV) detector and a NaI/PMT-radiodetector (Flow-RAM, LabLogic, Sheffield, UK). Radioactive semi-preparative HPLC was performed with a Sykam S-1122 pump (Eresing, Germany) and a Knauer K-2001 single-wavelength UV detector (Berlin, Germany). Following the elution and collection of [^{18}F]FtRGD, the semi-preparative HPLC column was washed and stored in 70% ethanol.

Levels of residual solvents were analyzed using a Varian 430-GC (Palo Alto, CA, USA) with a flame ionization detector and a Varian FactourFour™ VF-200 ms column (30 m, 0.32 mm, 1 μm).



Scheme 1. Radiosynthesis of [^{18}F]FtRGD.

Mass spectrometry analysis was performed on an Agilent 6224 system (electrospray time-of-flight, Santa Clara, CA, USA), with a 1200 capillary HPLC, column Vydac C18 0.5 x 150 mm (Grace Davison Discovery Sciences, Deerfield, IL, USA), 20 μL/min, gradient in 5 min from 10% acetonitrile, 0.1% formic acid, water to 80% acetonitrile, 0.1% formic acid, water, injection 5 μL, column temperature 45°C). Data are recalibrated using the internal standards.

The fluorous SPE (FSPE) cartridges were prepared in-house with 100-mg fluorous silica gel (40 μm, Fluorous Technologies, Pittsburgh, PA, USA) loaded into an empty polypropylene tube fitted with a 20-μm polyethylene frit (empty Chromafix 30-PS-HCO_3 tube, Macherey-Nagel, Düren, Germany), washed with 2-mL ethanol, 2-mL acetone, and conditioned with 3-mL water.

Chemistry

*Preparation of 3-(1-(2-fluoroethyl)-1H-*1,2,3-triazol-4-yl)propanoic c(RGDyK): FtRGD

To pentynoic c(RGDyK) (5 mg, FutureChem) in N,N-dimethylformamide (100 μL) were added a 0.1-M aqueous solution of copper sulfate (200 μL), a 0.1-M buffered sodium ascorbate solution (200 μL, in 0.1-M potassium carbonate buffer, pH 8.0), and a 0.3-M solution of 2-fluoroethylazide in THF (200 μL).[24] The reaction mixture was stirred overnight at room temperature, diluted with 1.2-mL water, and purified by semi-preparative HPLC (Phenomenex Luna C18(2), 150 x 10mm, 5 μm, 100 Å, Torrance, CA, USA; eluent: 1 mL/min acetonitrile : water : trifluoroacetic acid (TFA): 50:950:1 for 5 min, 50:950:1 to 130:870:1 linear gradient over 5 min, 130:870:1 to 180:820:1 linear gradient over 50 min). The collected fractions were combined, analyzed using electrospray time of flight mass spectrometry, and lyophilized to give FtRGD as a white fluffy powder (est. 3 mg).

^{1}H NMR (600 MHz, D_2O) δ (ppm) 7.85 (s, 1H), 7.11 (d, J = 8.5 Hz, 2H), 6.82 (d, J = 8.5 Hz, 2H), 4.83 (dt, J_1 = 47 Hz, J_2 = 4.6 Hz, 2H), 4.73 (dt, J_1 = 28 Hz, J_2 = 4.6 Hz, 2H), 4.56 (dd, J_1 = 10.3 Hz, J_2 = 6.0 Hz, 1H), 4.37 (dd, J_1 = 9.1 Hz, J_2 = 5.7 Hz, 1H), 4.22 (d, J = 14.9 Hz, 1H), 3.81 (dd, J_1 = 10.7 Hz, J_2 = 4.2 Hz, 1H), 3.50 (d, J = 14.9 Hz, 1H), 3.24–3.13 (m, 2H), 3.08–2.98 (m, 3H), 3.03 (t, J = 7.2 Hz, 2H), 2.93 (dd, J_1 = 16.7 Hz, J_2 = 7.9 Hz, 1H), 2.87 (dd, J_1 = 13.3 Hz, J_2 = 10.5 Hz, 1H), 2.75 (dd, J_1 = 16.7 Hz, J_2 = 6.5 Hz, 1H), 2.61 (t, J = 7.2 Hz, 2H), 1.90–1.83 (m, 1H), 1.69–1.57 (m, 2H), 1.55–1.28 (m, 2H), 1.47–1.40 (m, 1H), 1.26–1.20 (m, 2H), 0.88–0.82 (m, 2H).^{19}F NMR (376 MHz, D_2O) δ (ppm) −223.2 (tt, J_1 = 47 Hz, J_2 = 28 Hz). HRMS (ESI+) *m/z* calcd 789.3802; found 789.3802 $C_{34}H_{50}FN_{12}O_9$ $[M+H]^+$.

