In Vivo Positron Emission Tomography (PET) Imaging with an $\alpha_v\beta_6$ Specific Peptide Radiolabeled using ^{18}F-"Click" Chemistry: Evaluation and Comparison with the Corresponding 4-[^{18}F]Fluorobenzoyl- and 2-[^{18}F]Fluoropropionyl-Peptides

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Experimental procedures

General information. Solvents and chemicals were purchased from Aldrich (Milwaukee, WI) unless stated otherwise. Racemic (±)-2-fluoropropionic acid was obtained from Matrix Scientific (Columbia, SC). Fmoc-protected amino acids and coupling reagents were purchased from NovaBiochem (San Diego, CA) or GL Biochem (Shanghai, China). Rink Amide NovaGel HL resin (0.63 mmol/g) for solid-phase peptide synthesis was obtained from NovaBiochem (San Diego, CA). Sep-Pak SPE cartridges were obtained from Waters (Milford, MA) and ^{18}F Trap & Release Columns were purchased from ORTG, Inc. (Oakdale, TN). Mass spectrometry analysis was performed using an ABI 4700 MALDI TOF/TOF spectrometer. NMR spectra were recorded using a Bruker Avance 500 spectrometer. [^{18}F]Fluoride was produced from the ^{18}O(p,n)^{18}F nuclear reaction on [^{18}O]H_2O purchased from Marshall Isotopes Ltd. (Tel Aviv, Israel) using a CTI RDS 111 negative ion cyclotron (Knoxville, TN). Biodistribution samples were analyzed on a Wizard 1470 γ-counter (Perkin-Elmer, Waltham, MA). MicroPET scans were conducted on a Focus120 microPET scanner (Siemens Medical Solutions USA, Malvern, PA).

Reversed-phase HPLC was used to purify and analyze the products; solvent A: 0.05% TFA in water (v/v); solvent B: acetonitrile. HPLC systems were equipped with both UV absorbance detector (UV, 220 nm) and a radioactivity detector (PMT) connected in series, which accounts for the slight difference between detected retention times for corresponding ^{18}F- and ^{19}F-compounds.

- Analytical HPLC system A: Phenomenex Jupiter 4 μ Proteo 90 Å column (250 × 4.6 mm, 4 μm), solvent B isocratic 9% for 2 min, then linear gradient to 81% over 30 min, flow rate 1.5 mL/min;
- Semi-preparative HPLC system B: Phenomenex Jupiter 10 μ Proteo 90 Å (250 × 10 mm, 10 μm), solvent B isocratic 9% for 2 min, then linear gradient to 81% over 30 min, flow rate 3 mL/min;
- Isocratic HPLC system C: Phenomenex Jupiter 10 μ C18 300 Å (250 × 10 mm, 10 μm), isocratic 60% acetonitrile - 0.05% TFA in water (v/v), flow rate 3 mL/min;
- Isocratic HPLC system D: Phenomenex Jupiter 10 μ C18 300 Å (250 × 10 mm, 10 μm), isocratic 35% acetonitrile - 0.05% TFA in water (v/v), flow rate 3 mL/min.

Synthesis of 5-fluoro-1-pentyne. A solution of 4-pentyn-1-ol (1 g, 12 mmol) in CH_2Cl_2 (5 mL) was added dropwise over 5 min to a cooled (-78 °C) solution of (diethylamino)sulfur trifluoride (DAST, 2.3 g, 14 mmol) in CH_2Cl_2 (5 mL) under a nitrogen atmosphere. The resulting mixture was stirred at -78 °C for 1 h and at room temperature for an additional 3 h. The mixture was cooled to -10 °C and the excess of DAST was quenched with 10% aq. Na_2CO_3. The aqueous phase was extracted with CH_2Cl_2 (3 × 5 mL) and the collected organic extracts were washed with 10% aq. Na_2CO_3, water, and dried over $MgSO_4$. The solvent was removed and the product was purified by distillation at atmospheric pressure. According to the NMR analysis the highly volatile product was obtained as a 20% solution in CH_2Cl_2 and used for next reaction without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.77-1.89 (m, 2H), 1.91 (t, J = 2.5 Hz, 1H), 2.28 (td, J = 7.0, 2.5 Hz, 2H), 4.49 (dt, J = 47.0 Hz, J = 6.0 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.43 (d, J = 5.9 Hz), 29.26 (d, J = 20.2 Hz), 68.94, 82.30 (d, J = 164.9 Hz), 82.91; ^{19}F NMR (470 MHz, $CDCl_3$) δ -220.3.

