A Novel Method for Direct site-specific Radiolabeling of Peptides Using [^{18}F]FDG

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Experimental Procedures

General: Cyclic peptide *cyclo*[Arg(Pbf)-Gly-Asp(tBu)-^{D}Tyr(But)-Lys] was purchased from Peptides International (Louisville, KY). All other N-α-Fmoc (Fluorenylmethoxycarbonyl)-protected amino acids, O-benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), O-(7-azabenzotriazol-1-yl)-N,N,N',N'-trimethyluronium hexafluorophosphate (HATU), 1-hydroxy-7-Azabenzotriazole (HOAt), trifluoroacetic acid (TFA), and rink amide resin (100-200 mesh, 1% DVB, 0.5 mmol/g) were purchased from Advanced Chemtech (Louisville, KY). Dimethylformamide (DMF) and methylene chloride were obtained from Fisher Scientific (Fair Lawn, NJ). 20% piperidine in DMF was obtained from Protein Technologies Inc. (Tucson, AZ). Pyridine, acetic anhydride, acetic acid, and anhydrous ether were obtained from J.T.Baker (Phillipsburg, NJ). N-methylmorpholine (NMM), Boc-(aminooxy)acetic acid, N,N-diisopropylethylamine, 1, 2-Ethanedithiol (EDT), and triisopropylsilane (TIPS) were purchased from Aldrich Chemical Company (Milwaukee, WI). HPLC grade acetonitrile (CH_3CN) and Millipore 18 mΩ water were used for peptide purifications. ^{125}I-Echistatin labeled by the lactoperoxidase method with specific activity of 2,000 Ci/mmol was purchased from Amersham Biosciences (Piscataway, NJ). Purification and analysis of the peptides was done on a Dionex Summit HPLC system (Dionex Corporation, Sunnyvale, CA) equipped with a 340 U 4-Channel UV-Vis

absorbance detector and radioactivity detector (Carroll & Ramsey Associates, model 105S, Berkeley, CA). UV detection wavelengths were 218 nm, 254 nm and 280 nm for all the experiments and HPLC reveres phase column (Vydac, Hesperia, CA. 218TP510-C18, 5μ, 10 mm × 250 mm) was used for analysis of labeled peptides. The mobile phase was 0.1% TFA in water , and 0.1% TFA in acetonitrile. Radioactivity measurements were performed by A CRC-15R PETdose calibrator (Capintec Inc., Ramsey, NJ). Electron spray ionization (ESI) mass spectrometry was done by Vincent Coates Foundation Mass Spectrometry Laboratory, Stanford University or an electrospray ionization time of flight mass spectrometer (ESI-TOF-MS, model: JMS-T100LC) (JEOL, Tokyo, Japan) was performed by the Mass Spectrometry Lab at GE Global Research (Niskayuna, NY).

Linear RGD (Arg-Gly-Asp) peptide was synthesized using standard solid phase techniques with N-Fmoc-protected amino acids using substitution 2,4-dimethoxybenzhydrylamine resin (Rink Resin LS, Advanced Chemtech) on a 100 μmol scale. The synthesis was performed in a Rainin/Protein Technology, Inc. Symphony solid phase peptide synthesizer. N-terminal functionalized with aminooxy acetyl group was achieved by using Boc-(aminooxy)acetic acid. Cleavage from the solid support and removal of the acid-labile protecting groups was performed by 95% TFA: 2.5% TIPS (triisopropylsilae): 2.5% water (v/v/v) and purified to afford aminooxy-RGD (RGD-ONH_2) in 72 % yield. Analysis of the peptide by HPLC followed by ESI-MS showed *m/z* = 419.22 for $[M+H]^+$ ($C_{14}H_{27}N_8O_7$, Calculated $[M+H]^+$ = 419.19). For preparation of aminooxyacetyl Cyclo(RGD^{D}YK) [Cyclo(RGD^{D}YK)-ONH_2], initially the protected cyclo[Arg (pbf)-Gly-Asp(tBU)-^{D}Tyr(But)-Lys]-ONH-Boc was prepared by the reaction

