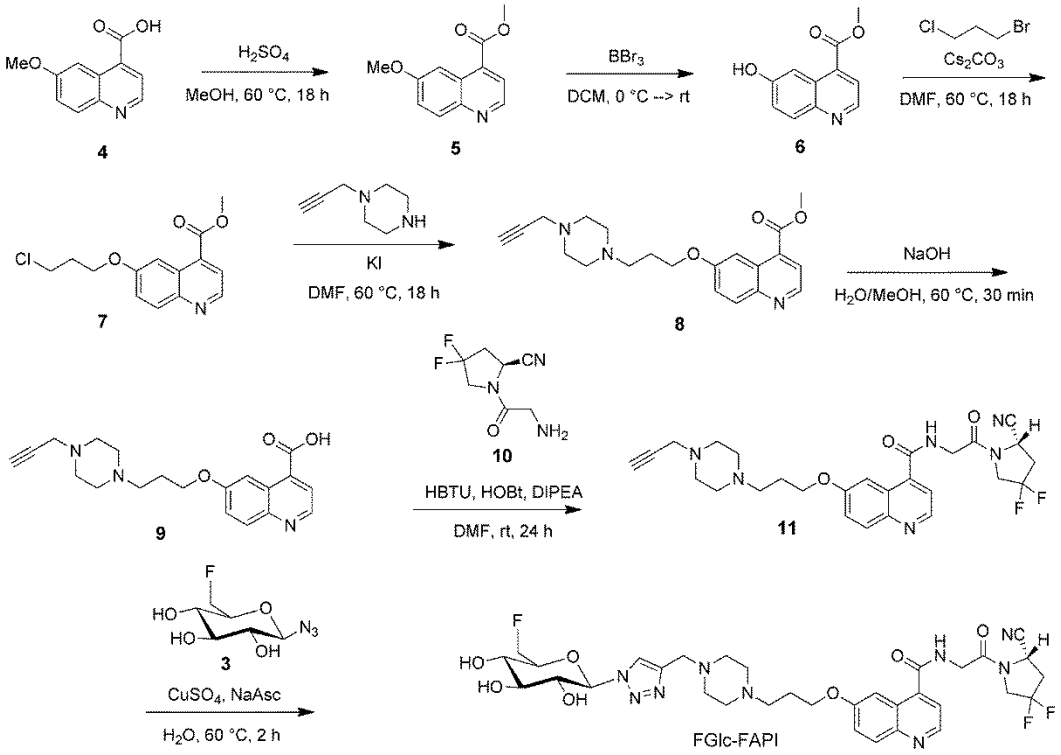
Compound Syntheses

General

All chemicals were purchased in the highest available quality and used without further purification. NMR spectra were acquired on a Bruker Avance Nanobay V3-I 400 MHz or a Bruker Avance III HD 600 MHz spectrometer. ESI mass spectra were recorded on Bruker amaZon SL or Bruker ESI timsTOF mass spectrometers. The FPLC system (FlashPure, Büchi Labortechnik AG) was equipped with a UV-detector (254 nm) and C-18 columns.

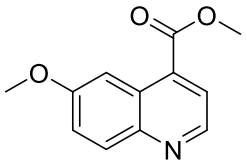
Synthesis of FGlc-FAPI

Precursor **1** as well as compounds **2** and **3** were synthesized according to Maschauer et al (*27*). Compound **10** was synthesized as described by Jansen et al (*20*). The analytical data was in agreement with the literature. For the synthesis of compounds **4** to **11** the route was adapted from Lindner et al (*22*) and is described below (Supplemental Figure 1). Since the reaction was up-scaled compared to the previous procedure, the carboxylic acid moiety was methylated to provide simplified workup without the use of a preparative HPLC system. Finally, FGlc-FAPI was obtained by copper-catalyzed alkyne azide cycloaddition with compounds **3** and **11** according to Maschauer et al (*27*).



Supplemental Figure 1. Synthesis route of alkyne **11** and reference substance FGlc-FAPI.

