Supporting Information

Synthesis, *in vitro* and *in vivo* evaluation of an ^{18}F-labeled neuropeptide Y analog for imaging of breast cancer by PET

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METHODS

Synthesis

*General.*Canonical N^{α}-9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids as well as noncanonicalpropargylglycine (Fmoc-L-Pra-OH) were purchased from Iris Biotech GmbH. Reactive side chains were protected by *tert*-butoxycarbonyl (Boc, for Lys and Trp), *tert*-butyl (tBu, for Tyr and Asp), 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf, for Arg) and trityl (Trt, for Asn, His and Gln). Rink amide resin (loading 0.63 mmol/g) was obtained from Novabiochem. Amino acid coupling reagents N,N´-diisopropylcabodiimide (DIC) and hydroxybenzotriazole (HOBt) were purchased from Iris Biotech GmbH and Fluka, respectively. For fluoroglycosylation ascorbic acid, copper(II) sulfate pentahydrate ($Cu(II)SO_4 \bullet 5H_2O$), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \bullet H_2O$) and

disodium hydrogen phosphate dihydrate ($NaH_2PO_4 \bullet 2H_2O$) were purchased from Sigma-Aldrich. For peptide synthesis and reversed-phase high-performance liquid chromatography (RP-HPLC) HPLC grade solvents were used. Peptides were analyzed by analytical RP-HPLC using columns as follows: Grace Vydac C18-column (250 x 4.6 mm; 5 µm; 300 Å), Grace Vydac C8-column (250 x 4.6 mm; 5 µm; 300 Å), Phenomenex Jupiter 5µ C18 (250 x 4.6 mm; 5 µm; 300 Å) and VariTide RPC (250 x 4.6 mm; 6 µm; 200 Å). Analytical chromatograms were recorded on a Merck-Hitachi LaChrom system equipped with a L-7455 diode array detector and a D-7000 HSM-Sys software or a VWR-Hitachi ELITE LaChrome system equipped with a L-2455 diode array detector and EZChrom Elite v. 3.3.1 software. A Shimadzu device equipped with a SPD-20A VP prominence UV/VIS detector and LCsolution v. 1.25 software was used for semi-preparative and preparative peptide purification on two respective Phenomenex C18-columns (250×10 mm, 5 µm, 300 Å or 250×21 mm, 10 µm; 90 Å). The binary solvent system was composed of solvent A 0.1% trifluoroactetic acid (TFA) in bi-distilled water and solvent B 0.08% TFA in acetonitrile (ACN). Peptide identity was confirmed by both matrix-assisted laser desorption/ionization time of flight/time of flight mass spectrometry (MALDI-ToF/ToF MS) on a Bruker DaltonicsUltraflex III and electrospray ionization mass spectrometry (ESI-MS) on a Bruker Daltonics HCT. Monoisotopic molecular weights were determined by Peptide Companion v. 1.2.4. Radio-HPLC was performed on an Agilent 1100 system with a quarternary pump and variable wavelength detector and radio-HPLC detector D505TR (Canberra Packard). Computer analysis of the HPLC data was performed using FLO-One software (Canberra Packard).

Solid Phase Peptide Synthesis. NPY analogs were synthesized by a combination of automated synthesis on a MultiSynTech Syro II peptide synthesizer and manual elongation steps. Peptide elongation was performed in iterative cycles of Fmoc cleavage and subsequent coupling of the next amino acid. For Fmoc/tBu coupling strategy 10 equivalents of Fmoc-protected amino acids were *in situ* pre-activated with equimolar amounts of HOBt/DIC and

coupled on Rink amide resin (15 µmol scale). After double-coupling procedure Fmoc was cleaved under basic conditions with 20% piperidine in dimethylformamide (DMF). The alkyne function for click chemistry was manually introduced using 3 equivalents of Fmoc-Pra-OH and *in situ* pre-activation with equimolar amounts of HOBt/DIC. Peptides were cleaved as carboxamides from Rink amide resin in 90% aqueous TFA and 10% thioanisole/thiocresole (1:1 v/v) scavenger mixture. After precipitation from cold diethylether (Et_2O) crude peptides were iteratively washed in Et_2O and dried under reduced pressure. Crude peptides were analyzed by RP-HPLC and MALDI-MS and purified in preparative scale to homogeneity which was finally confirmed by two different RP-HPLC systems. The identity of the pure peptides was verified by MALDI- and ESI-MS.

