**SUPPLEMENTARY MATERIALS**

**General Methods**

1-(1,1-Dimethylethyl) butanedioate (**1**), (*S*)-*N*-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)-2‐[[2‐[[(*tert*‐butoxy)carbonyl]methylamino]ethyl]methylamino]pyridine‐4‐ carboxamide (**3**), AV02070, Ga-AV02070, [68Ga]Ga-AV02070, PSMA-617, Ga-PSMA-617, and [68Ga]Ga-PSMA-617 were synthesized following literature procedure [1–4]. All other chemicals and solvents were obtained from commercial sources and used without further purification. Purification and quality control of DOTA-conjugated ligands and their natGa/68Ga-complexed analogs were performed on Agilent (Santa Clara, CA, USA) HPLC systems equipped with a model 1200 quaternary pump, a model 1200 UV absorbance detector (220 nm), and a Bioscan (Washington, DC, USA) NaI scintillation detector. The HPLC columns used were a semi-preparative column (Luna C18, 5 µm, 250 × 10 mm) and an analytical column (Luna C18, 5 µm, 250 × 4.6 mm) purchased from Phenomenex (Torrance, CA, USA). The collected HPLC eluates containing the desired products were lyophilized using a Labconco (Kansas City, MO, USA) FreeZone 4.5 Plus freeze-drier. 1H NMR acquisitions were taken by a Bruker (Billerica, MA, USA) Avance 300 MHz NMR spectrometer. MS analyses were conducted using the Waters (Milford, MA, USA) Acquity QDa mass spectrometer with the equipped 2489 UV/Vis detector and e2695 Separations module. C18 Sep-Pak cartridges (1 cm3, 50 mg) were purchased from Waters (Milford, MA, USA). 68Ga was eluted from an ITM Medical Isotopes GmbH (Munich, Germany) generator, and purified according to the previously published procedures using a DGA resin column from Eichrom Technologies LLC (Lisle, IL, USA) [5]. The radioactivity of 68Ga-labeled tracers was measured using a Capintec (Ramsey, NJ, USA) CRC®-25R/W dose calibrator and the radioactivity of mouse tissues collected from biodistribution studies were counted using a Perkin Elmer (Waltham, MA, USA) Wizard2 2480 automatic gamma counter.

**Synthesis of the FAP-targeted Ligand**

Synthesis of *tert*-butyl (2,3,5,6-tetrafluorophenyl) succinate (**2**)



A solution of 1-(1,1-dimethylethyl) butanedioate (**1**, 1.87 g, 10.7 mmol) in dichloromethane (DCM) were added 2,3,5,6-tetrafluorophenol (TFP, 2.32 g, 13.9 mmol) and *N*-ethyl-*N’*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC.HCl, 2.26 g, 11.8 mmol) and stirred at room temperature for 20 h. The solution was evaporated under reduced pressure and the residue was dissolved in ethyl acetate (100 mL), washed with H2O (100 mL × 2). The organic phase was collected, dried over anhydrous MgSO4 and evaporated under reduced pressure to yield **2** (7.5 mmol, 71% yield) as a light yellow oil which was used for the next step without further purification. MS (ESI) calculated for C14H14F4O4 322.1, found [M+Na]+ 345.1. 1H NMR (300 MHz, DMSO-d6) δ 7.94 (tt, *J* = 11.0, 7.4 Hz, 1H), 3.02 – 2.92 (m, 2H), 2.67 – 2.57 (m, 2H), 1.39 (s, 9H).

Synthesis of *tert*-butyl (*S*)-4-((2-((4-((2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)pyridin-2-yl)(methyl)amino)ethyl)(methyl)amino)-4-oxobutanoate (**4**)