Methyl 6-methoxyquinoline-4-carboxylate (5)

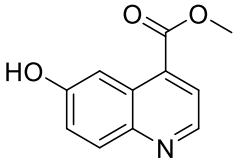


6-Methoxyquinoline-4-methyl carboxylic acid **4** (0.291 g, 1.43 mmol) was suspended in MeOH (2 mL). H_2SO_4 (0.382 mL, 7.16 mmol) was added to the reaction while cooling to 0°C. The reaction was stirred at 60 °C for 18 hours. The solvent was evaporated under reduced pressure. Saturated sodium bicarbonate solution (10 mL) was added and extracted three times with dichloromethane. The combined organic phases were washed three times with brine and dried over sodium sulfate. The solvent was evaporated and the residue was dried under vacuum. The product **5** was obtained as light brown solid (0.303 g, 1.40 mmol, 97%).

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.89 (d, *J* = 4.4 Hz, 1H), 8.08 (d, *J* = 2.8 Hz, 1H), 8.05 (d, *J* = 9.2 Hz, 1H), 7.94 (d, *J* = 4.4 Hz, 1H), 7.52 (dd, *J* = 9.2, 2.8 Hz, 1H), 3.99 (s, 3H), 3.92 (s, 3H).

LC-MS (ESI): m/z 217.77 $[M+H]^+$, calculated: 218.07 $[M+H]^+$

Methyl 6-hydroxyquinoline-4-carboxylate (6)

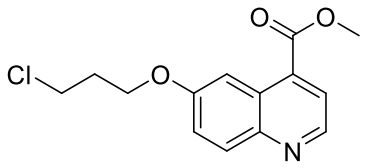


Methyl 6-methoxyquinoline-4-carboxylate **5** (0.155 g, 0.71 mmol) was dissolved in dry dichloromethane (4 mL) under argon atmosphere. BBr_3 (1 M solution, 1.43 mL, 1.43 mmol) was added dropwise to the reaction while cooling to 0 °C. The reaction was allowed to warm to room temperature and was stirred overnight. Saturated sodium bicarbonate solution (10 mL) was added and the solution was extracted with ethyl acetate three times. The combined organic phases were dried over sodium sulfate. The solvent was evaporated and the residue was dried under vacuum. The product **6** was obtained as yellow solid (0.120 g, 0.59 mmol, 82%).

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.89 (d, *J* = 4.6 Hz, 1H), 8.03 (d, *J* = 9.1 Hz, 1H), 7.99 (d, *J* = 2.6 Hz, 1H), 7.96 (d, *J* = 4.6 Hz, 1H), 7.48 (dd, *J* = 9.1, 2.7 Hz, 1H), 3.99 (s, 3H).

LC-MS (ESI): m/z 203.72 $[M+H]^+$, calculated: 204.06 $[M+H]^+$

Methyl 6-(4-chloropropoxy)quinoline-4-carboxylate (7)

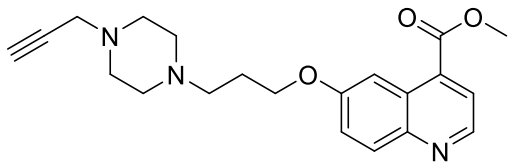


Methyl 6-hydroxyquinoline-4-carboxylate **6** (0.105 g, 0.56 mmol) and Cs_2CO_3 (0.906 g, 2.78 mmol) were dissolved in DMF (3 mL) and 1-bromo-3-chloropropane (0.192 mL, 1.95 mmol) was added. The reaction was stirred at 60 °C overnight. Then the solvent was removed under reduced pressure. The residue was partitioned between ethyl acetate and brine and the aqueous phase was extracted with ethyl acetate two times. The combined organic phases were dried over sodium sulfate and the solvent was evaporated under reduced pressure. Finally, the crude product was purified by column chromatography (n-hexane/ethyl acetate, 2:1) using silica. The product **7** was obtained as yellow oil (0.055 g, 0.20 mmol, 35%).