Synthesis of 3-azidopropionic acid (Azpr). The compound was prepared according to the previously published procedure.[1] The final product was contaminated with approx. 10% of 3-hydroxypropionic acid according to the NMR analysis. The material was used for the next reaction without further purification due to safety concerns associated with the distillation of a potentially explosive organic azide. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.58 (t, J = 6.5 Hz, 2H), 3.54 (t, J = 6.5 Hz, 2H), 11.10 (bs, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 33.72, 46.35, 177.25.

Solid-phase peptide synthesis. Peptides were synthesized on Rink Amide NovaGel HL resin. Manual synthesis was performed using a 3-fold excess of amino acids and HATU/DIEA activation with a 2 h coupling time, followed by 30 min Fmoc deprotection with 20% piperidine in DMF. 2-Fluoropropionic acid (FPA), 4-fluorobenzoic acid (FBA) or 3-azidopropionic acid (Azpr) were coupled to the peptide using a 3-fold excess of the acid and HATU/DIEA activation for 30 min. The progress of the reaction was monitored by the ninhydrin based Kaiser test[2] or the picrylsulfonic acid test.[3] Side-chain protecting groups were removed and the peptides were cleaved off the solid support using TFA/1,2-ethanedithiol/TIPS/water 94:2.5:1:2.5 (v/v/v/v). The crude peptides were washed three times with diethyl ether before purification on semi-preparative HPLC (system B) and characterization using mass spectrometry. Analytical data for peptides: FBA-A20FMDV2: HPLC (system A, UV 220 nm) retention time 16.4 min; MS

(MALDI) *m/z* 2284.1841 $[M+H]^+$, calcd ($C_{100}H_{168}N_{32}O_{28}F$) 2284.26899; FPA-A20FMDV2: HPLC (system A, UV 220 nm) retention time 14.6 min; MS (MALDI) *m/z* 2236.1794 $[M+H]^+$, calcd ($C_{96}H_{168}N_{32}O_{28}F$) 2236.26899; Azpr-A20FMDV2: HPLC (system A, UV 220 nm) retention time 15.9 min; MS (MALDI) *m/z* 2259.4126 $[M+H]^+$, calcd ($C_{96}H_{168}N_{35}O_{28}$) 2259.27981.

Synthesis of FC5-A20FMDV2. A solution of 5-fluoro-1-pentyne (2 mg, 0.013 mmol) in CH_2Cl_2 was added to a solution of sodium ascorbate (25 mg, 0.13 mmol), CuI (2.5 mg, 0.013 mmol), Azpr-A20FMDV2 (3 mg, 0.0013 mmol), DIEA (25 μL, 0.14 mmol) in a mixture of DMF (0.2 mL), water (0.25 mL) and acetonitrile (0.25 mL) at 0 °C. The resulting orange solution was stirred vigorously at 0 °C and the progress of the reaction was monitored by HPLC. After about 0.5 h the solution was filtered and the product was isolated using semi-preparative HPLC (system B). HPLC (system A, UV 220 nm) retention time 14.6 min; MS MALDI *m/z* 2345.0784 $[M+H]^+$, calcd ($C_{101}H_{175}N_{35}O_{28}F$) 2345.33299.