Production of [^{18}F]Fluoride. No-carrier-added (n.c.a.) [^{18}F]fluoride was produced by the $^{18}O(p,n)^{18}F$ reaction in ^{18}O-enriched (97%) water using a proton beam of 11 MeV generated by a RDS 111e cyclotron (CTI-Siemens) and trapped on an anion exchange cartridge (QMA, Waters).

In Vitro Characterization

Reagents and Cell Culture. Cell culture reagents were purchased from PAA Laboratories GmbH and cell plastics from Greiner Bio-One GmbH. African green monkey cells (COS-7), stably expressing either the EYFP-tagged human Y receptor subtype 1 (Y_1R) or Y_2R and the chimeric $G\alpha_{\Delta 6qi4myr}$-protein, were grown in standard Dolbecco's modified Eagle's medium (DMEM) containing 4.5 g L^{-1} glucose and L-glutamine. DMEM medium was supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin, 150 µg/mL hygromycin and 1.5 mg /mL G418-sulfate. Human embryonic kidney cells (HEK293), each stably expressing either the EYFP-tagged hY_1R or hY_2R [1], were grown in DMEM/Ham's F12 (1:1 v/v) with L-glutamine containing 15% (v/v) heat-inactivated FCS. Human Y_1R expressing MCF-7 cells were grown in

DMEM/Ham's F 12 (1:1 v/v) supplemented with 10% FCS and 1% Glutamin. Cells were cultured as monolayers in T75 culture flasks at 37 °C under humidified atmosphere enriched with 5% CO_2. Cells were routinely subcultured every 3-4 days.

*Determination of Distribution Coefficient at pH 7.4 ($logD_{7.4}$).*The partition ratio of **[^{18}F]3b** between water and 1-octanol was determined as the distribution coefficient at pH 7.4. **[^{18}F]3b** (10 µl in saline, 30 kBq) was added to a tube containing 1-octanol (0.5 mL) and PBS (0.5 mL, pH 7.4), and vigorously mixed for 1 min at room temperature. The tubes were centrifuged and triplicate samples of 100 µL of each layer were counted in a γ-counter (Wallac Wizard). The distribution coefficient was determined by calculating the ratio of radioactivity concentrations in the two phases, and expressed as $logD_{7.4}$ ($log(cpm_{1\text{-}octanol}/cpm_{buffer})$). Three independent experiments were performed in triplicate.

Determination of Tracer Stability in Human Serum. An aliquot of **[^{18}F]3b** or **[^{18}F]4b** in saline (35 µL, pH 7.4) was added to human serum (200 µL) and incubated at 37 °C. Aliquots (20 µL) were taken at various time intervals (5, 15, 30, 45, 60, 150 min) and quenched in methanol/water (1:1 v/v, 200 µL). The samples were centrifuged, and the supernatants were analyzed by radio-HPLC (Chromolith RP-18e, 100×4.6 mm, 10–100% acetonitrile (0.1% TFA) in water (0.1% TFA) in a linear gradient over 5 min, 4 mL/min, t_R(**[^{18}F]3b**)= 2.29 ± 0.04 min, t_R(**[^{18}F]4b**)= 2.45 ± 0.02 min; Kromasil C8 250×4.6 mm, 20–70% acetonitrile (0.1% TFA) in water (0.1% TFA) in a linear gradient over 30 min, 1.5 mL/min, t_R(**[^{18}F]3b**)= 14.60 ± 0.05 min, t_R(**[^{18}F]4b**)= 15.70 ± 0.05 min).