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.90 (dd, *J* = 4.4, 3.2 Hz, 1H), 8.05 (d, *J* = 9.2 Hz, 1H), 8.01 (d, *J* = 4.4 Hz, 1H), 7.95 (d, *J* = 4.4 Hz, 1H), 7.57 – 7.51 (m, 1H), 4.26 (t, *J* = 6.0 Hz, 2H), 3.99 (s, 3H), 3.91 – 3.81 (m, 2H), 2.32 – 2.23 (m, 2H).

LC-MS (ESI): m/z 279.82 $[M+H]^+$, calculated: 280.07 $[M+H]^+$

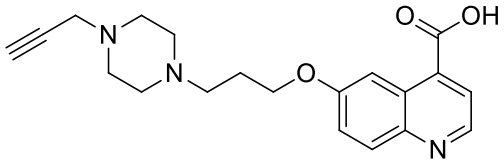
Methyl 6-(3-(1-piperazinyl-4-prop-2-ynyl)propoxy)quinoline-4-carboxylate (8)



Methyl 6-(4-chloropropoxy)quinoline-4-carboxylate **7** (0.050 g, 0.18 mmol) and 1-prop-2-ynyl-piperazine (0.077 g, 0.62 mmol) were dissolved in DMF (2.5 mL) together with potassium iodide (0.206 g, 1.24 mmol). The reaction was stirred under argon atmosphere at 60 °C overnight. The solvent was evaporated under reduced pressure. Saturated sodium bicarbonate solution (10 mL) was added and extracted three times with ethyl acetate. The combined organic phases were dried over sodium sulfate and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography (dichloromethane/methanol, 15:1 + 1% ammonia solution) using silica. The product **8** was obtained as yellow oil (0.033 g, 0.09 mmol, 50%).

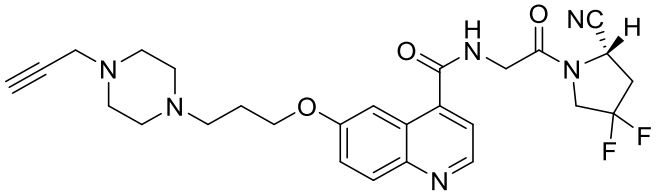
^{1}H NMR (600 MHz, DMSO-d_6) δ 8.89 (d, *J* = 4.4 Hz, 1H), 8.08 (d, *J* = 2.8 Hz, 1H), 8.04 (d, *J* = 9.2 Hz, 1H), 7.94 (d, *J* = 4.4 Hz, 1H), 7.52 (dd, *J* = 9.2, 2.8 Hz, 1H), 4.17 (t, *J* = 6.4 Hz, 2H), 3.99 (s, 3H), 3.29 (m, 2H), 3.25 (d, *J* = 2.4 Hz, 2H), 3.13 (t, *J* = 2.4 Hz, 1H), 2.50 – 2.38 (m, 8H), 2.00 – 1.92 (m, 2H) ppm.
LC-MS (ESI): m/z 368.20 $[M+H]^+$, calculated: 368.19 $[M+H]^+$

6-(3-(1-Piperazinyl-4-prop-2-ynyl)propoxy)quinoline-4-carboxylic acid (9)



Methyl 6-(3-(1-piperazinyl-4-prop-2-ynyl)propoxy)quinoline-4-carboxylate **8** (0.033 g, 0.09 mmol) was dissolved in a mixture of methanol (1.5 mL) and water (0.5 mL) and sodium hydroxide solution (2 M, 0.2 mL) was added. The reaction was stirred at 60 °C for 30 min. The reaction was neutralized with HCl and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography using a FPLC system with a C18-column (acetonitrile/H_2O 1:3 + 0.1% TFA). The product **9** was obtained as a light yellow solid which could be a hydrochloride or sodium salt (0.049 g, 0.13 mmol, quantitative).
^{1}H NMR (600 MHz, D_2O) δ 8.82 (d, *J* = 5.1 Hz, 1H), 8.06 (d, *J* = 9.3 Hz, 1H), 7.73 (d, *J* = 5.1 Hz, 1H), 7.63 (dd, *J* = 9.3, 2.7 Hz, 1H), 7.57 (d, *J* = 2.7 Hz, 1H), 4.33 (t, *J* = 5.8 Hz, 2H), 3.90 – 2.52 (m, 8H), 3.46 (d, *J* = 2.4 Hz, 2H), 3.44 (t, *J* = 7.9 Hz, 2H), 2.77 (t, *J* = 2.4 Hz, 1H), 2.34 (p, *J* = 7.8, 5.9 Hz, 2H) ppm.
LC-MS (ESI): m/z 354.13 $[M+H]^+$, calculated: 354.17 $[M+H]^+$

