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Click Chemistry for ^{18}F -Labeling of RGD Peptides and microPET Imaging of Tumor Integrin $\alpha_v\beta_3$ Expression

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Abstract

The cell adhesion molecule integrin $\alpha_v\beta_3$ plays a key role in tumor angiogenesis and metastasis. A series of ^{18}F -labeled RGD peptides have been developed for PET of integrin expression based on primary amine reactive prosthetic groups. In this study, we report the use of the Cu(I)-catalyzed Huisgen cycloaddition, also known as a click reaction, to label RGD peptides with ^{18}F by forming 1,2,3-triazoles. Nucleophilic fluorination of a toluenesulfonic alkyne provided ^{18}F -alkyne in high yield (nondecay-corrected yield: $65.0 \pm 1.9\%$, starting from the azeotropically dried ^{18}F -fluoride), which was then reacted with an RGD azide (nondecay-corrected yield: $52.0 \pm 8.3\%$ within 45 min including HPLC purification). The ^{18}F -labeled peptide was subjected to microPET studies in murine xenograft models. Murine microPET experiments showed good tumor uptake ($2.1 \pm 0.4\%$ ID/g at 1 h postinjection (p.i.)) with rapid renal and hepatic clearance of ^{18}F -fluoro-PEG-triazoles-RGD2 (^{18}F -FPTA-RGD2) in a subcutaneous U87MG glioblastoma xenograft model (kidney $2.7 \pm 0.8\%$ ID/g; liver $1.9 \pm 0.4\%$ ID/g at 1 h p.i.). Metabolic stability of the newly synthesized tracer was also analyzed (intact tracer ranging from 75% to 99% at 1 h p.i.). In brief, the new tracer ^{18}F -FPTA-RGD2 was synthesized with high radiochemical yield and high specific activity. This tracer exhibited good tumor-targeting efficacy and relatively good metabolic stability, as well as favorable in vivo pharmacokinetics. This new ^{18}F labeling method based on click reaction may also be useful for radiolabeling of other biomolecules with azide groups in high yield.

INTRODUCTION

The vitronectin receptor integrin $\alpha_v\beta_3$ has been the focus of intensive research because of its major role in several distinct processes, particularly osteoclast-mediated bone resorption (1), angiogenesis and pathological neovascularization (2), and tumor metastasis (3). Integrin $\alpha_v\beta_3$ has distinct functional properties that are mediated through interactions with a variety of extracellular matrix (ECM) proteins (4, 5) and a number of important intracellular signaling molecules (including paxillin, focal adhesion kinase caspase-8, and others) (6–8).

These interactions play a part in regulating intracellular signaling, cell migration, cell proliferation, and cell survival.

Since integrin $\alpha_v\beta_3$ plays a key role in multiple physiological and pathological processes, a method to noninvasively visualize and quantify $\alpha_v\beta_3$ integrin expression levels will provide a means to document integrin levels, wisely choose patients for anti-integrin treatment, and monitor treatment efficacy in integrin-positive patients. Multimodality approaches have been applied to image integrin expression in vivo, including magnetic resonance (MR) (9, 10), ultrasound (11, 12), near-infrared fluorescence (NIRF) (13, 14), single photon emission computed tomography (SPECT), and positron emission tomography (PET) (15–17). Due to the high sensitivity and reasonably good spatial/temporal resolution, PET probe development for targeting integrin expression continues to be an area of great interest.