[^{18}F]FtRGD

No-carrier-added [^{18}F]fluoride was produced by the $^{18}O(p,n)^{18}F$ nuclear reaction with a 16-MeV proton beam generated by a GE PETtrace cyclotron in a silver target using [^{18}O]H_2O (Singapore Radiopharmaceuticals Pte Ltd). A TRACERlab FX_{FN} radiochemistry module (GE Healthcare) was used for the 2-[^{18}F]fluoroethyl-triazole-c(RGDyK) two-step synthesis and purification. A 5-mL V vial (Wheaton, Millville, NJ, USA) was used as a second reaction vessel in place of the HPLC loading vessel, and a remotely controlled Elite dry bath incubator fitted with a 20-mm-well heating block (Major Science, Saratoga, CA, USA) was used as the second reaction vessel heater.

Prior to the radiopharmaceutical synthesis, in the 5-mL V vial, pentynoic c(RGDyK) (5 mg) was dissolved in a solution of gentisic acid (4 mg) in potassium carbonate buffer (100 μL, pH 8.0, 1.0 M). To this solution were added a solution of bathophenanthroline disulfonate (16 μL, 0.1 M) and a solution of sodium ascorbate (12 μL, 1.0 M). The mixture was purged with a gentle stream of helium for 5 min, before adding a solution of copper(II) sulfate (16 μL, 0.1 M).

Isolation of the [^{18}F]fluoride from [^{18}O]H_2O was achieved by trapping on a Chromafix 30-PS-HCO_3^- cartridge (Macherey-Nagel) and further elution of [^{18}F]fluoride, into the glassy carbon reaction vessel, with a solution of TBA·HCO_3 (10 μmol) in 1.0-mL methanol : water (9:1) (vial 1). Simultaneously, 1.0-mL acetonitrile (vial 2) was added to the glassy carbon reaction vessel, and anhydrous [^{18}F]fluoride was obtained after azeotropic evaporation for 7 min at 60°C under a flow of helium and 7 min at 100°C under vacuum.

A 0.8-mL solution of 2-azidoethyl 1*H*,1*H*,2*H*,2*H*-perfluorodecane-1-sulfonate (2 mg) in acetonitrile (vial 3) was added to the reaction vessel, and fluorination was performed at 90°C for 10 min. The crude solution was cooled down to 40°C, diluted with 0.35-mL water (vial 4), and eluted through a neutral alumina cartridge (Sep-Pak light, Waters, Milford, MA, USA) and an FSPE cartridge connected in series between the valves V14 and V12. The reactor was washed with 0.35-mL water (vial 5) and eluted through the same cartridges into the second reaction vessel containing the peptide and the copper catalytic system. The 1,3-dipolar cycloaddition was performed in the second reaction vessel for 5 min at 60°C. Acetonitrile was further evaporated for 5 min at 60°C with a stream of helium, and the reaction mixture was diluted with water (4.5 mL, 0.1% TFA, vial 6).