In vitro Autoradiography (Y_1R detection in MCF-7 tumor slices). Nude mice bearing MCF-7 tumors were killed while anaesthetized. Tumors were quickly removed and frozen in *n*-hexane at -70°C. Sections of 20 µm of the tumor were cut using a cryostat microtome

(HM550, Microm, Germany), three sections thaw-mounted on glass slides (Histobond) and stored at -20 °C. For binding of **[^{18}F]3b** the sections were thawed, dried at room temperature and preincubated for 10 min at room temperature in assay buffer (10 mM HEPES, 150 mMNaCl, 25 mM $NaHCO_3$, 5 mMKCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.2% BSA, 50 μg/L bacitracin, pH 7.4). Subsequently, the slides were transferred into new chambers containing **[^{18}F]3b** or **[^{18}F]3b** and **3a** (1.25 μM) or **[^{18}F]3b** and BIBP3226 (1 μM) and incubated in assay buffer at room temperature for 45 min. Slide-mounted sections were washed (3×2 min) in ice-cold assay buffer, dipped in ice-cold water, and dried under a stream of cool air, and exposed overnight to a phosphor screen (FUJI Imaging Plate BAS-IP SR). Exposed screens were analyzed with a high-resolution radioluminography laser scanner (DÜRR Medical HD-CR 35 Bio, Raytest) using the software AIDA Image Analyzer to quantitate radioligand binding.

Stability in Mouse Blood ex vivo. Healthy female athymic nude mice (without tumor) were injected under isoflurane anesthesia (4%) with 3MBq of [**^{18}F]3b** in the tail vein. After 10 and 20 min the mice were killed by cervical dislocation and blood was collected in Li-heparine coated vials. The vials were centrifuged immediately, the supernatant (50 μL) was added to 50 μL of mathanol to precipitate plasma proteins and this mixture was centrifuged again. The supernatant (50 μL) was analyzed by radio-HPLC (PerfectSil 300 C4, 125×8 mm, 30-50% acetonitrile (0.1% TFA) in water (0.1% TFA) in a linear gradient over 20 min, 4 mL/min, t_R=8.5 ± 0.1 min).

References

[1] Mäde V, Babilon S, Jolly N, Wanka L, Bellmann-Sickert K, Diaz Gimenez LE, Mörl K, Cox HM, Gurevich VV, Beck-Sickinger AG. Peptide Modifications Differentially Alter G Protein-Coupled Receptor Internalization and Signaling Bias. *Angew. Chem. Int. Ed.* 2014; 126(38), 10231–10235.

Table S1. Analytical data of precursors [Pra^4,F^7,P^{34}]NPY (**3a**) and [Pra^4,F^7,A^{33},P^{34},A^{35}]NPY (**4a**) as well as the corresponding fluoroglycosylated analogs [Pra^4(F-Glc),F^7,P^{34}]NPY (**3b**) and [Pra^4(F-Glc), F^7,A^{33},P^{34},A^{35}]NPY (**4b**).

		HPLC			MALDI-MS	ESI-MS	
No.	Peptide	Purity[a] [%]	1st System [% Solvent B]	2nd System [% Solvent B]	Calcd [g/mol]	Obsd (z)[h] [m/z]	Obsd (z)[h] [m/z]
3a	[Pra^4,F^7,P^{34}] NPY	>95	46.5[b]	47.4[d]	4220.1	4221.2 (+1) 2111.2 (+2)	1408.4 (+3) 1056.5 (+4) 845.5 (+5) 704.8 (+6)
4a	[Pra^4,F^7,A^{33}, P^{34},A^{35}]NPY	>95	55.0[e]	47.4[f]	4050.0	4051.5 (+1) 2026.2 (+2)	1351.5 (+3) 1014.0 (+4) 811.4 (+5)
3b	[Pra^4**(F-Glc)**, F^7,P^{34}]NPY	>94	54.9[f]	45.5[g]	4427.2	4428.1 (+1) 2214.5 (+2)	1108.4 (+4) 887.0 (+5) 739.4 (+6) 633.8 (+7)
4b	[Pra^4**(F-Glc)**, F^7,A^{33},P^{34},A^{35}] NPY	>94	56.1[f]	53.5[c]	4257.1	4258.8 (+1) 2130.2 (+2)	1421.2 (+3) 1065.8 (+4) 852.7 (+5)