Radiochemical synthesis of [^{18}F]FBA. The compound was prepared according to the previously published procedure using a modified Siemens/CTI Chemistry Process Control Unit (CPCU) double vessel [^{18}F]FDG module.[4]

Briefly, the [^{18}F]fluoride was delivered to the CPCU unit, captured on a ^{18}F-fluoride Trap & Release Column cartridge and eluted with a solution of 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane (K222; 15 mg)/potassium carbonate (3 mg) in acetonitrile/water (1.5 mL; 6% v/v). The acetonitrile was evaporated and the K^{18}F/K222 complex was dried by azeotropic distillation of the water using an additional 2 mL acetonitrile. A solution of ethyl 4-(trimethylammoniumtriflate)benzoate (5 mg) in anhydrous DMSO (0.75 mL) was added and the resulting mixture was heated to 115 °C (15 min). Aqueous NaOH (0.5 N, 1.5 mL) was added and the resulting mixture was heated to 115 °C (9 min). The crude product was diluted with water (10 mL) and 1N HCl (3 mL). The solution was passed through a C18 Sep-Pak cartridge. The product, trapped on the cartridge, was washed with water (5 mL) and dried with air. The product was eluted with acetonitrile (2 mL). The synthesis time from EOB was 50 minutes and the decay corrected yield was 70 ± 5% (n = 5).

Radiochemical synthesis of [^{18}F]FBA-A20FMDV2 (1). The compound was prepared by the reaction of [^{18}F]FBA with the N-terminally deprotected peptide on solid phase as previously described.[5,6]

Briefly, the [^{18}F]FBA in DMF (50 μL) was added to the peptide resin (5 mg) swollen in DMF, followed by HATU (5 mg) in DMF (30 μL) and DIEA (10 μL) in DMF (30 μL). The reaction mixture was shaken at 30 °C for 30 min. Next, the solvent was removed and the solid support was washed with DMF (3 × 0.5 mL) and methanol (3 × 0.5 mL). Then, the TFA cleavage mixture (0.7 mL; trifluoroacetic acid/triisopropylsilane/water 95:2.5:2.5 v/v/v) was drawn into the syringe and the reaction mixture was incubated at 30 °C for 2 × 10 min. The product was collected and the resin was washed with dichloromethane (0.5 mL). Following evaporation of the cleavage solvents and purification by semipreparative HPLC (system D; retention time 12.0 min) the product was obtained in a decay corrected yield of 7.8 ± 2.2% (n = 5) based on K^{18}F. Analytical HPLC (system A, PMT) retention time 16.6 min; purity >99%. Synthesis time: 87 min.

Radiochemical synthesis of (±)-2-[^{18}F]fluoropropionic acid ([^{18}F]FPA). The compound was prepared according to the previously published procedure.[7,8]

Briefly, the [^{18}F]fluoride was captured on a ^{18}F-fluoride Trap & Release Column cartridge and eluted with a solution of 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane (K222; 10 mg)/potassium carbonate (2 mg) in acetonitrile/water (1 mL; 6% v/v). The acetonitrile was evaporated at 100 °C under a gentle stream of nitrogen and the K^{18}F/K222 complex was dried by azeotropic distillation of the water using an additional 3 × 1 mL acetonitrile. A solution of 9-methylanthranyl 2-bromopropionate (10 mg) in acetonitrile (2 mL) was added and the mixture was heated in a sealed vial to 100 °C for 15 min. The product was isolated on semi-preparative HPLC (system C; retention time 18-20 min) and further purified using a C18 Sep-Pak cartridge. The product, trapped on the cartridge, was washed with water (5 mL) and dried with a stream of nitrogen. The 9-methylanthranyl 2-[^{18}F]fluoropropionate was eluted with dichloromethane (3 mL) and the solvent was evaporated under a stream of nitrogen. The methylanthranyl ester was hydrolyzed using 200 μL of hydrolysis mixture (9.5 mL water, 0.5 mL triethylamine), acetonitrile (200 μL) and DMF (100 μL) in a closed vial at 100 °C for 10 min. The water and acetonitrile were evaporated under a gentle stream of nitrogen and the DMF-solution was dried using azeotropic distillation with acetonitrile (3 × 1 mL). The synthesis time from EOB was 95 minutes and the decay corrected yield was of 49 ± 3% (n = 3).