The resulting solution was purified by HPLC (Phenomenex Synergy Polar-RP 250 × 10 mm, 4 μm, 80 Å, guard column; eluent water : acetonitrile : TFA (850:150:1), 5 mL/min; λ = 280 nm). [^{18}F]FtRGD was collected at 18–19 min into the round-bottomed dilution flask, containing 40-mL water (0.1% TFA). The resulting solution was eluted through an hydrophilic–lipophilic balance (HLB) cartridge (Oasis Plus Light, Waters); the cartridge was then washed with water (4 mL, vial 9), and [^{18}F]FtRGD was finally eluted with 50% ethanol (0.4 mL, vial 8) followed by 1.6-mL phosphate buffered saline (pH 7.0, vial 7). The radiopharmaceutical solution was then sterile filtered into a sterile vial through a 0.2-μm mixed cellulose esters membrane (Millex-OR, Millipore, Bedford, MA, USA).

Typical yields of [^{18}F]FtRGD were 10–30% (not decay-corrected), and the total synthesis and reformulation time was 70–75 min. The radiopharmaceutical identity was confirmed by HPLC (Phenomenex Gemini NX C18 150 × 4.6 mm, 5 μm, guard column; eluent: acetonitrile : water : TFA 100:900:1 to 300:700:1 gradient over 10 min, 1 mL/min) and indicated >98% radiochemical purity. The specific activity was 28 ± 13 GBq/μmol at end of synthesis (EOS) (n = 11). Contaminants were less than 5 μg/mL, estimated from their absorbance at 276 nm and the molar extinction coefficient of pentynoic c(RGDyK). The final product was a clear and colorless solution (1.5 ± 0.8 GBq/mL at EOS) at pH 6.5–7.5.

Solid-phase receptor binding assay

The saturation binding assay was performed as described previously.[25] Purified $\alpha_V\beta_3$- and $\alpha_V\beta_5$-integrins (Millipore) were diluted at 0.1 μg/mL in coating buffer (20-mM Tris pH 7.4, 150-mM NaCl, 2-mM $CaCl_2$, 1-mM $MgCl_2$, 1-mM $MnCl_2$). A 100 μl/well was added to 96-well plates (high binding polystyrene, Corning Stripwell) and incubated overnight at 4°C. The coating solution was removed, and 200 μL of blocking/binding solution (50-mM Tris pH 7.4, 100-mM NaCl, 2-mM $CaCl_2$, 1-mM $MgCl_2$, 1-mM $MnCl_2$, 1%

bovine serum albumin) was added to the wells and incubated for an additional 2 h at room temperature. The plates were rinsed with 200 μL of blocking/binding solution and incubated for 3 h at room temperature with increasing concentration (30 pM–2 nM) ^{125}I-echistatin (81.4 GBq/μmol, PerkinElmer, Boston, MA, USA) in blocking/binding solution. After incubation for 3 h at room temperature, the plates were washed twice with blocking/binding buffer. The wells were separated and counted with an automatic gamma counter (2470 Wizard^2, Perkin Elmer). Bound radioactivity was plotted versus the radioligand concentration. The equilibrium dissociation constant K_d and the density of receptors B_{max} were obtained by nonlinear regression analysis of the total binding, accounting for ligand depletion (Prism 5.0c, GraphPad Software, La Jolla, CA, USA).[26]

The competition-binding assay was performed as described previously.[25,27] Purified $\alpha_V\beta_3$- and $\alpha_V\beta_5$-integrins (Millipore) were diluted at 0.5 and 1.0 μg/mL, respectively, in coating buffer. A 100 μL/well was added to 96-well plates (high binding polystyrene, Corning Stripwell, Corning, NY, USA) and incubated overnight at 4°C. The coating solution was removed, and 200 μL of blocking/binding solution was added to the wells and incubated for an additional 2 h at room temperature. The plates were rinsed with 200 μL of blocking/binding solution and incubated for 3 h at room temperature with 0.05-nM ^{125}I-echistatin (PerkinElmer) and increasing concentrations (1 pM–10 μM) of the c(RGDyK) derivatives in blocking/binding solution. After incubation for 3 h at room temperature, the plates were washed twice with blocking/binding buffer. The wells were separated and counted with an automatic gamma counter (2470 Wizard^2, Perkin Elmer). Bound radioactivity was plotted versus the logarithm of the competitor concentration. The inhibition constant K_i was determined by nonlinear regression analysis with hillslope, accounting for ligand depletion (Prism 5.0c, GraphPad Software).