(S)-N-(2-(2-Cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)-6-(3-(1-piperazinyl-4-prop-2-ynyl)propoxy)quinoline-4-carboxamide (11)



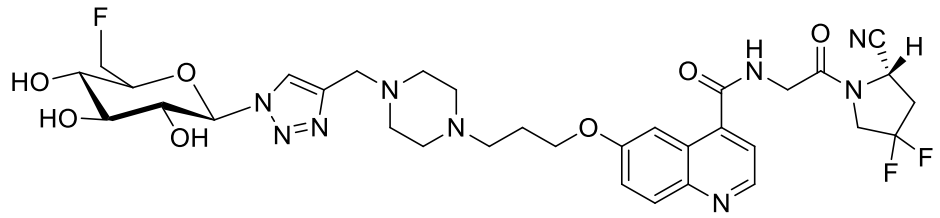
6-(3-(1-Piperazinyl-4-prop-2-ynyl)propoxy)quinoline-4-carboxylic acid **9** (0.065 g, 184 µmol) and HOBt (0.037 g, 276 µmol) were dissolved in DMF (1.2 mL). Then DIPEA (78 µL, 460 µmol) and HBTU (0.084 g, 221 µmol) were added to the reaction and stirred for 10 min before addition of compound **10** (0.083 g,

230 µmol) dissolved in DMF (0.6 mL) and DIPEA (78 µL, 460 µmol). The reaction was stirred for 24 hours at room temperature before quenching it with H_2O. The solvent was evaporated and the residue purified by column chromatography (dichloromethane:methanol 9:1) using silica. This gave a light colored solid which was again purified using a FPLC system with a C18-column (acetonitrile/H_2O 1:9 + 0.1% TFA). The product **11** was obtained as slightly yellow oil (0.094 g, 179 µmol, 97%).

^{1}H NMR (600 MHz, DMSO-d_6) δ 9.13 (t, *J* = 6.0 Hz, 1H), 8.87 (d, *J* = 4.4 Hz, 1H), 8.04 (d, J = 9.2 Hz, 1H), 7.92 (d, *J* = 2.8 Hz, 1H), 7.59 (d, *J* = 4.4 Hz, 1H), 7.50 (dd, *J* = 9.2, 2.8 Hz, 1H), 5.15 (dd, *J* = 9.4, 2.9 Hz, 2H), 4.38 – 4.20 (m, 2H), 4.30 – 4.11 (m, 2H), 4.25 (t, *J* = 6.0 Hz, 2H), 3.50 (s, 2H), 3.36 (s, 1H), 3.30 (t, *J* = 8.1 Hz, 2H), 3.18 –2.80 (m, 8H), 2.22 (p, *J* = 14.9, 7.0 Hz, 2H).

LC-MS (ESI): m/z 525.18 $[M+H]^+$, calculated: 525.23 $[M+H]^+$

_N_-(2-((_R_)-2-Cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)-6-(3-(4-((1-((2_R_,3_R_,4_S_,5_S_,6_S_)-6-(fluoromethyl)-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl)-1H-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)propoxy)quinoline-4-carboxamide (FGlc-FAPI)



Compound **11** (3.15 mg, 6.01 µmol) was dissolved in H_2O (300 µL) and 6-deoxy-6-fluoro-β-D-glucopyranosyl-1-azide **3** (1.57 mg, 7.58 µmol) dissolved in H_2O (150 µL) was added. A $CuSO_4$ pentahydrate solution (75 µL, 0.2M) was added and the reaction was started by addition of a sodium ascorbate solution (75 µL, 0.6M) and heating to 60 °C. After stirring for 2 hours the reaction was diluted with H_2O and purified by HPLC (method 1) followed by lyophilization to obtain **FGlc-FAPI** as a pale white solid (2.90 mg, 3.96 µmol, 66%).