Because ^{18}F is readily available from most small medical cyclotrons and has almost 100% positron efficiency, the physical half-life of ^{18}F (109.7 min) is well-suited for routine clinical use and is well-matched to the biological half-life (blood clearance) of peptides (usually minutes to less than a few hours). ^{18}F -labeled target-specific peptides are becoming widely used as in vivo imaging agents, a few of which have entered early-phase clinical trials (15). Different from small organic molecules, direct labeling of peptides via either SN_1 or SN_2 nucleophilic substitution is usually not an option, as peptides will not tolerate the harsh reaction conditions associated with these procedures. Radiofluorination of peptides thus generally uses ^{18}F -prosthetic groups (synthons) that will form stable chemical bonds on the peptides. ^{18}F labeling could be accomplished through the amino group at the N terminus or the lysine side chain using *N*-succinimidyl-4- ^{18}F -fluorobenzoate (^{18}F -SFB) (18, 19), 4- ^{18}F -fluorobenzaldehyde (^{18}F -FBA, via oxime formation and reductive amination) (20, 21), 3- ^{18}F -fluoro-5-nitrobenzimidate (^{18}F -FNB, via imidation reaction) (22), 4-azidophenacyl ^{18}F -fluoride (^{18}F -APF, via photochemical conjugation) (23), and 4- ^{18}F -fluorophenacyl bromide (^{18}F -FPB, via alkylation reactions) (22). ^{18}F labeling of peptide or protein via the carboxylic acid group at the C terminus or glutamic/aspartic acid side chain is less common, and only a few reports exist (24). We have previously reported the Michael addition reaction for labeling thiolated RGD peptides (25). However, most of these procedures suffer from lengthy and tedious multistep synthetic procedures. As a result, these long, difficult processes make them a challenge to automate and adversely decrease the overall radiolabeling yield.

The recent discovery that Cu(I) catalyzes the Huisgen 1,3-dipolar cycloaddition of organo azides with terminal alkynes to form 1,2,3-triazoles (26, 27), often referred to as click chemistry (28), has led wide-ranging applications in combinatorial chemistry (29–31). This reaction could be carried out in high yields under mild conditions, and the 1,2,3-triazole formed has similar polarity and size with an amide bond (32). Due to these favorable aspects with click chemistry, the use of this reaction for making ^{18}F -labeled model peptides have been recently reported (33, 34). However, no in vivo PET study has been reported on ^{18}F -labeled tracers synthesized by click chemistry. Moreover, distillation was required for some of the reported methods (34), which is unfortunately difficult to automate. In this study, we labeled dimeric-RGD peptide with our newly developed ^{18}F synthon based on click

chemistry and studied the tumor targeting efficacy, in vivo kinetics, and metabolic stability of this tracer in tumor-bearing mice.

MATERIALS AND METHODS

All chemicals obtained commercially were of analytical grade and used without further purification. No-carrier-added $^{18}\text{F-F}^-$ was obtained from a PETtrace cyclotron (GE Healthcare). Reversed-phase extraction C-18 Sep-Pak cartridges were obtained from Waters and were pretreated with ethanol and water before use. The syringe filter and polyethersulfone membranes (pore size, 0.22 μm ; diameter, 13 mm) were obtained from Nalge Nunc International. ^{125}I -Echistatin, labeled by the lac-toperoxidasemethod to a specific activity of 74 000 GBq/mmol (2000 Ci/mmol), was purchased from GE Healthcare. Analytical as well as semipreparative reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Dionex 680 chromatography system with a UVD 170U absorbance detector and model 105S single-channel radiation detector (Carroll & Ramsey Associates). The recorded data were processed using *Chromeleon* v 6.50 software. Isolation of peptides and ^{18}F -labeled peptides was performed using a Vydac protein and peptide column (218TP510; 5 μm , 250 $\times$ 10 mm). The flow rate was set at 5 mL/min, with the mobile phase starting from 95% solvent A (0.1% trifluoroacetic acid [TFA] in water) and 5% solvent B (0.1% TFA in acetonitrile [ACN]) (0–2 min) to 35% solvent A and 65% solvent B at 32 min. The analytical HPLC was performed using the same gradient system, but with a Vydac column (218TP54, 5 μm , 250 $\times$ 4.6 mm) and a flow rate of 1 mL/min. The UV absorbance was monitored at 218 nm, and the identification of the peptides was confirmed by separate standard injection.