Animals

Outbred male nude CD1 mice were purchased from the Biological Resources Centre (Singapore). Animal experiments were performed in accordance with the Institutional Animal Care and Use Committee guidelines under ethics number IACUC 090437.

U87-MG cancer cells

Human U87-MG glioma cancer cells were purchased from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37°C in a humidified atmosphere, with 5% of CO_2.

Treatment studies

At 3-week post-inoculation of U87-MG cells, the tumors reached a volume of 200–250 mm^3, and the mice were randomly divided into two treatment groups. One group received daily administrations of Axitinib (25 mg/kg) by intraperitoneal injection, whereas the second group received administrations of the polyethylene glycol/H_2O (30:70) vehicle alone. Animals were imaged on the day prior to initiation of the dosing regimen (day 0), and the same animals were imaged after 10 days of therapy (day 10).

In vivo imaging studies

The U87-MG tumor-bearing mice ($n = 4$ per group) were imaged on days 0 and 10 with [^{18}F]FtRGD. The mice were injected with a solution of [^{18}F]FtRGD (~10 MBq in 0.2 mL) via the lateral tail vein, and the animals imaged under isoflurane anesthesia (2% alveolar concentration) and biological monitoring for respiration, and temperature was performed using a BioVet system (m2m imaging, Cleveland, OH). Small-animal PET imaging was performed from 70 to 90 min p.i., on an Inveon PET/CT system (Siemens Inc., Washington DC). Images were generated from sinogram data, rebinned to two-dimensional format by the Fourier rebinning algorithm, followed by two-dimensional filtered back projection. Low-dose CT images (40 kV, 500 μA; 4 × 4 binning, 200-μm resolution) were acquired for anatomical information. Images were reconstructed using the image reconstruction, visualization, and analysis program supplied by the manufacturer. PET and CT data were analyzed by using the Amide software (Sourceforge 10.1, http://amide.sourceforge.net). The PET and CT images were coregistered, to confirm anatomical location of the tumors. Uptake of radioactivity in the tumor was determined by placement of a region of interest around the tumor border delineated using the CT images. The tissue concentrations were measured using region of interest analysis and are presented as percent injected dose/gram (%ID/g) for tumor, muscle (lower left hind limb), liver, kidneys, and heart and brain.

Plasma metabolites analysis

After intravenous injection of the radiopharmaceutical into tumor-bearing mice, blood samples (50–200 μL) from the tail vein were manually collected into 0.5-mL heparinized polypropylene centrifuge tubes, at 2, 30, 60, and 90 min p.i.. Blood samples were centrifuged at 2500 g for 10 min. The supernatant plasma samples were diluted with 1-mL water (0.1% TFA) and analyzed using on-line HLB SPE and reversed-phase HPLC, as described by Chitneni *et al.*[28] The diluted plasma samples were (i) while bypassing the HPLC column: injected onto a preconditioned Oasis® HLB column (20 × 3.9 mm, 5 μm, Waters) and rinsed with water for 3 min; (ii) with the HLB column connected to the HPLC column: eluted with the mobile phase (acetonitrile : water : TFA 0:1000:1 to 300:700:1 gradient over 10 min, 1 mL/min). All eluates were analyzed with an online NaI/PMT radioactivity detector (IN/US).

Results and discussion

Radiochemistry

We recently developed an FSPE technique for the separation of the radiolabeled synthon from its precursor: the fluorous tagged precursor is detagged upon nucleophilic ^{18}F-fluorination, and on the basis of the unique separation properties of highly polyfluorinated molecules (fluorous), the resulting ^{18}F-labeled synthon is separated by simple elution although a fluorous stationary phase.[23,24] This technique allowed us to obtain ^{18}F-labeled synthons reliably and with a minimum of purification effort. For instance, 2-[^{18}F]fluoroethyl azide was synthesized by nucleophilic ^{18}F-fluorination of the polyfluorinated sulfonate precursor and purified rapidly by simple elution through alumina and fluorous silica gel: alumina traps excess [^{18}F]fluoride, whereas fluorous silica gel traps excess precursor and polyfluorinated by-products.