^{1}H NMR (600 MHz, D_2O) δ 8.87 (d, J = 4.8 Hz, 1H), 8.27 (s, 1H), 8.11 (d, J = 9.3 Hz, 1H), 7.79 (d, J = 4.8 Hz, 1H), 7.70 (d, J = 2.7 Hz, 1H), 7.62 (dd, J = 9.3, 2.7 Hz, 1H), 5.82 (d, J = 9.3 Hz, 1H), 5.16 (dd, J = 9.0, 3.9 Hz, 1H), 4.68 (d, J = 3.7 Hz, 1H), 4.43 – 4.35 (d, 2H), 4.33 (t, J = 6.2, 2H), 4.35 – 4.13 (ddt, J = 20.3, 10.5 Hz, 1H), 4.06 (t, J = 9.2 Hz, 1H), 3.98 – 3.88 (m, 1H), 3.94 (s, 2H), 3.75 (t, J = 9.2 Hz, 1H), 3.70 (t, J = 9.6 Hz, 1H), 3.41 (t, J = 7.8 Hz, 2H), 3.20 – 2.80 (m, 8H), 3.05 – 2.89 (m, 2H), 2.32 (p, J = 6.1 Hz, 2H).

LC-MS (ESI): m/z 732.23 $[M+H]^+$, calculated: 732.30 $[M+H]^+$

HRMS (ESI): m/z 732.3076 $[M+H]^+$, calculated: 732.3071 $[M+H]^+$

Radiochemistry

General

The HPLC-System (Series 1100, Agilent) was equipped with a VWD UV-Lamp (detection at 214 or 254 nm) and was for radio-HPLC additionally connected to a radio-detector (500 TR Series, Packard). Computer analysis of the HPLC data was performed using FLO-One software (Canberra Packard). The conditions used for preparative and analytical HPLC are described in methods 1-5.

Method 1: Kromasil C8, 125 × 8 mm, 0-30% acetonitrile (0.1% trifluoroacetic acid (TFA)) in water (0.1% TFA) in a linear gradient over 20 min, 4 mL/min.

Method 2: Chromolith RP-18e, 100 × 4.6 mm, 0-30% acetonitrile (0.1% TFA) in water (0.1% TFA) in a linear gradient over 5 min, 4 mL/min.

Method 3: Kromasil C8, 250 × 4.6 mm, 0-30% acetonitrile (0.1% TFA) in water (0.1% TFA) in a linear gradient over 20 min, 1.5 mL/min.

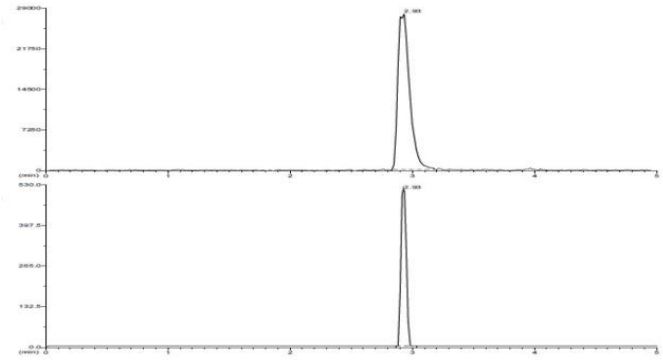
Method 4: Chromolith RP-18e, 100 × 4.6 mm, 10-50% acetonitrile (0.1% TFA) in water (0.1% TFA) in a linear gradient over 5 min, 4 mL/min.

Method 5: Chromolith RP-18e, 100 × 4.6 mm, 0-70% acetonitrile (0.1% TFA) in water (0.1% TFA) in a linear gradient over 5 min, 4 mL/min.