Radiochemical synthesis of [^{18}F]FPA-A20FMDV2 (2). The compound was prepared by the reaction of [^{18}F]FPA with the N-terminally deprotected peptide on solid phase as described for [^{18}F]FBA-A20FMDV2 (**1**).[8] Following purification by semipreparative HPLC (system D; retention time 7.5 min) the product was obtained in a decay corrected yield of 4.6 ± 0.8% (n = 3) based on K^{18}F. Analytical HPLC (system A, PMT) retention time 14.7 min; purity >99%. Synthesis time: 76 min.

Radiochemical synthesis of 5-[^{18}F]fluoro-1-pentyne. The compound was prepared according to the previously published procedure.[9]

Briefly, the [^{18}F]fluoride was captured on a ^{18}F-fluoride Trap & Release Column cartridge and eluted with a solution of 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane (K222; 10 mg)/potassium carbonate (2 mg) in acetonitrile/water (1 mL; 6% v/v). The acetonitrile was evaporated at 100 °C under a gentle stream of nitrogen and the K^{18}F/K222 complex was dried by azeotropic distillation of the water using an additional 3 × 1 mL acetonitrile. A solution of 4-pentynyl *p*-toluenesulfonate (5 μL) in anhydrous acetonitrile (1.5 mL) was added and the sealed vial was connected to another vial *via* silicone tubing (20 cm × 2 mm inner diameter); the second vial was vented using a short drying tube and placed in a -78 ºC bath. The first vial was heated to 100 ºC and the 5-[^{18}F]fluoro-1-pentyne was distilled into the second vial together with acetonitrile over a period of 10 minutes. The

synthesis time from EOB was 22 minutes and decay corrected yield was 86 ± 2% (n = 3). Analytical HPLC (system A, PMT) retention time 17.1 min; purity >99%.

Radiochemical synthesis of [^{18}F]FC5-A20FMDV2 (3). The compound was prepared by the reaction of 5-[^{18}F]fluoro-1-pentyne in acetonitrile (0.3 mL) with *N*-(3-azidopropionyl)-A20FMDV2 (Azpr-A20FMDV2, 1.0 mg, 0.0004 mmol) in the presence of sodium ascorbate (25 mg, 0.13 mmol), CuI (2.5 mg, 0.013 mmol), DIEA (25 μL, 0.14 mmol) in a mixture of DMF (0.2 mL) and water (0.25 mL).[9] The resulting orange solution was stirred vigorously at room temperature for 10 minutes. The reaction mixture was then drawn into a syringe containing water (10 mL) and passed through a C18 Sep-Pak cartridge. The product, trapped on the cartridge, was washed with water (5 mL) and eluted with 1% acetic acid in ethanol (v/v). The solvent and any remaining 5-[^{18}F]fluoro-1-pentyne were evaporated by a gentle stream of nitrogen. Following purification by semipreparative HPLC (system D; retention time 8.0 min) the product was obtained in a decay corrected yield of 8.7 ± 2.3% (n = 2) based on K^{18}F. Analytical HPLC (system A, PMT) retention time 14.7 min; purity >98%. Synthesis time: 44 min.

Animal Studies

General information. All animal experiments were conducted under a protocol approved by the University of California, Davis, Animal Use and Care Committee. Male athymic mice (nu/nu; Charles River Laboratories, Wilmington, MA) were inoculated s.c. on opposite flanks in the shoulder region with 3×10^6 DX3puro or DX3puroβ6 cells in 100 μL serum free DMEM (Mediatech, Manassas, VA). Cell lines were analyzed by flow cytometry before injection to confirm levels of integrin expression. Food and water were available *ad libitum*. Imaging was conducted 2-5 weeks after injection.