It is worth noting that tetrabutylammonium was used as a phase-transfer catalyst for the nucleophilic fluorination.[29,30] It

proved superior to the classical mixture of potassium carbonate and Kryptofix® 2.2.2 (K_{222}) cryptand, providing less by-products and the expected ^{18}F-labeled synthon with a moderate specific activity. It should actually be noted that the fluorous purification technique usually suffers from a decrease of the specific activity possibly from leaching of [^{19}F]fluoride ion from the precursor tag.[31] Solvent evaporation and [^{18}F]fluoride drying could then be performed with the addition of acetonitrile forming the ternary azeotrope methanol–acetonitrile–water. The resulting tetrabutylammonium [^{18}F]fluoride was then used to provide 2-[^{18}F]fluoroethyl azide with high radiochemical yield and higher specific activity. Furthermore, the reaction mixture after nucleophilic fluorination was less troublesome: the obtained solution was colorless and fully soluble, whereas brown-colored solution with insoluble residues could be obtained with K_{222}.

The Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition between an alkyne and an azide is being used increasingly in drug discovery and radiopharmaceutical development.[32–34] The efficient, water-compatible and orthogonal reaction is actually one of the main technique for the preparation of RGD-containing peptide conjugates.[35]

Once purified, 2-[^{18}F]fluoroethyl azide could be conjugated to the alkyne-functionalized peptide, such as the pentynoic derivative of c(RGDyK), commercially available from FutureChem. The cyclic pentapeptide c(RGDyK) was chosen over its popular analogue c(RGDfK) because of its increase hydrophilicity: the former was actually reported with lower liver uptake.[36] Dimeric or tetrameric ligands based on the pentapeptides could also be used to improve the binding affinity of the radiopharmaceutical; however, their commercial availability is still limited.

The Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition was performed following an optimized procedure based on those reported in the literature. In details, the copper(I)-catalyzed reaction was performed using the mixture of copper sulfate, bathophenantroline disulfonate, sodium ascorbate, and gentisic acid described by Gill *et al.*[37] Excess ascorbate was used to reduce Cu(II) to Cu(I), and the addition of an equimolar amount of the bathophenantroline disulfonate ligand stabilized the oxidation state and promotes the solubilization of Cu(I). The reaction mixture improved both the conjugation reaction (increased efficiency, reduced amount of peptide, and reduced reaction time) and the purification step (better solubility and less clogging). As recommended by Glaser *et al.*, the reaction was performed at pH 8.0, in a buffered carbonate solution.[18] It is noteworthy that the pentynoic derivative of c(RGDyK) is water soluble; hence, the use of dimethylformamide or dimethyl sulfoxide was avoided.

Reaction monitoring indicated that the reaction was almost complete (>90% conversion) in 5 min at 60°C. Higher temperature was not investigated because of the volatility of the ^{18}F-labeled synthon. Prior to HPLC purification, acetonitrile is evaporated with a gentle stream of helium at 60°C, and the diluted crude reaction mixture is purified by semi-preparative HPLC. The collected fraction is reformulated into a volume as low as possible: using an HLB SPE cartridge, the radiopharmaceutical is separated from the diluted HPLC eluate and further eluted into the final vial with only 0.4 mL 50% aqueous ethanol. It is noteworthy that only 80–90% of the radiopharmaceutical was eluted from the HLB cartridge, but attempts for a higher recovery rate led to excessive dilution of the final solution. The solution is further made isotonic and buffered with the addition of 1.6-mL saline and phosphate buffer solution (pH 7.0, 10 mM). A 0.5 mL was used for quality control, affording 1.5-mL solution for injection (10% ethanol).