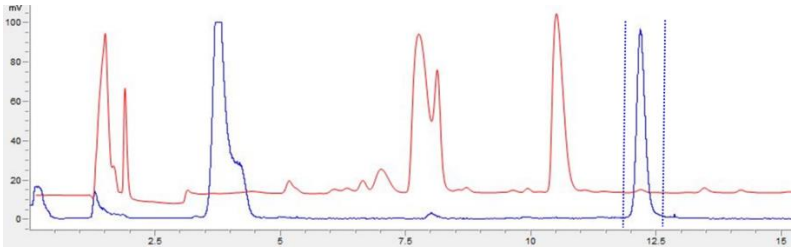
The time difference between UV detector and radio-detector was 0.06 min for method 2 (flow: 4 mL/min) and 0.15 min for method 3 (flow: 1.5 mL/min).

A γ-counter (Wallac Wizard, Perkin Elmer, Waltham, MA, USA) was used to measure the radioactivity of samples generated in cell-based experiments, in vitro experiments and the determination of the biodistribution.

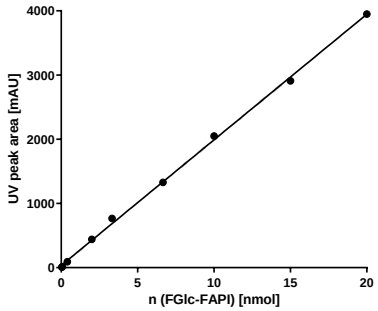
No-carrier-added [^{18}F]fluoride was produced through the ^{18}O(p,n)^{18}F reaction on a PETtrace 800 cyclotron using H_2[^{18}O]O as a target at the University Hospital Würzburg (Center of Radiopharmacy, Würzburg, Germany). [^{177}Lu]$LuCl_3$ was obtained from Isotope Technologies Garching (ITG, Garching). [^{68}Ga]Ga^{3+} was obtained by elution from a ^{68}Ge/^{68}Ga-generator (Eckert & Ziegler, Berlin).



Supplemental Figure 2. Characterization of **[^{18}F]FGlc-FAPI**: Coinjection of [^{18}F]FGlc-FAPI (above) and non-radioactive reference FGlc-FAPI (below) was performed using method 2 on the HPLC system (t_R= 2.90 min). The time delay of 0.06 min between the radio and the UV detector was corrected by the processing program. A coinjection using method 3 was additionally performed, demonstrating identical retention times for radio and UV peak (t_R= 9.9 min, data not shown).



Supplemental Figure 3. Chromatogram of a representative isolation of the fraction containing [^{18}F]FGlc-FAPI (dashed lines) by semi-preparative HPLC (method 1).



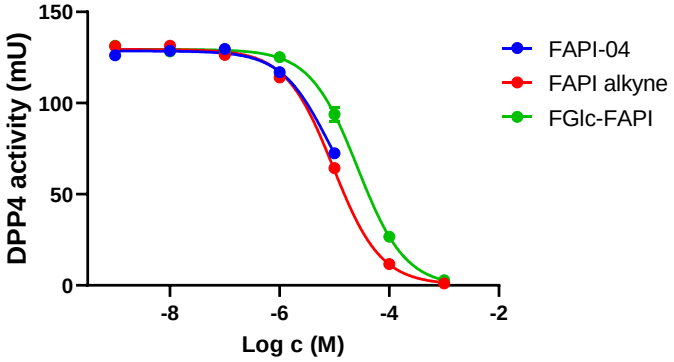
Supplemental Figure 4. Calibration curve of the HPLC UV detector for the quantification of the amount of FGlc-FAPI (linear regression of the amount of FGlc-FAPI vs. peak area (UV, 214 nm)).