Preparation of Alkyne Tosylate (1)

The alkyne tosylate (**1**) (Figure 1) was prepared by using the modified method reported by Burgess (35). In brief, sodium hydride (1 g, 25 mmol, 60%) was slowly added to the THF solution of triethylene glycol (5.8 g, 38 mmol) at 0 °C. The mixture was stirred for 30 min and propargyl bromide (2.1 mL, 19 mmol) was then added dropwise. The mixture was stirred at room temperature for 18 h, and the triethylene glycol alkyne was obtained as a light yellow oil after purification by chromatography (2.5 g, 70%). ^1H NMR (400 MHz, CDCl_3) δ 4.13 (d, J = 2.5 Hz, 2H), 3.61–5.8 (m, 10H), 3.52–3.50 (m, 2H), 2.75 (br, 1H), 2.38 (t, J = 2.5 Hz, 1H). After the triethylene glycol alkyne (1 g, 5.4 mmol) was reconstituted in ACN (15 mL) and triethylamine (2 mL, 14 mmol), *p*-toluenesulfonyl chloride (2.1 g, 11 mmol) was added slowly, and the mixture was stirred at room temperature for 16 h. After the reaction was quenched followed by general workup, the crude product was purified by flash chromatography to afford the alkyne tosylate (**1**) (1.5 g, 81%) as a colorless oil. ^1H NMR (400 MHz, CDCl_3) δ 7.75 (d, J = 8.4 Hz, 2H), 7.30 (d, J = 8.4 Hz, 2H), 4.14–4.06 (m, 4H), 3.65–3.58 (m, 6H), 3.55–3.52 (m, 4H), 2.38 (s, 3H), 2.37 (t, J = 2.5 Hz, 1H).

Preparation of Azido-RGD2

The 5-azidopentanoic acid was obtained as colorless oil according to the procedure published by Carrié (36). ^1H NMR (400 MHz, CDCl_3) δ 3.25 (t, J = 6.5 Hz, 2H), 2.34 (t, J = 7.1 Hz, 2H), 1.68–1.59 (m, 4H). The azido-RGD2 was prepared from cyclic RGD dimer

E[c(RGDyK)]₂ (denoted as RGD2). To a solution of 5-azidopentanoic acid (18.6 mg, 0.13 mmol) and 20 μ L DIPEA in ACN (0.5 mL), *O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU, 27 mg, 0.09 mmol) was added. The reaction mixture was stirred at room temperature for 0.5 h and then added to E[c(RGDyK)]₂ (20 mg, 14.8 μ mol) in *N,N'*-dimethylformamide (DMF). The reaction was stirred at room temperature for another 2 h, and the desired product azido-RGD2 was isolated by preparative HPLC. The collected fractions were combined and lyophilized to give a white fluffy powder (12 mg, 57% yield) with a retention time of 14.8 min on analytical HPLC. MALDI-TOF-MS: *m/z* 1475.87 for [MH]⁺ (C₆₄H₉₅N₂₂O₁₉, calculated molecular weight [MW] 1475.71).

Preparation of Fluoro-PEG-Triazole-E(RGDyK)₂ (FPTA-RGD2)

To a solution of alkyne tosylate (**1**) (6.8 mg, 0.02 mmol) in ACN, powdered potassium fluoride (6 mg, 0.10 mmol), potassium carbonate (3 mg), and Kryptofix 222 (15 mg) were added, and the mixture was heated at 90 °C for 40 min. The reaction mixture was evaporated to dryness, and the residue was redissolved in 0.4 mL water and 0.4 mL THF. Azido-RGD2 (1 mg, 0.7 μ mol) was then added, followed by CuSO₄ (100 μ L, 0.1 N) and sodium L-ascorbate (100 μ L, 0.3 N) solution. The resulting mixture was stirred at room temperature for 24 h, and the reaction was then quenched and purified by semi-preparative HPLC. The final product fluoro-PEG-triazole-E(RGDyK)₂ (FPTA-RGD2) was obtained in 81% yield (0.91 mg) with a retention time of 13.4 min on analytical HPLC. MALDI-TOF-MS: *m/z* 1665.82 for [MH]⁺ (C₇₃H₁₁₀FN₂₂O₂₂, calculated [MW] 1665.81).