The total synthesis, purification, and formulation time for 2-[^{18}F]fluoroethyl-triazole c(RGDyK) was only 70–75 min. The overall nondecay-corrected radiochemical yield was 10–30% ($n = 11$), providing 1.5 ± 0.8 GBq/mL radiopharmaceutical. The specific activity was moderate: 28 ± 13 GBq/μmol at the EOS. As mentioned previously, the moderate specific activity was due to the leaching of fluorine from the fluorous tag of the precursor. Interestingly, the specific activity obtained with TBA·HCO_3 was one order of magnitude higher than that obtained with K_{222}/K_2CO_3. Finally, it is worth mentioning that the final radiopharmaceutical solution contained less than 5 μg/mL of UV-absorbing contaminant. Residual organic solvents in the final product were analyzed using gas chromatography, and the highest concentrations measured were well below the specifications for radiopharmaceuticals (acetone: $55 < 5000$ ppm; acetonitrile: $6 < 410$ ppm).

Table 1. Dissociation constants of c(RGDyK) derivatives

	K_i ($\alpha_V\beta_3$) (nM)	K_i ($\alpha_V\beta_5$) (nM)
c(RGDyK)	46 ± 11	3.7 ± 0.4
Ga-NOTA-Bn-c(RGDyK)	69 ± 19	5.4 ± 0.5
FtRGD	54 ± 14	1.7 ± 0.2

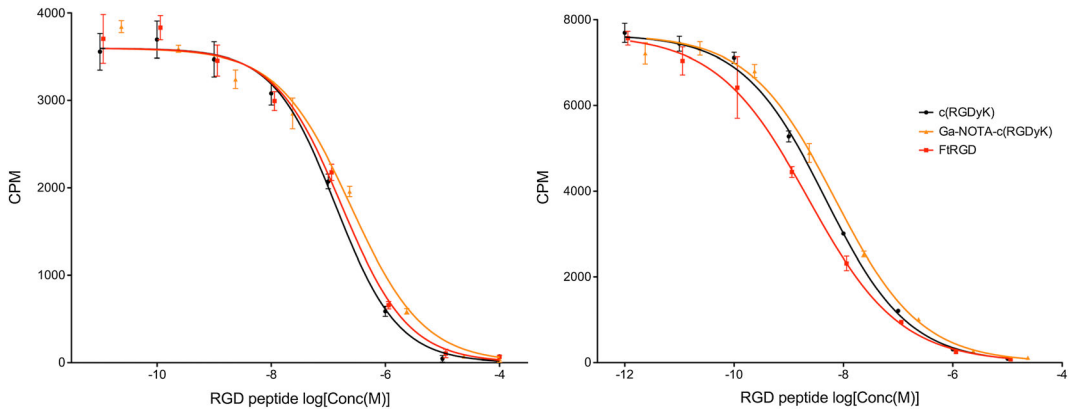


Figure 1. Competition binding assay against [^{125}I]echistatin with $\alpha_V\beta_3$ (left) and $\alpha_V\beta_5$ (right).

In vitro validation

The competitor concentrations required for half-maximal binding of to $\alpha_V\beta_3$- and $\alpha_V\beta_5$-integrins were determined in solid-phase receptor binding assays.[25,27] The dissociation constant (K_i) of the inhibitors was further determined from the binding affinity (K_d) of ^{125}I-echistatin (Table 1, Figure 1). For comparison, the competition assay was performed with the fluoroethyl conjugate of c(RGDyK)—FtRGD—with the reference peptide—c(RGDyK)—and the gallium complex derivative—Ga-NOTA-Bn-c(RGDyK).[38] We could observe that the binding affinity of our fluorinated conjugate is similar to that of the c(RGDyK) and its Ga-NOTA-derivative. As expected, the fluoroethyl-triazole scaffold has minimal effect on the binding affinity of the cyclic pentapeptide.