Radiotracers were formulated for injection as follows: After HPLC purification, the fractions containing the product were collected, diluted with water to a total volume of 20 mL and passed through a C18 Sep-Pak cartridge. The product, trapped on the cartridge, was washed with water (5 mL) and eluted with 1% acetic acid in ethanol (v/v). The solvent was removed by a stream of nitrogen and the radiotracer was reconstituted in saline/PBS, followed by pH-adjustment.

MicroPET imaging. The [^{18}F]-peptide (approx. 100-250 μCi) in isotonic solution (150-200 μL) was injected i.v. into the tail vein *via* a catheter in mice (n = 3/tracer) anesthetized with 3% isoflurane. The animals were placed in a head-first, prone position on the scanner bed and maintained under 1.5-2.0% isoflurane. Body temperature was measured by a rectal probe and maintained with a heating pad, supplemented by heating lamp. Dynamic 4 × 15 min scans were acquired starting 15 min after injection. Full body image reconstructions were obtained using a maximum *a posteriori* algorithm (MAP) using ASIPro software (Siemens Medical Solutions). For time-activity curves, regions of interest (ROI) were drawn using ASIPro. Decay-corrected radioactivity concentrations are expressed as percent of injected dose per volume of ROI (% ID/cm^3).

Biodistribution. The [^{18}F]-peptide (approx. 15-30 μCi) in isotonic solution (150-200 μL) was injected i.v. into the tail vein *via* a catheter in mice anesthetized with 3% isoflurane. Following a 1 h conscious uptake period, the mice were anesthetized (4% isoflurane), sacrificed and dissected (n = 3 per tracer). Organs were rapidly collected and radioactivity measured in a γ-counter. Calibrated, decay-corrected radioactivity concentrations are expressed as percent of injected dose per gram of tissue (% ID/g).[6]

Urine analysis. Aliquots of the urine samples collected 1 h after injection as part of the biodistribution study were analyzed by HPLC (system A).

Statistical analysis. Two-tailed Student's *t* tests were carried out to evaluate tumor uptake ratios for statistical significance.

Supporting information references

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Table S1: List of biodistribution data of tracers **1-3** obtained 1 h after injection (n = 3/tracer). Levels of radioactivity are expressed as % ID/g ± SD.

Organ	1	2	3
Heart	0.31 ± 0.13	0.62 ± 0.02	0.47 ± 0.17
Lung	1.39 ± 0.34	2.06 ± 0.24	1.83 ± 0.54
Spleen	0.07 ± 0.02	0.32 ± 0.04	0.13 ± 0.01
Brain	0.02 ± 0.02	0.28 ± 0.03	0.05 ± 0.04
Muscle	0.56 ± 0.21	0.82 ± 0.25	1.01 ± 0.39
Bone	0.29 ± 0.11	0.38 ± 0.16	0.39 ± 0.18
Kidneys	3.56 ± 1.38	4.87 ± 0.52	9.50 ± 1.54
Urine	1082 ± 279	311 ± 133	501 ± 332
Bladder	8.25 ± 5.44	5.30 ± 2.94	6.24 ± 0.50
Liver	0.63 ± 0.18	0.51 ± 0.04	1.12 ± 0.09
Gall Bladder	15.05 ± 17.19	2.13 ± 0.59	4.72 ± 2.50
Blood	0.27 ± 0.08	0.64 ± 0.04	0.43 ± 0.05
Pancreas	0.23 ± 0.12	0.39 ± 0.05	0.30 ± 0.02
Stomach	1.33 ± 0.22	3.61 ± 1.46	0.97 ± 0.20
DX3puroβ6	0.66 ± 0.09	1.18 ± 0.28	1.01 ± 0.09
DX3puro	0.21 ± 0.07	0.61 ± 0.03	0.31 ± 0.05