Radiochemistry

[¹⁸F] Fluoride was prepared by the ¹⁸O(p,n)¹⁸F nuclear reaction, and it was then adsorbed onto an anion exchange resin cartridge. Kryptofix 222/K₂CO₃ solution (1 mL 9:1 ACN/water, 15 mg Kryptofix 222, 3 mg K₂CO₃) was used to elute the cartridge, and the resulting mixture was dried in a glass reactor. A solution of alkyne tosylate (**1**) (4 mg in 1 mL ACN/DMSO) was then added, and the resulting mixture was heated at the desired temperature (Table 1). After cooling, the reaction was quenched, and the mixture was injected onto a semipreparative HPLC for purification. The collected radioactive peak was diluted in water (10 mL) and passed through a C18 cartridge. The trapped activity was then eluted off the cartridge with 1 mL THF and used for the next reaction. To the reactor vial with azido-RGD2 (1 mg), 37 MBq activity, CuSO₄ (100 μ L, 0.1 N), and sodium L-ascorbate (100 μ L, 0.3 N) were added sequentially. The resulting mixture was heated at 40 °C for 20 min, and the reaction was then quenched and purified by semipreparative HPLC. The final product ¹⁸F-FPTARGD2 (*R*_t 13.4 min; decay-corrected yield 69 = 11%; radio-chemical purity >97%) was concentrated and formulated in saline (0.9%, 500 μ L) for in vivo studies.

Octanol–Water Partition Coefficient

Approximately 111 kBq of ¹⁸F-FPTA-RGD2 in 500 μ L of PBS (pH 7.4) were added to 500 μ L of octanol in an Eppendorf microcentrifuge tube (model 5415R, Brinkman). The mixture was vigorously vortexed for 1 min at room temperature and centrifuged at 12 500 rpm for 5 min. After centrifugation, 200 μ L aliquots of both layers were measured using a γ -counter (Packard Instruments). The experiment was carried out in triplicate.

Cell Line and Animal Models

U87MG human glioblastoma cells were grown in Dulbecco's medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Invitrogen Co.). Animal procedures were performed according to a protocol approved by the Stanford University Institutional Animal Care and Use Committee. A U87MG xenograft model was generated by subcutaneous (s.c.) injection of 1×10^7 U87MG cells (integrin $\alpha_v\beta_3$ -positive) into the front flank of female athymic nude mice. Three to four weeks after inoculation (tumor volume: 100–400 mm³), the mice (about 9–10 weeks old with 20–25 g body weight) were used for microPET studies.

Cell Integrin Receptor-Binding Assay

In vitro integrin-binding affinity and specificity of E[c(RGDyK)]₂ and FPTA-RGD2 were assessed via competitive cell binding assays using ¹²⁵I-echistatin as the integrin $\alpha_v\beta_3$ -specific radioligand (17). The best-fit 50% inhibitory concentration (IC₅₀) values for U87MG cells were calculated by fitting the data with nonlinear regression using *GraphPad Prism* (GraphPad Software, Inc.). Experiments were performed with triplicate samples.

In Vivo Metabolic Stability Studies

The metabolic stability of ¹⁸F-FPTA-RGD2 was evaluated in an athymic nude mouse bearing a U87MG tumor. 60 min after intravenous injection of 2 MBq of ¹⁸F-FPTA-RGD2, the mouse was sacrificed, and relevant organs were harvested. The blood was collected and immediately centrifuged for 5 min at 13 200 rpm. Liver, kidneys, and tumor were homogenized and then centrifuged for 5 min at 13 200 rpm. After removal of the supernatants, the pellets were washed with 1 mL PBS. For each sample, supernatants of both centrifugation steps of blood, liver, and kidneys were combined and passed through C18 Sep-Pak cartridges. The urine sample was directly diluted with 1 mL of PBS and passed through a C18 Sep-Pak cartridge. The cartridges were each washed with 2 mL of water and eluted with 2 mL of ACN containing 0.1% TFA. After evaporation of the solvent, the residues were redissolved in 1 mL PBS and were injected onto the analytical HPLC. The eluent was collected with a fraction collector (0.5 min/fraction), and the radioactivity of each fraction was measured with the γ -counter.