[a] As quantified by analytical RP-HPLC at 220 nm with a minimum of two different HPLC column systems.Percental elution concentration of solvent B determined by analytical HPLC at 220 nm using following columns and gradients:
[b] Grace Vydac C18-column with a linear gradient of 20% to 50% solvent B over 30 min or
[c] 20% to 70% solvent B over 40 min,
[d] Grace Vydac C8-column with a linear gradient of 20% to 70% solvent B over 40 min,
[e] Phenomenex C18-column with a linear gradient of 20% to 80% solvent B over 30 min or
[f] 20% to 70% over 40 min and
[g] VariTide RPC column with a linear gradient of 20% to 70% solvent B over 40 min.
[h] charge number of the respective molecule ions measured by MALDI-ToF/ToF and ESI-MS.

Table S2. Biodistribution of **[^{18}F]3b** in MCF-7 Tumor-bearing Nude Mice 20 min, 60 min and 120 min postinjection[a].

% ID/g[a]	20 min	60 min	120 min
blood	1.47 ± 0.44	0.82 ± 0.33	0.27 ± 0.05
lung	1.68 ± 0.39	0.97 ± 0.31	0.80 ± 0.23
liver	6.88 ± 0.46	5.99 ± 0.54	5.58 ± 0.43
kidney	62.45 ± 4.93	58.64 ± 7.86	29.55 ± 9.65
heart	1.35 ± 0.44	0.77 ± 0.29	0.50 ± 0.09
spleen	1.87 ± 0.52	0.99 ± 0.12	0.99 ± 0.35
MCF-7 tumor	1.82 ± 0.66	1.42 ± 0.64	0.66 ± 0.03
brain	0.33 ± 0.12	0.07 ± 0.01	0.05 ± 0.01
muscle	0.61 ± 0.24	0.37 ± 0.17	0.28 ± 0.12
duodenum	1.09 ± 0.29	1.01 ± 0.67	0.73 ± 0.40
femur	0.57 ± 0.16	0.25 ± 0.19	0.23 ± 0.04
pancreas	1.20 ± 0.27	1.14 ± 0.11	1.74 ± 0.13
adrenal glands	2.41 ± 0.46	0.91 ± 0.25	0.65 ± 0.16
thyroid	1.05 ± 0.33	0.63 ± 0.15	0.52 ± 0.08
gall bladder	4.35 ± 1.76	3.56 ± 0.51	2.77 ± 0.54

[a]Data are expressed in percent injected dose per gram of tissue (%ID/g) and provided as mean ± SD (n = 3-4).