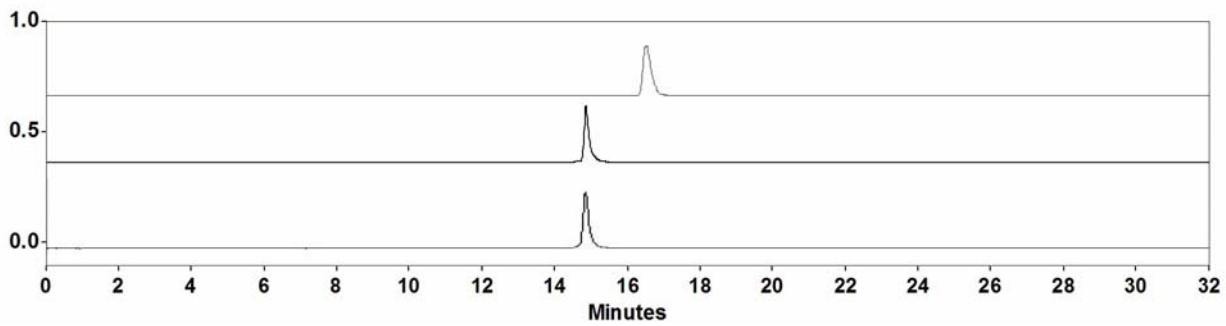

Figure S1: Radio-HPLC traces (system A) of [^{18}F]FBA-A20FDMV2 (**1**, top, retention time 16.6 min), [^{18}F]FPA-A20FDMV2 (**2**, middle, retention time 14.7 min), and [^{18}F]FC5-A20FDMV2 (**3**, bottom, retention time 14.7 min). By comparison, the HPLC retention times (system A) for H_2N-A20FMDV2 (precursor of **1** and **2**; cleaved and fully deprotected) and *N*-(3-azidopropionyl)-A20FMDV2 (precursor of **3**) were 13.5 min and 15.9 min, respectively.