microPET Studies

PET scans and image analysis were performed using a microPET R4 rodent model scanner (Siemens Medical Solutions) as previously reported (17, 19). About 2 MBq of ¹⁸F-FPTA-RGD2 was intravenously injected into each mouse ($n = 3$) under isoflurane anesthesia (1–3%) and then subjected to a 30-min dynamic scan (1 $\times$ 1 min, 1 $\times$ 1.5 min, 1 $\times$ 3.5 min, 3 $\times$ 5 min, 1 $\times$ 6 min, total of 7 frames) starting from 1 min p.i. 5 min static PET images were also acquired at 1 and 2 h p.i. For each microPET scan, regions of interest (ROIs) were drawn over the tumor, normal tissue, and major organs on decay-corrected whole-body coronal images. The radioactivity concentration (accumulation) within a tumor was obtained from the mean value within the multiple ROIs and then converted to %ID/g (17). For a receptor-blocking experiment, mice bearing U87MG tumors on the front left flank were scanned (5 min static) after coinjection with ¹⁸F-FPTA-RGD2 (2 MBq) and c(RGDyK) (10 mg/kg).

Statistical Analysis

Quantitative data were expressed as mean $\pm$ SD. Means were compared using one-way ANOVA and Student's *t* test. *P* values of <0.05 were considered statistically significant.

RESULTS

Chemistry and Radiochemistry

Both alkyne tosylate (**1**) and azido-RGD2 were obtained in high yields (Figure 1). The alkyne fluoride was prepared in situ and could be used directly for the reaction with azido-RGD2 to make the cold standard, which was purified by HPLC and confirmed by MALDI-TOF mass spectrometry. ^{18}F -alkyne was also obtained in high yield at various conditions (Table 1). The presence of acetonitrile may lower the labeling yield to some extent (Table 1, entries 1–3). Although entry 5 gave the highest decay-corrected yield ($84.3 \pm 2.1\%$), the non-decay-corrected yield was 69.8%, which is actually slightly lower than the non-decay-corrected yield from entry 4 (71.4%). Thus, the condition from entry 4 was used for the subsequent studies. We also noted that the ^{18}F -alkyne intermediate had to be purified before the conjugation with azido-RGD2 to guarantee high labeling yield (this might due to the removal of a large excess amount of unreacted alkyne). The radiochemical purity of the ^{18}F -labeled peptide ^{18}F -FPTA-RGD2 was higher than 97% according to analytical HPLC. The specific radioactivity of ^{18}F -FPTA-RGD2 was determined to be about 100–200 TBq/mmol based on the labeling agent ^{18}F -SFB, as the unlabeled azido-RGD2 was efficiently separated from the product.

The octanol/water partition coefficient ($\log P$) for ^{18}F -FPTA-RGD2 was -2.71 ± 0.006 , indicating that the tracer is slightly more hydrophilic than ^{18}F -FB-RGD2 (^{18}F -FRGD2, -2.103 ± 0.030) and ^{18}F -FB-PEG3-RGD2 (^{18}F -FPRGD2, -2.280 ± 0.054) (18).

In Vitro Cell Integrin Receptor-Binding Assay

The receptor-binding affinity of RGD2 and FPTA-RGD2 was determined by performing competitive displacement studies with ^{125}I -echistatin. All peptides inhibited the binding of ^{125}I -echistatin (integrin $\alpha_v\beta_3$ -specific) to U87MG cells in a concentration-dependent manner. The IC_{50} values for RGD2 and FPTA-RGD2 were 79.2 ± 4.2 and 144 ± 6.5 nM, respectively ($n = 3$) (Figure 2). In a parallel experiment, the IC_{50} value for FPRGD2 was 97 ± 4.8 nM. The comparable IC_{50} values of these compounds suggest that the introduction of miniPEG linker and triazole group had little effect on the receptor-binding affinity.