A solution of (*S*)-*N*-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)-2‐[[2‐[[(tert‐butoxy)-carbonyl]methylamino]ethyl]methylamino]pyridine‐4‐carboxamide (**3**) (1.49 g, 3.1 mmol) in DCM (20 mL) was added TFA (20 mL) and stirred at room temperature for 2 h. After evaporation, the residue was dissolved in CH3CN (50 mL), and compound **2** (2.44 g, 7.5 mmol) and triethylamine (TEA, 2.8 mL, 20 mmol) were added. After stirring at 50 °C for 19 h, the volatile solvent was evaporated under reduced procedure, and the residue was purified with flash column chromatography eluted with ethyl acetate (0.6 L), followed by 19:1 (v/v) ethyl acetate/methanol (1.8 L) and 9:1 (v/v) ethyl acetate/methanol (1 L), consecutively. The product eluate fractions were combined and evaporated under reduced pressure to yield **4** as a yellow powder (750 mg, 45% yield). MS (ESI) calculated for C25H34F2N6O5 536.3, found [M+H]+ 537.3. 1H NMR (300 MHz, DMSO-d6) δ 7.39 (d, *J* = 8.1 Hz, 1H), 7.15 (s, 1H), 7.06 (t, *J* = 6.0 Hz, 2H), 6.88 (t, *J* = 6.4 Hz, 1H), 5.07 (dd, *J* = 9.1, 3.0 Hz, 2H), 4.45 (dd, *J* = 9.6, 4.2 Hz, 1H), 4.26 – 3.88 (m, 5H), 3.77 (d, *J* = 6.1 Hz, 4H), 3.70 (dd, *J* = 17.3, 5.6 Hz, 1H), 2.90 (dt, *J* = 14.3, 9.1 Hz, 1H), 2.83 – 2.67 (m, 3H), 1.38 (s, 9H).

Synthesis of (*S*)-4-((2-((4-((2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)pyridin-2-yl)(methyl)amino)ethyl)(methyl)amino)-4-oxobutanoic acid (**5**)



Compound **4** (737 mg, 1.4 mmol) was dissolved in DCM (10 mL), and triethylsilane (481 mg, 4.1 mmol) and TFA (10 mL) were added. After stirring for 2 h, the resulting solution was evaporated and the residue was dissolved in diethyl ether (250 mL). The diethyl ether solution was stirred for 2 h and the resulting yellow powder was filtered and dried under reduced pressure to yield 670 mg of **5** (100% yield). MS (ESI) calculated for C21H26F2N6O5 480.2, found [M+H]+ 481.2. 1H NMR (300 MHz, DMSO-d6) δ 9.03 (dt, *J* = 27.2, 5.8 Hz, 1H), 8.27 – 8.12 (m, 3H), 7.25 (s, 1H), 7.11 – 6.97 (m, 2H), 5.10 (dd, *J* = 9.1, 2.9 Hz, 1H), 4.38 – 4.19 (m, 1H), 4.18 – 4.05 (m, 1H), 3.76 (s, 1H), 3.09 (s, 2H), 2.98 (s, 3H), 2.85 (s, 2H), 2.80 (s, 1H), 2.36 (dd, *J* = 8.7, 4.8 Hz, 3H).

**Synthesis of DOTA-conjugated Peptides**

AV01084 and AV01088 were synthesized on solid phase using Fmoc peptide chemistry. Fmoc-Lys(ivDde)-Wang resin (0.05 mmol, 0.081 g) was treated with 20% piperidine in DMF to remove the Fmoc protecting group. The isocyanate derivative of Glu(tBu)-OtBu (3 eq.) was synthesized following previously published procedures [6] and was added to the lysine-immobilized resin, with *N*,*N*-diisopropylethylamine (DIEA, 2 eq.) as the base and reacted for 16 h to form the Lys-urea-Glu moiety. The ivDde-protecting group was then removed with 2% hydrazine in DMF (5 × 5 min). Fmoc-2-Nal-OH (4 eq.), Fmoc-tranexamic acid (3 eq.), Fmoc-Gly-OH (4 eq.), and Fmoc-Lys(ivDde)-OH (4 eq.) were pre-activated with HATU (4 eq.) and DIEA (7 eq.) before being sequentially coupled to the resin. Following the removal of Fmoc protecting group, the FAP-targeted ligand (compound **5)** or DOTA-tris(*t*-butyl)ester (3 eq.) was pre-activated with HATU (3 eq.) and DIEA (7 eq.) and coupled to the α-amino group of lysine for AV01084 or AV01088, respectively. Finally, the ivDde-protecting group on lysine was removed and coupled with DOTA-tris(*t*-butyl)ester or compound **5** activated with HATU (3 eq.) and DIEA (7 eq.) for AV01084 and AV01088, respectively.

The peptides were deprotected and simultaneously cleaved from the resin with a mixture of trifluoroacetic acid (TFA, 95%), triisopropylsilane (TIS 2.5%) and water (2.5%) for 4 h at room temperature. The cleaved peptides were filtrated and then precipitated by the addition of cold diethyl ether. The crude peptides were collected by centrifugation and purified with HPLC (semi-preparative column; flow rate: 4.5 mL/min). The eluates containing the desired peptides were collected and lyophilized. The HPLC conditions, retention times, isolated yields and MS confirmations of DOTA-conjugated peptides are provided in the Table S1.