of Boc-(aminooxy)acetic acid, O-(7-azabenzotriazol-1-yl)-N,N,N',N'-trimethyluronium hexafluorophosphate (HATU) and Hunig's base with commercially available cyclo[Arg (pbf)-Gly-Asp(tBU)-^{D}Tyr(But)-Lys] in DMF. The protected Cyclo[Arg(pbf)-Gly-Asp(tBU)-^{D}Tyr(But)-Lys]-ONH-Boc was purified by HPLC (white powder,78 % yield). Analysis of peptide by ESI-MS showed *m/z* = 1157.48 for $[M+H]^+$ ($C_{55}H_{85}N_{10}O_{15}S$, calculated $[M+H]^+$ = 1157.58). Removal of the acid-labile protecting groups of cyclo[Arg(pbf)-Gly-Asp(tBU)-^{D}Tyr(But)-Lys]-ONH-Boc was performed as above and purified by HPLC to afford Cyclo(RGD^{D}YK)-ONH_2 in 92 % yield. Analysis of the peptide by ESI-MS showed *m/z* = 693.26 for $[M+H]^+$ ($C_{29}H_{45}N_{10}O_{10}$, calculated

$[M+H]^+$ =693.32).

Synthesis of FDG-RGD: The linear aminooxy-RGD (RGD-ONH_2, 2 mg) was incubated with 3.7 equivalents cold FDG in 16 % ethanol in saline (120 µl) in the presence of 0.4 % TFA at 100 °C for 40 min. After cooling to room temperature, the reaction mixture was diluted to 300 µL with water. The resulting solution was injected into a HPLC column (Vydac 218TP54, C18, 5 u, 4.6 X 250 mm, 1mL/min flow rate). The eluent changed from 95% solvent A (0.1 % TFA in H_2O) and 5 % solvent B (0.1% TFA in acetonitrile) (0-3 min) to 35 % solvent A and 65 % solvent B at 33 min. The E and Z oximes were collected (retention time 6.0-7.0 min) and solvents were removed under reduced pressure to yield FDG-RGD (1.1 mg, 39.5 %). The mass spectrometry (ESI-MS) analysis of the isolated product showed two identical mass peaks of 584.2 ($[M+H]^+$) which correspond to FDG-RGD (E- and Z-oximes) (cal. [M+H] for FDG-RGD is 584.5.).

Synthesis of [^{18}F]FDG-RGD: Aminooxy-RGD (RGD-ONH_2, 2 mg) was reacted with 4-6 mCi of [^{18}F]FDG in 100 µl saline in the same condition as above but in 30 minutes.

The poducts (E and Z oximes) were isolated by the above HPLC conditions but with a different gradients. The eluent changed from 97% solvent A (0.1 % TFA in H_2O) and 3 % solvent B (0.1% TFA in acetonitrile) (0-3 min) to 85 % solvent A and 15 % solvent B at 33 min. After products collection, solvents were removed under reduced pressure to afford [^{18}F]FDG-RGD (E and Z) in 27.5 % radiochemical yields based on [^{18}F]FDG (decay corrected).

Synthesis of FDG-Cyclo(RGD^{D}YK): Cyclo(RGD^{D}FK)-ONH_2 (2 mg) was incubated with 5.7 equivalents of FDG in 16 % ethanol in saline (120 µl) and 0.4 % TFA at 100 °C for 60 min. After cooling to room temperature, the reaction mixture was diluted to 300 µL with water. The resulting solution was injected into a HPLC column (above). The eluent changed from 95% solvent A (0.1 % TFA in H_2O) and 5 % solvent B (0.1% TFA in acetonitrile) (0-3 min) to 35 % solvent A and 65 % solvent B at 33 min. The products were collected (retention times 17.5-19 min) and solvents were removed under reduced pressure to yield (E and Z) FDG-cyclo(RGDDYK) (1.03 mg, 41.4 %). The purified FDG-cyclo(RGD^{D}YK) showed two identical mass peaks of 857.35 ($[M+H]^+$) in ESI-MS which are corresponding to FDG-cyclo(RGD^{D}YK) (E- and Z-oximes) (cal. M+H for FDG-cyclo(RGD^{D}YK) is 857.47.).