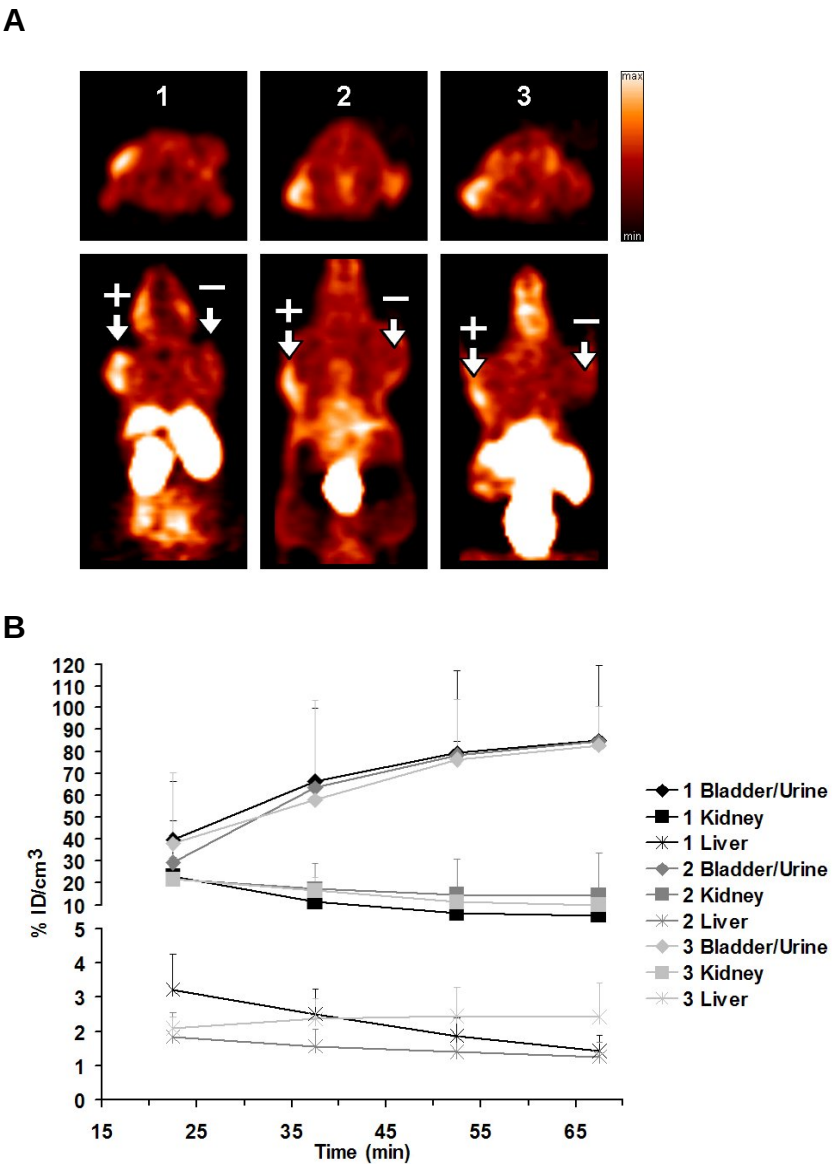

Figure S2: (A) Transaxial sections (Figure 1) of microPET scans obtained 60-75 min after injection of tracer, along with the corresponding coronal sections. The coronal sections, obtained through the plane of the tumors, show the high levels of activity in the excretory organs (chiefly kidneys and urinary bladder), as also seen in the biodistribution data. (B) Time-activity curves (lines; bars: SD) for major excretory organs derived from PET scans (n = 3/tracer), corroborating the renal pathway as major route of elimination from the body, as also seen in the biodistribution data. The PET data were acquired as dynamic 4 × 15 min scans, beginning 15 min after injection. The animals were continuously kept under anesthesia from time of injection until the end of the PET scan.

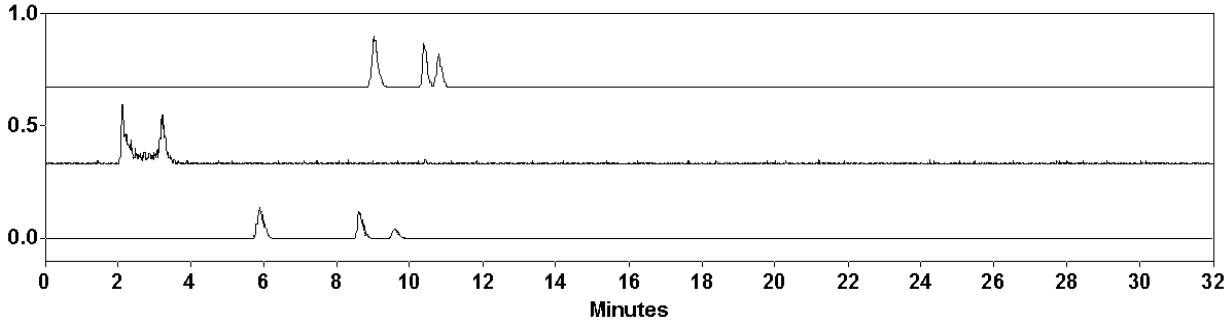

Figure S3: Radio-HPLC traces (system A) of urine samples collected one hour after injection of [^{18}F]FBA-A20FDMV2 (**1**, top, retention times 9.0, 10.4, and 10.8 min), [^{18}F]FPA-A20FDMV2 (**2**, middle, retention times 2.1 and 3.2 min), and [^{18}F]FC5-A20FDMV2 (**3**, bottom, retention times 5.9, 8.6, and 9.6 min).