Radiosynthesis of [^{177}Lu]Lu-FAPI-04

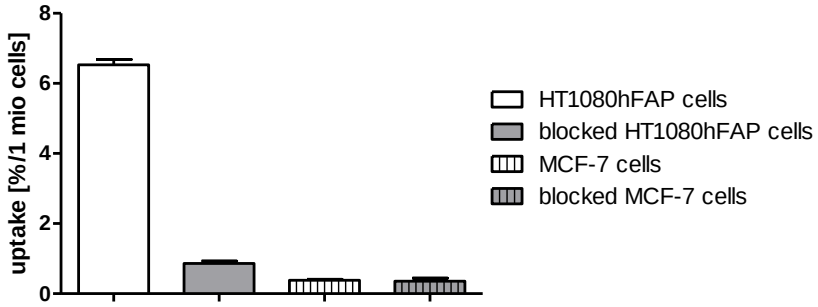
For the radiosynthesis of ^{177}Lu-labeled FAPI-04 2-12 µL [^{177}Lu]$LuCl_3$-solution (2-12 µL, 20-120 MBq) was diluted with HEPES-solution (0.5M, 200 µL, pH 5) and FAPI-04 precursor (4 µL, 4.6 nmol) was added. The reaction was heated to 99 °C in an Eppendorf tube and the reaction progress was monitored on HPLC (t_R = 1.19 min, method 4). After 15-20 min, the radiochemical yield was 92 ± 5% (n = 5), as determined by integration of the radio-HPLC peaks and [^{177}Lu]Lu-FAPI-04 was used without further purification or was isolated on a C18-cartridge and eluted with ethanol, followed by evaporation and formulation with cell culture media for the use of [^{177}Lu]Lu-FAPI-04 as a radioligand in the competitive FAP binding assay.

Radiosynthesis of [^{68}Ga]Ga-FAPI-04

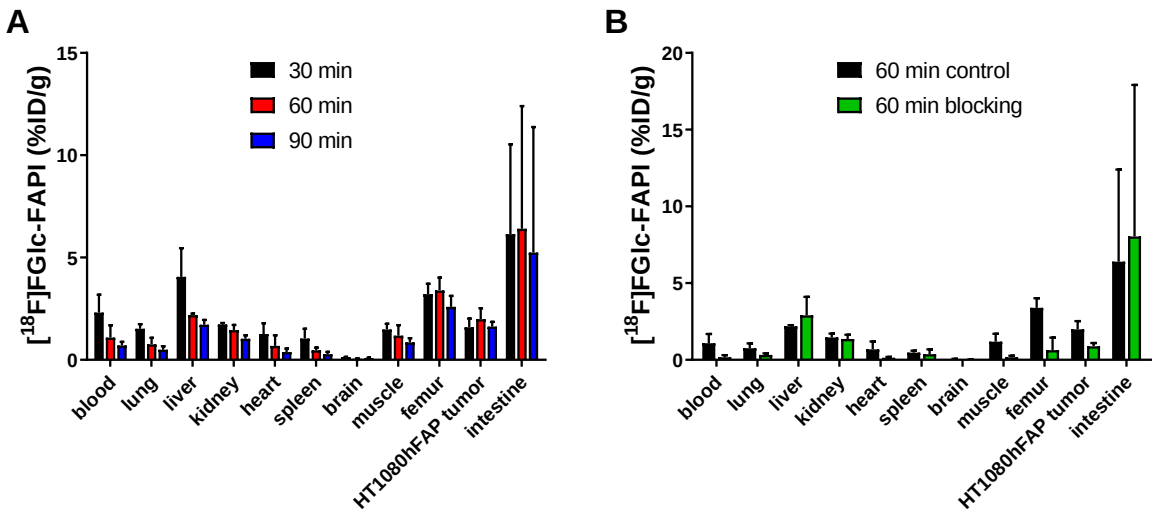
[^{68}Ga]Ga^{3+} was eluted with 0.1M HCl (10 mL) and trapped on a PS-H^+-cartridge (Chromafix, Machery-Nagel) which was then eluted with a NaCl solution (5M, 1 mL). The FAPI-04 precursor (3 µL, 3.44 nmol) was diluted with HEPES solution (2.5M, 300 µL, pH 5) and the ^{68}Ga-eluate was added (500 µL, 100 - 250 MBq). The reaction was incubated in an Eppendorf tube for 10 min at 99 °C to obtain a radiochemical yield of 86 ± 11% (n = 3) as determined by HPLC (t_R = 1.10 min, method 4). The reaction mixture was fixed on a C18-cartridge and eluted with ethanol, which was finally evaporated. The radiotracer was obtained in a radioactivity yield of 53 ± 2% in a total synthesis time of 30 min, with a radiochemical purity of >97% and an apparent molar activity of approximately 15-40 GBq/µmol.