In vivo study

The biology studies described here were performed to investigate the performance of ^{18}F-labeled peptide for imaging angiogenesis in an U87-MG xenograft model in vivo using PET. Previously, other ^{18}F-labeled peptides have been used successfully to image angiogenesis and early response to antiangiogenic therapy using the U87-MG model.[39] U87-MG cells form solid tumors when implanted subcutaneously in nude mice and have been shown

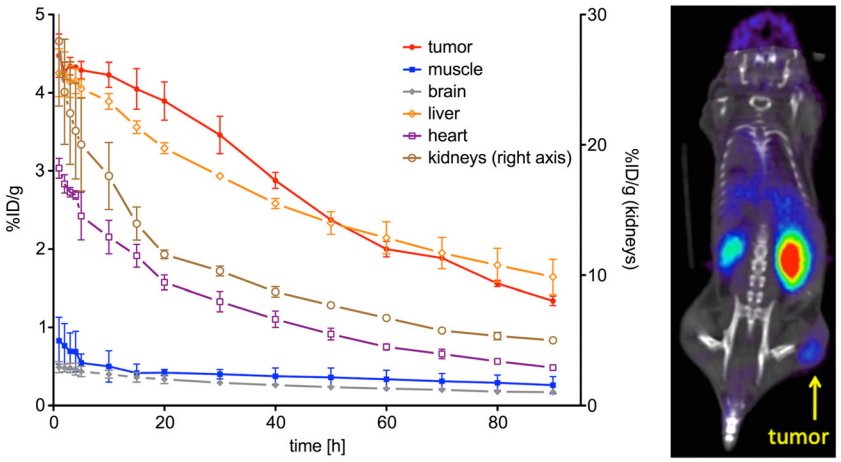


Figure 2. Time-activity curves of [^{18}F]FtRGD (%ID/g ± standard error of the mean) in the tumor, muscle, brain, liver, kidneys, and the heart, quantified using region of interest analysis of positron emission tomography images (left) and coronal image of a mice at 70–90 min post-injection (right).

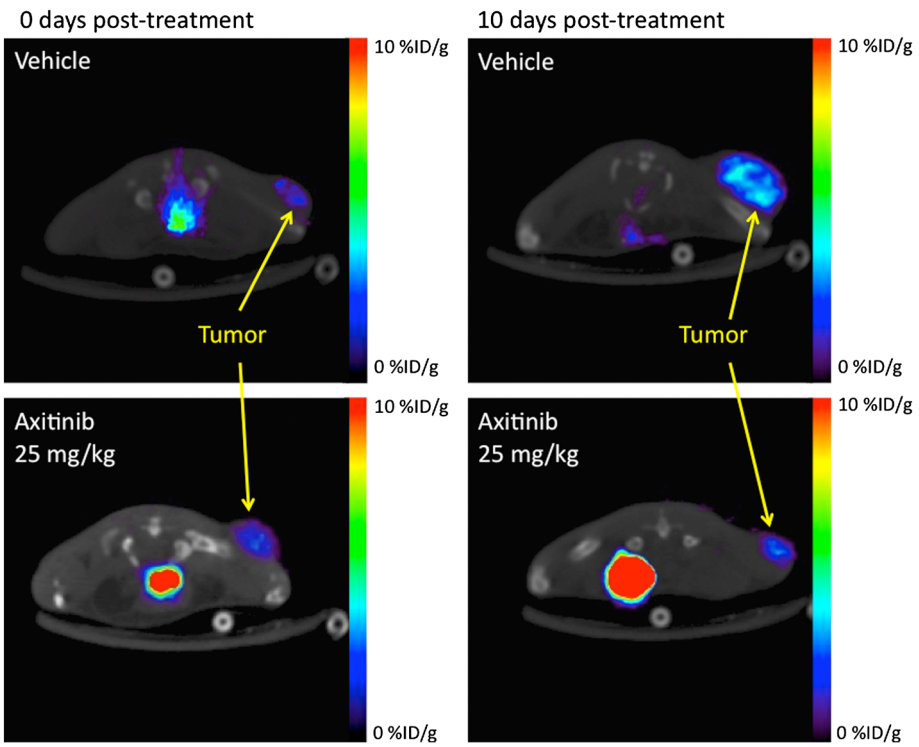


Figure 3. Tumor uptake of [^{18}F]FtRGD following intravenous bolus injection. Images show static acquisition data from 70–90 min post-injection of [^{18}F]FtRGD indicating no change in tumor uptake in the Axitinib-treated U87-MG tumors, but a significant increase in tumor retention in the vehicle-treated tumors is observed. This figure is available in colour online at www.interscience.wiley.com/journal/jlcr