microPET Imaging of U87MG Tumor-Bearing Mice

Dynamic microPET scans were performed on U87MG xenograft model and selected coronal images at different time points after injecting ^{18}F -FPTA-RGD2 were shown in Figure 3A. Good tumor-to-contralateral background contrast was observed as early as 10 min after injection ($5.4 \pm 0.7\%$ ID/g). The U87MG tumor uptake was 3.1 ± 0.6 , 2.1 ± 0.4 , and $1.3 \pm 0.4\%$ ID/g at 0.5, 1, and 2 h p.i., respectively ($n = 3$). Most activity in the nontargeted tissues and organs was cleared by 1 h p.i. For example, the uptake values in the kidney, liver, and muscle were as low as 2.7 ± 0.8 , 1.9 ± 0.4 , and $1.0 \pm 0.3\%$ ID/g, respectively, at 1 h p.i. The averaged time-activity curves (TACs) for the U87MG tumor, liver, kidney, and muscle were

shown in Figure 4. ^{18}F -FPTA-RGD2 was cleared mainly through the kidneys. Some hepatic clearance was also observed. The integrin $\alpha_v\beta_3$ specificity of ^{18}F -FPTA-RGD2 in vivo was confirmed by a blocking experiment where the tracer was coinjected with c(RGDyK) (10 mg/kg). As can be seen from Figure 3B, the U87MG tumor uptake in the presence of nonradiolabeled RGD peptide ($0.9 \pm 0.3\%$ ID/g) is significantly lower than that without RGD blocking ($2.1 \pm 0.4\%$ ID/g) ($P < 0.05$) at 1 h p.i.

The comparison of tumor and various organ uptake of ^{18}F -FPTA-RGD2 with ^{18}F -FPRGD2 and ^{18}F -FRGD2 was shown in Figure 5. The uptake in the U87MG tumor was slightly lower for ^{18}F -FPTA-RGD2, which might be caused by integrin $\alpha_v\beta_3$ binding affinity difference (Figure 5A). The kidney uptake for these three tracers was comparable (Figure 5B), and the clearance rate was highest for ^{18}F -FPTA-RGD2. ^{18}F -FPTA-RGD2 had lowest liver uptake, which was consistent with the hydrophilic sequence of these three compounds (Figure 5C). The nonspecific uptake in the muscle was at a very low level for all three compounds (Figure 5D).

In Vivo Metabolic Stability Studies

The metabolic stability of ^{18}F -FPTA-RGD2 was determined in mouse blood and urine and in the liver, kidney, and tumor homogenates at 1 h after intravenous injection of radiotracer into a U87MG tumor-bearing mouse. The extraction efficiency of all organs was between 86% and 99% (Table 2). The lowest extraction efficiency was found for the kidney. 1% to 41% of the total activity could not be trapped on the C-18 cartridges, which can be related to very hydrophilic metabolites and protein-bound activity. After ACN elution, the radioactivity of each sample was injected onto an analytical HPLC, and the HPLC chromatograms are shown in Figure 6. The fraction of intact tracer was between 75% and 99% (Table 2). Although we did not identify the metabolites, we found that all metabolites eluted earlier from the HPLC column rather than the parent compound (Figure 6), which behaved similarly to ^{18}F -FRGD2 (19) and ^{18}F -FPRGD2 (18).

DISCUSSION

^{18}F -labeling of cyclic RGD peptide was first reported by Haubner et al. (37). A monomeric glycopeptide based on c(RGDfK) was ^{18}F -radiolabeled via ^{18}F -2-fluoropropionate prosthetic group and the resulting ^{18}F -galacto-RGD exhibited integrin $\alpha_v\beta_3$ specific tumor uptake in integrin-positive xenograft models. Initial clinical trials in a limited number of cancer patients revealed that this tracer can be safely given to patients and is able to delineate certain lesions that are integrin-positive (15). We have ^{18}F -radiolabeled both monomeric and dimeric RGD peptides using an ^{18}F -4-fluorobenzoyl (^{18}F -FB) prosthetic group (16, 19). The dimeric RGD peptide tracer, ^{18}F -FB-E[c(RGDyK)]₂ (denoted ^{18}F -FRGD2), exhibited excellent integrin $\alpha_v\beta_3$ -specific tumor imaging with favorable in vivo pharmacokinetics (19, 38). The binding potential extrapolated from Logan plot graphical analysis of the PET data correlated well with the receptor density measured by SDS-PAGE/autoradiography in various xenograft models. The tumor-to-background ratio at 1 h after injection of ^{18}F -FRGD2 also gave a good linear relationship with the tumor tissue integrin $\alpha_v\beta_3$ expression level (19). We have also reported a thiol-reactive synthon, *N*-[2-(4- ^{18}F -