**Table S1:** HPLC purification conditions and MS characterizations of DOTA-conjugated precursors.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Compoundname | HPLC conditions | Retention time (min) | Yield (%) | Calculated mass (m/z) | Found (m/z) |
| AV01084 | 26% CH3CN and 0.1% TFA in H2O | 14.1 | 12 | [M+2H]2+845.4 | [M+2H]2+845.8 |
| AV01088 | 26% CH3CN and 0.1% TFA in H2O | 13.7 | 7.2 | [M+2H]2+845.4 | [M+2H]2+845.8 |

**Synthesis of Nonradioactive Ga-complexed Standards**

The nonradioactive Ga-complexed standards were prepared by reacting the DOTA-conjugated precursors with GaCl3 (5 eq.) in NaOAc buffer (0.1 M, 500 µL, pH 4.2 – 4.5) at 80 °C for 15 min. The reaction mixture was then purified via HPLC (semi-preparative column, flow rate: 4.5 mL/min). The HPLC eluates containing the desired products were collected and lyophilized. The HPLC conditions, retention times, isolated yields and MS confirmations of these nonradioactive Ga-complexed standards are provided in Table S2.

**Table S2:** HPLC purification conditions and MS characterizations of nonradioactive Ga-complexed standards.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Compoundname | HPLC conditions | Retention time (min) | Yield(%) | Calculated mass (m/z) | Found (m/z) |
| Ga-AV01084 | 27% CH3CN and 0.1% TFA in H2O | 10.8 | 60 | [M+2H]2+878.9 | [M+2H]2+879.8 |
| Ga-AV01088 | 26% CH3CN and 0.1% TFA in H2O | 14.1 | 21 | [M+2H]2+878.9 | [M+2H]2+879.8 |

**Synthesis of 68Ga-labeled Tracers**

The radiolabeling experiments were performed according to previously published procedures [3-4]. Purified 68GaCl3 in 0.5 mL water was added to a 4-mL glass vial preloaded with 0.7 mL of HEPES buffer (2 M, pH 5.0) and 10 μL precursor solution (1 mM). The radiolabeling reaction was carried out under microwave heating for 1 min, followed by purification using the semi-preparative HPLC column. The eluate fraction containing the radiolabeled product was collected, diluted with water (50 mL), and passed through a C18 Sep-Pak cartridge that was pre-washed with ethanol (10 mL) and water (10 mL). The 68Ga-labeled product was eluted off the cartridge with ethanol (0.4 mL), and diluted with PBS for imaging and biodistribution studies. Quality control was performed using the analytical column. The HPLC conditions and retention times are provided in Table S3. The tracers were obtained in 33 - 64% decay-corrected radiochemical yields with ≥ 44 GBq/µmol molar activity and > 95% radiochemical purity.

**Table S3:** HPLC conditions for the purification and quality control of 68Ga-labeled tracers.

|  |  |  |
| --- | --- | --- |
| Tracer name | HPLC conditions | Retention time (min) |
| [68Ga]Ga-AV01084 | Semi-Prep | 28% CH3CN and 0.1% TFA in H2O; flow rate 4.5 mL/min | 16.8 |
| QC | 31.5% CH3CN and 0.1% TFA in H2O; flow rate 2.0 mL/min | 6.3 |
| [68Ga]Ga-AV01088 | Semi-Prep | 28% CH3CN and 0.1% TFA in H2O; flow rate 4.5 mL/min | 16.7 |
| QC | 31.5% CH3CN and 0.1% TFA in H2O; flow rate 2.0 mL/min | 6.3 |