to express $\alpha_V\beta_3$ and $\alpha_V\beta_5$ on both the tumor cells and vasculature.[40] The acquired [^{18}F]FtRGD PET data demonstrate uptake into untreated U87-MG tumors of 1.6 ± 0.3 %ID/g and skeletal muscle of 0.24 ± 0.07 %ID/g at ~70–90 min p.i., displaying tumor uptake values consistent with previously published ^{18}F-labeled angiogenesis imaging peptides such as [^{18}F]fluciclitide (~1.5 ± 0.5 %ID/g) and [^{18}F]FPP(RGD)$_2$ (~1.85 ± 0.3 %ID/g).[39,41] The excretion profile of [^{18}F]FtRGD was also similar to previously published results with other RGD-containing radiopharmaceuticals, displaying high initial kidney accumulation and rapid urinary excretion (Figure 2). Liver uptake was relatively low with some hepatobiliary excretion; muscle retention was also low consistent with the low lipophilicity of the RGD peptides. The tumor-to-muscle ratio was sufficiently high to be able to determine therapy efficacy using angiogenesis inhibitors such as Axitinib (Pfizer). Axitinib is an orally bioavailable, receptor tyrosine kinase inhibitor that exhibits potent antiangiogenic and antitumor activity at VEGFR-1, VEGFR-2, VEGFR-3, cKit, and PDGFR.[42] In this study, Axitinib was well tolerated, with no significant effects on animal health observed. Previous data have shown that Axitinib displays treatment-related tumor growth inhibition within 10 days using calipers; therefore, animals were imaged the day prior to initiation of the dosing regimen and after 10 days.[42] As can be seen in Figure 3, the tumor retention of [^{18}F]FtRGD stayed constant from day 0 (1.62 ± 0.32 %ID/g) in the Axitinib treated group reaching 1.65 ± 0.25 %ID/g ($p > 0.89$) at day 10 posttreatment. However, the tumor retention of [^{18}F]FtRGD significantly increased in the vehicle treated group from day 0 (1.49 ± 0.25 %ID/g) to day 10 (3.33 ± 0.63 %ID/g ($p < 0.001$)).

Plasma metabolites analysis

The time-course stability of the ^{18}F-labeled peptide was examined by reversed-phase HPLC. In detail, plasma samples, at 2, 30, 60, and 90 min p.i., were analyzed by HPLC using the HLB SPE technique described by Chitneni *et al.*[28] No metabolites could be detected in the plasma: the only radioactive signal that could be observed was from the parent peptide: [^{18}F]FtRGD.

Conclusion

A novel ^{18}F-labeled c(RGDyK) peptide derivative ([^{18}F]FtRGD) was developed on the basis of the fluorous methodology we reported recently. The approach allowed for a quick and easy purification of radiolabeled synthons by simple elution through alumina and fluorous silica gel, and could be readily implemented with a standard nucleophilic radiosynthesizer to perform a two-step, two-pot process: that is, nucleophilic ^{18}F-fluorination and CuAAC conjugation. The procedure including the radiolabeling of 2-[^{18}F]fluoroethyl azide, the FSPE purification, the CuAAC conjugation with the alkyne-functionalized c(RGDyK) peptide, the HPLC purification, and the reformulation of the radiopharmaceuticals provided a reliable method to obtain [^{18}F]FtRGD for preclinical PET. [^{18}F]FtRGD could be obtained in less than 75 min and in good radiochemical yield. The radiochemical purity, specific activity, and reformulation solution proved suitable for preclinical imaging. The evaluation of [^{18}F]FtRGD in mice confirmed the affinity of the radiopharmaceutical for integrins and exhibited a favorable metabolic profile (high stability and fast renal clearance) for angiogenesis PET imaging with 1–2 h p.i.. Further studies are ongoing using [^{18}F]FtRGD as a tool for preclinical PET monitoring of antiangiogenic therapies.

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Conflict of Interest

The authors did not report any conflict of interest.

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