fluorobenzamido)ethyl]maleimide (^{18}F -FBEM), for labeling monomeric and dimeric sulfhydryl-RGD peptides (25). To extend our efforts of ^{18}F -radiolabeling strategies, we explored and reported the possibility of labeling dimeric RGD peptide E[c(RGDyK)]₂ using Hsuigen 1,3-dipolar cycloaddition reaction (one of the click chemistry reactions) and evaluated the ability of the new PET tracer for integrin $\alpha_v\beta_3$ targeting in vitro and in vivo.

Alkyne tosylate (1) was designed as the labeling precursor that allowed nucleophilic fluorination and displacement of the tosyl group to occur in high yield under mild conditions (15 min, $78.5 \pm 2.3\%$ yield). A triethylene glycol linker was employed in the structure to reduce volatility and obtain water solubility. The azido group was introduced to RGD dimer RGD2 by reacting the glutamate amine group with the azido-NHS ester. A robust catalytic system, Cu^{2+} /ascorbate, was used for the labeling reaction (27). In comparison with the SFB labeling procedure (starting from $^{18}\text{F}\text{-F}^-$, the total synthesis time of ^{18}F -FPRGD2 was about 180 min with an overall non-decay-corrected yield of 12.9% (decay-corrected yield 40%)) (18), click-labeled ^{18}F -FPTA-RGD2 could be obtained in 110 min with 26.9% non-decay-corrected yield (decay-corrected yield 53.8%). The reduced reaction time and increased labeling yield make click chemistry a valuable method for labeling RGD peptide with ^{18}F .

We also studied the application of ^{18}F -FPTA-RGD2 for in vivo imaging. We found that this tracer had good tumor-to-muscle ratio and predominant renal excretion. Compared with ^{18}F -FPRGD2 and ^{18}F -FRGD2, the tumor-targeting efficacy of ^{18}F -FPTA-RGD2 was decreased to some extent, which might be caused by the slightly decreased integrin binding affinity based on cell binding assay. The unspecific blood pool activity could be another factor. However, no significant difference was observed for these compounds ($P > 0.5$) (Figure 5E). ^{18}F -FPTA-RGD2 also had a faster clearance rate and lower liver uptake which might due to the increased hydrophilicity of this tracer ($\log P = -2.710 \pm 0.006$), after the replacement of the benzoic group with a short PEG linker. A metabolic stability study revealed that the triazoles unit, formed by click chemistry in ^{18}F -FPTA-RGD2, has comparable in vivo stability compared with the amide bound made from SFB in the case of ^{18}F -FRGD2 and ^{18}F -FPRGD2 (18, 19).

This study demonstrated that RGD peptide can be labeled efficiently through click chemistry. The major advantage of ^{18}F -FPTA-RGD2 would be shortened reaction time, increased labeling yield, and comparable in vivo stability. The tumor-targeting efficacy of this tracer was comparable with SFB-labeled RGD peptides and can be further improved. First, the relatively long linker (triethylene glycol plus four methylene groups) in ^{18}F -FPTA-RGD2 might account for the decreased integrin binding affinity. Our future work will focus on the development of various linkers suitable for this new labeling method and study the in vivo pharmacokinetics of the resulting tracers. Second, high $\alpha_v\beta_3$ binding affinity is needed to afford high tumor uptake and retention. Based on polyvalency effect, tetrameric RGD peptide (17), labeled with the synthon described here, would have more effective binding to integrin $\alpha_v\beta_3$ and better tumor targeting efficacy. Third, the click labeling method developed here could also be applied to label a variety of other peptides, proteins, antibodies, or oligonucleotides after the introduction of the azido group. Due to the mild labeling conditions, ^{18}F might be easily engineered to incorporate the organo azide residue without compromising the biological activity.