**Table S4:** Biodistribution (mean ± SD, n = 4) and uptake ratios of 68Ga-labeled PSMA/FAP bispecific tracers, PSMA-617, and AV02070 in LNCaP tumor-bearing mice. The mice in the blocked group were co-injected with 2-PMPA (500 µg/mouse). The biodistribution data of [68Ga]Ga-PSMA-617 have been reported previously by our group [2], and are included here for comparison.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Tissue (%ID/g) | [68Ga]Ga-AV01084 |   | [68Ga]Ga-AV01088 |   | [68Ga]Ga-PSMA-617 |  | [68Ga]Ga-AV02070 |
| 1 h |   | 1 h |   | 1 h blocked |   | 1 h |  | 1 h |
| Blood | 2.26 ± 0.49 |   | 2.18 ± 0.22 |   | 1.45 ± 0.53 |   | 0.63 ± 0.13 |   | 0.75 ± 0.21 |
| Fat | 1.44 ± 0.15 |  | 0.70 ± 0.36 |  | 0.29 ± 0.20 |  | 0.25 ± 0.13 |  | 0.17 ± 0.16 |
| Testes | 0.66 ± 0.06 |  | 0.40 ± 0.13 |  | 0.34 ± 0.10 |  | 0.26 ± 0.06 |  | 0.38 ± 0.40 |
| Small intestine | 0.63 ± 0.15 |  | 0.52 ± 0.06 |  | 0.44 ± 0.21 |  | 0.31 ± 0.15 |  | 0.54 ± 0.17 |
| Stomach | 0.23 ± 0.06 |  | 0.20 ± 0.02 |  | 0.18 ± 0.01 |  | 0.08 ± 0.01 |  | 0.11 ± 0.05 |
| Spleen | 4.35 ± 1.25 |  | 1.14 ± 0.16 |  | 0.40 ± 0.09 |  | 1.18 ± 0.37 |  | 0.25 ± 0.07 |
| Liver | 0.49 ± 0.01 |  | 0.49 ± 0.08 |  | 0.40 ± 0.13 |  | 0.62 ± 0.15 |  | 0.47 ± 0.08 |
| Pancreas | 0.99 ± 0.07 |  | 0.70 ± 0.04 |  | 0.48 ± 0.20 |  | 0.19 ± 0.01 |  | 0.31 ± 0.21 |
| Adrenal glands | 8.22 ± 0.90 |  | 2.10 ± 0.48 |  | 1.19 ± 0.40 |  | 1.20 ± 0.57 |  | 0.36 ± 0.17 |
| Kidney | 84.3 ± 8.82 |  | 28.6 ± 10.4 |  | 2.91 ± 0.90 |  | 29.2 ± 5.13 |  | 2.57 ± 0.58 |
| Lungs | 2.09 ± 1.39 |  | 0.94 ± 0.64 |  | 1.03 ± 0.42 |  | 1.03 ± 0.32 |  | 0.63 ± 0.22 |
| Heart | 0.85 ± 0.08 |  | 0.57 ± 0.03 |  | 0.40 ± 0.14 |  | 0.23 ± 0.04 |  | 0.22 ± 0.05 |
| LNCaP tumor | 9.05 ± 1.54 |   | 8.85 ± 1.25 |   | 2.95 ± 1.13 |   | 16.7 ± 2.30 |   | 0.74 ± 0.21 |
| Muscle | 1.00 ± 0.35 |  | 0.77 ± 0.27 |  | 0.39 ± 0.08 |  | 0.12 ± 0.02 |  | 0.31 ± 0.14 |
| Bone | 2.47 ± 0.22 |  | 2.47 ± 0.50 |  | 1.97 ± 1.15 |  | 0.23 ± 0.05 |  | 0.56 ± 0.28 |
| Brain | 0.11 ± 0.05 |  | 0.06 ± 0.02 |  | 0.03 ± 0.01 |  | 0.02 ± 0.00 |  | 0.03 ± 0.01 |
| Tumor/bone | 3.70 ± 0.83 |  | 3.75 ± 1.28 |  | 1.78 ± 0.98 |  | 96.5 ± 47.6 |  | 1.63 ± 0.95 |
| Tumor/muscle | 9.57 ± 2.73 |  | 12.7 ± 5.32 |  | 7.25 ± 1.77 |  | 133 ± 19.5 |  | 2.76 ± 1.44 |
| Tumor/blood | 4.09 ± 0.77 |  | 4.10 ± 0.88 |  | 2.06 ± 0.34 |  | 27.7 ± 6.28 |  | 0.99 ± 0.12 |
| Tumor/kidney | 0.10 ± 0.02 |  | 0.34 ± 0.13 |  | 1.00 ± 0.12 |  | 0.63 ± 0.09 |  | 0.29 ± 0.08 |