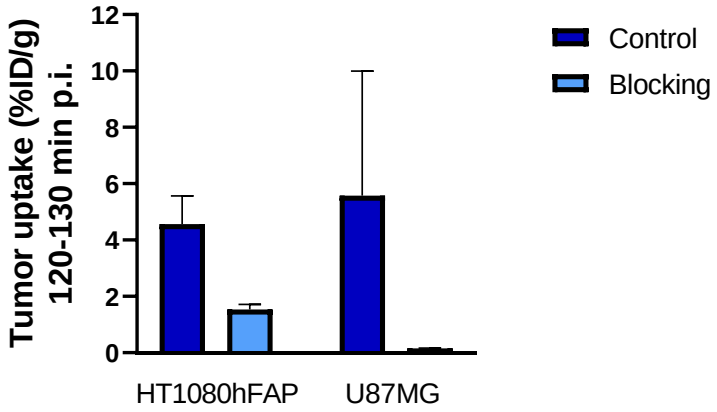
Supplemental Figure 5. Inhibition of DPP4 activity by FAPI-04, FAPI alkyne 11, and FGlc-FAPI (n = 2).



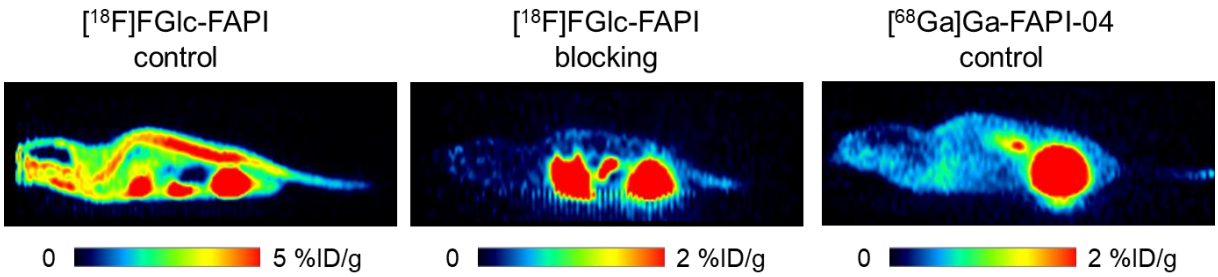
Supplemental Figure 6. Uptake of [^{18}F]FGlc-FAPI in HT1080hFAP cells (with FAP expression) and MCF-7 cells (without FAP expression) after 60 min-incubation at 37°C. Nonspecific uptake was defined in the presence of an excess of FAPI alkyne **11** in a concentration of 1 µM. Each bar represents the mean ± SD (n = 4).



Supplemental Figure 7. A) Biodistribution of [^{18}F]FGlc-FAPI in HT1080hFAP xenografts after 30, 60, and 90 min p.i. (n = 2). B) Comparison of biodistribution of [^{18}F]FGlc-FAPI in HT1080hFAP xenografts at 60 min p.i., without (control) and with coinjection of **11** (blocking, 30 nmol/mouse, n = 2).



Supplemental Figure 8. Tumor uptake values [^{18}F]FGlc-FAPI at 120–130 min p.i. derived from PET scans (additional data for Fig. 4 and Fig. 5 of the main manuscript).



Supplemental Figure 9. Representative µPET images of U87MG xenografted mice in sagittal view using [^{18}F]FGlc-FAPI (left), [^{18}F]FGlc-FAPI together with alkyne **11** (middle), and [^{68}Ga]Ga-FAPI (right).