CONCLUSIONS

The new tracer ^{18}F -FPTA-RGD2 was synthesized with high specific activity based on click chemistry. This tracer exhibited good tumor-targeting efficacy, relatively good metabolic stability, as well as favorable in vivo pharmacokinetics. The new ^{18}F labeling method developed in this study could also have general application in labeling azido-containing bioactive molecules in high radiochemical yield and high specific activity for successful PET applications.

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Figure 1. (A) Radiosynthesis of ^{18}F -fluoro-PEG-alkyne intermediate and 1,3-dipolar cycloaddition with terminal azide. R = targeting biomolecule (peptides, proteins, antibodies, etc.). (B) Structure of ^{18}F -fluoro-PEG-alkyne labeled $\text{E}[\text{c}(\text{RGDyK})]_2$: ^{18}F -fluoro-PEG-triazole- $\text{E}(\text{RGDyK})_2$ (^{18}F -FPTA-RGD2).

Figure 2.

Cell binding assay of $\text{E}[\text{c}(\text{RGDyK})]_2$ and FPTA-RGD2 using U87MG cells with competitive displacement studies using ^{125}I -echistatin. The IC_{50} values for $\text{E}[\text{c}(\text{RGDyK})]_2$ and FPTA-RGD2 were 79.2 ± 4.2 and 144 ± 6.5 nM, respectively ($n = 3$).

Figure 3.

(A) Decay-corrected whole-body coronal microPET images of athymic female nude mice bearing U87MG tumor at 10, 20, 30, 60, and 125 min postinjection (p.i.) of about 2 MBq of ^{18}F -FPTA-RGD2. (B) Coronal microPET images of U87MG tumor-bearing mice at 30 and 60 min p.i. of ^{18}F -FPTA-RGD2 with (denoted as “blocking”) and without coinjection of 10 mg/kg mouse body weight of c(RGDyK). Tumors are indicated by arrows.

Figure 4. Time-activity curves of the U87MG tumor, liver, kidney, blood, and muscle after intravenous injection of ^{18}F -FPTA-RGD2. Data were derived from multiple time-point microPET study. ROIs are shown as the %ID/g $\pm$ SD ($n = 3$). Note that the kidney uptake in the figure is 1/4 of the actual value.

Figure 5. Comparison of ^{18}F -FPTA-RGD2, ^{18}F -FB-RGD2 (^{18}F -FRGD2), and ^{18}F -FB-PEG3-RGD2 (^{18}F -FPRGD2) in U87MG tumor, kidney, liver, muscle, and blood over time.

Figure 6. Metabolic stability of ^{18}F -FPTA-RGD2 in mouse blood and urine samples and in liver, kidney, and U87MG tumor homogenates at 1 h after injection. The HPLC profile of pure ^{18}F -FPTA-RGD2 (Standard) is also shown.

Table 1Radiolabeling Yields (decay-corrected) of ^{18}F -Fluoro-PEG-Alkyne Intermediate at Various Conditions ($n = 3$)

entry	solvent	temperature and time	yield (%)
1	ACN	90 °C for 15 min	61.2 $\pm$ 2.5
2	ACN	110 °C for 15 min	71.4 $\pm$ 3.0
3	ACN/DMSO	110 °C for 15 min	75.0 $\pm$ 1.8
4	DMSO	110 °C for 15 min	78.5 $\pm$ 2.3
5	DMSO	110 °C for 30 min	84.3 $\pm$ 2.1

Table 2

Extraction Efficiency, Elution Efficiency, and HPLC Analysis of Soluble Fraction of Tissue Homogenates at 1 h Postinjection of ^{18}F -FPTA-RGD2^a

fraction	blood	urine	liver	kidney	U87mg
	Extraction Efficiency (%)				
insoluble fraction	0.8	ND	10.3	13.3	7.5
soluble fraction	99.2	ND	89.7	86.7	92.5
	Elution Efficiency (%)				
unretained fraction	2.8	0.4	33.9	12.8	18.5
wash water	8.8	0.5	7.4	3.9	5.2
acetonitrile eluent	88.4	99.1	58.7	83.3	76.4
	HPLC Analysis (%)				
intact tracer	75.9	99.7	81.6	89.1	82.4

^a ND denotes not determined.