**Table S5:** Biodistribution (mean ± SD, n = 4) and uptake ratios of 68Ga-labeled PSMA/FAP bispecific tracers in HEK293T:hFAP tumor-bearing mice. The mice in the blocked group were co-injected with FAPI-04 (250 µg/mouse). The biodistribution data of [68Ga]Ga-AV02070 have been reported previously by our group [3], and are included here for comparison.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Tissue (%ID/g) | [68Ga]Ga-AV01084 |  | [68Ga]Ga-AV01088 |  | [68Ga]Ga-PSMA-617 |  | [68Ga]Ga-AV02070 |
|  | 1 h |  | 1 h |  | 1 h blocked |  | 1 h |  | 1 h |
| Blood | 1.53 ± 0.03 |  | 0.79 ± 0.43 |  | 0.68 ± 0.25 |  | 0.28 ± 0.02 |  | 0.36 ± 0.05 |
| Fat | 0.78 ± 0.16 |  | 0.20 ± 0.05 |  | 0.19 ± 0.09 |  | 0.09 ± 0.04 |  | 0.44 ± 0.81 |
| Testes | 0.54 ± 0.05 |  | 0.22 ± 0.05 |  | 0.33 ± 0.15 |  | 0.15 ± 0.03 |  | 0.17 ± 0.07 |
| Small Intestine | 0.45 ± 0.06 |  | 0.20 ± 0.02 |  | 0.26 ± 0.07 |  | 0.14 ± 0.02 |  | 0.42 ± 0.13 |
| Stomach | 0.11 ± 0.02 |  | 0.08 ± 0.01 |  | 0.08 ± 0.03 |  | 0.07 ± 0.08 |  | 0.08 ± 0.04 |
| Spleen | 3.89 ± 1.38 |  | 0.81 ± 0.15 |  | 0.63 ± 0.28 |  | 0.55 ± 0.13 |  | 0.25 ± 0.12 |
| Liver | 0.46 ± 0.17 |  | 0.17 ± 0.01 |  | 0.26 ± 0.09 |  | 0.11 ± 0.01 |  | 0.39 ± 0.05 |
| Pancreas | 0.71 ± 0.05 |  | 0.31 ± 0.04 |  | 0.21 ± 0.07 |  | 0.11 ± 0.03 |  | 0.34 ± 0.45 |
| Adrenal glands | 3.10 ± 0.62 |  | 0.96 ± 0.53 |  | 0.44 ± 0.06 |  | 0.32 ± 0.22 |  | 0.22 ± 0.15 |
| Kidney | 56.4 ± 16.1 |  | 10.5 ± 4.20 |  | 8.18 ± 3.98 |  | 12.4 ± 3.24 |  | 1.85 ± 0.21 |
| Lungs | 1.60 ± 0.10 |  | 0.55 ± 0.05 |  | 0.76 ± 0.26 |  | 0.34 ± 0.05 |  | 0.34 ± 0.06 |
| Heart | 0.56 ± 0.03 |  | 0.26 ± 0.04 |  | 0.18 ± 0.04 |  | 0.10 ± 0.00 |  | 0.12 ± 0.03 |
| HEK293T:hFAP tumor | 1.90 ± 0.41 |  | 1.20 ± 0.25 |  | 0.51 ± 0.14 |  | 0.26 ± 0.01 |  | 7.93 ± 1.88 |
| Muscle | 0.85 ± 0.30 |  | 0.41 ± 0.03 |  | 0.16 ± 0.12 |  | 0.08 ± 0.01 |  | 0.19 ± 0.10 |
| Bone | 1.44 ± 0.21 |  | 0.88 ± 0.22 |  | 0.20 ± 0.06 |  | 0.09 ± 0.02 |  | 0.23 ± 0.06 |
| Brain | 0.05 ± 0.00 |  | 0.40 ± 0.74 |  | 0.02 ± 0.01 |  | 0.01 ± 0.00 |  | 0.02 ± 0.02 |
| Tumor/bone | 1.02 ± 0.10 |  | 1.08 ± 0.31 |  | 2.58 ± 0.68 |  | 2.95 ± 0.55 |  | 34.3 ± 7.35 |
| Tumor/muscle | 1.88 ± 0.31 |  | 2.44 ± 1.08 |  | 4.07 ± 2.13 |  | 3.01 ± 0.66 |  | 45.7 ± 9.88 |
| Tumor/blood | 1.06 ± 0.21 |  | 2.26 ± 2.25 |  | 0.78 ± 0.09 |  | 0.94 ± 0.13 |  | 22.9 ± 10.1 |
| Tumor/kidney | 0.03 ± 0.02 |  | 0.15 ± 0.14 |  | 0.06 ± 0.01 |  | 0.02 ± 0.00 |  | 4.34 ± 1.36 |

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