Synthesis of [^{18}F]FDG-Cyclo(RGD^{D}YK): Cyclo(RGD^{D}FK)-ONH_2 (2 mg) was incubated with [^{18}F]FDG (4-6 mCi) in 16 % ethanol in saline (120 µl) and 0.4 % TFA at 100 °C for 45 min. After cooling to room temperature, the products (E and Z oximes) were isolated by the above HPLC conditions which used for the synthesis of FDG-cyclo(RGD^{D}YK). After products collection, solvents were removed under reduced pressure to afford [^{18}F]FDG-cyclo(RGD^{D}YK) (E and Z oximes) in 41 % radiochemical

yields based on [^{18}F]FDG (decay corrected). [^{18}F]FDG-cyclo(RGD^{D}YK) (E- and Z-) oximes were purified but not separated by HPLC and the two ^{18}F-labeled products (E- or Z-oximes) were isolated as a mixture and analyzed by analytical HPLC. The exact stereochemistry (E- and Z-) of these two products has not been determined yet.

U87MG Glioblastoma Xenograft Model

U87MG human glioblastoma cell line was obtained from American Type Culture Collection (Manassas, VA) and was maintained at 37 ºC in a humidified atmosphere containing 5% CO_2 in Iscove's modified Dulbecco's medium and 5% fetal bovine serum (Life Technologies, Inc., Grand island, NY). Animal studies were carried out according to a protocol approved by the Stanford University Administrative Panels on Laboratory Animal Care (A-PLAC). Female athymic nude mice (*nu/nu*), purchased from Charles River Laboratories, Inc. (Cambridge, MA) at 4-6 weeks of age, were inoculated subcutaneously in the flanks with 5×10^6 U87MG glioblastoma cells suspended in 100 μL of phosphate buffered saline (PBS). The tumor bearing mice were subjected to *in vivo biodistribution and* imaging studies when the tumors reached 0.4-0.6 cm in diameter (14-21 d after implant).

Biodistribution

For biodistribution studies, the U87MG tumor-bearing mice (20 g each, n = 3 for each group) were injected with radiolabeled [^{18}F]FDG-RGD or [^{18}F]FDG-cyclo(RGD^{D}YK) (10-30 μCi, 0.37–1.11 MBq) via tail vein and sacrificed and dissected at 30 min to 2 h after injection. Tumor, blood and major tissues of interest were removed

and weighed, and their radioactivity was measured in a gamma-counter. The radioactivity uptake in the tumor and normal tissues was expressed as a percentage of the injected radioactive dose per gram of tissue (% ID/g).

Integrin Binding Characteristics *in vitro*

The receptor binding affinity study of conjugate FDG-cyclo(RGD^{D}YK) for integrin $\alpha_v\beta_3$ was carried out using $\alpha_v\beta_3$ positive U87MG cells. We compared the receptor binding affinity of FDG-cyclo(RGD^{D}YK) by performing competitive displacement studies with ^{125}I-echistatin as radioligand. FDG-cyclo(RGD^{D}YK) inhibited the binding of ^{125}I-echistatin to U87MG cells in a dose dependent manner. The IC_{50} value for FDG-cyclo(RGD^{D}YK) was 0.66 ± 0.22 μM.

MicroPET Imaging

PET scans were performed on a microPET R4 rodent model scanner (Concorde Microsystem Inc.). The mice bearing U87MG were injected with [^{18}F]FDG-RGD or [^{18}F]FDG-cyclo(RGD^{D}YK) via the tail vein and the mice were imaged at 30 min to 2 h after post injection (pi). At different time p.i. (0.5, 1 and 2 h), the mice were anesthetized with 2% isoflurane, and placed in the prone position and near the center of the filed of view of microPET. The 5-min static scans were obtained and the images were reconstructed by a two-dimensional ordered subsets expectation maximum (OSEM) algorithm. Regions of interest (ROIs) were then drawn over the tumor on decay-corrected whole-body coronal images.