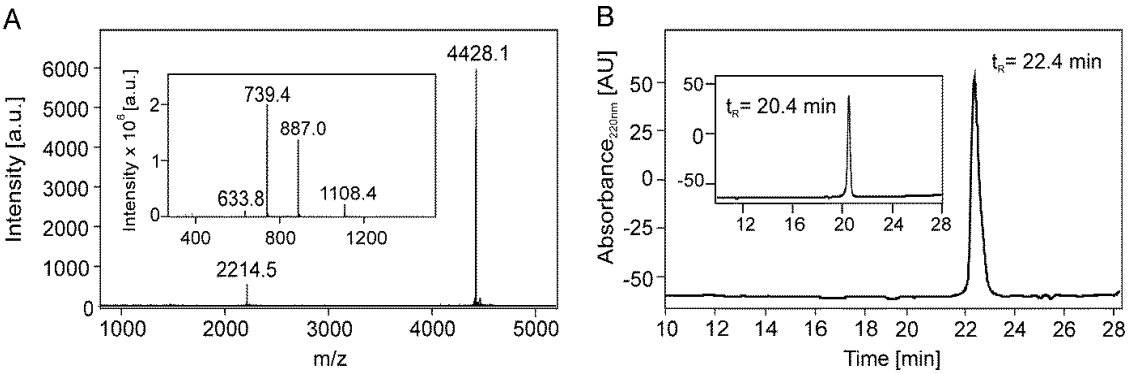

Figure S1. Analytical data of fluoroglycosylated [Pra^4(F-Glc),F^7,P^{34}]NPY analog **3b** (monoisotopic molecular weight 4427.2 g mol^{-1}). (**A**) MALDI mass spectrum (large spectrum) displaying the $[M+H]^+$ and $[M+H]^{2+}$ signal, ESI mass spectrum (inlet) exhibiting the serial ion charge series from $[M+H]^{4+}$ to $[M+H]^{7+}$. (**B**) Analytical RP-HPLC on a Phenomenex C18-column (large chromatogram) and on a VariTide RPC column (inlet) both with a linear gradient of 20% to 70% solvent B over 40 min.

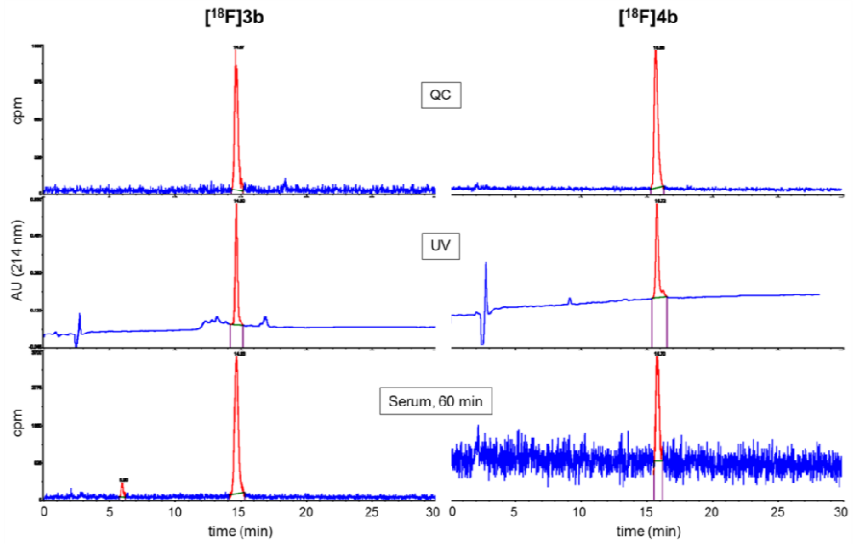

Figure S2. Purity, identity and stability of **[^{18}F]3b** and **[^{18}F]4b.** Top: Quality control (QC) of the radiotracers after isolation by HPLC, middle: reference compounds **3b** and **4b** (UV), bottom: stability of the radiotracers after incubation in human serum at 37°C for 60 min. Kromasil C8 250×4.6 mm, 20–70% acetonitrile (0.1% TFA) in water (0.1% TFA) in a linear gradient over 30 min, 1.5 mL/min, t_R (**[^{18}F]3b**) = 14.60 ± 0.05 min, t_R (**[^{18}F]4b**) = 15.70 ± 0.05 min.

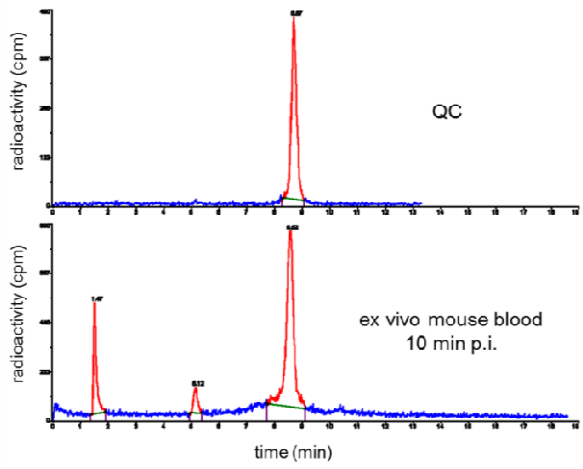

Figure S3. Stability of **[^{18}F]3b** in mouse blood at 10 min p.i. in comparison with the quality control (QC) of the radiotracer. PerfectSil 300 C4, 125×8 mm, 30-50% acetonitrile (0.1% TFA) in water (0.1% TFA) in a linear gradient over 20 min, 4 mL/min.

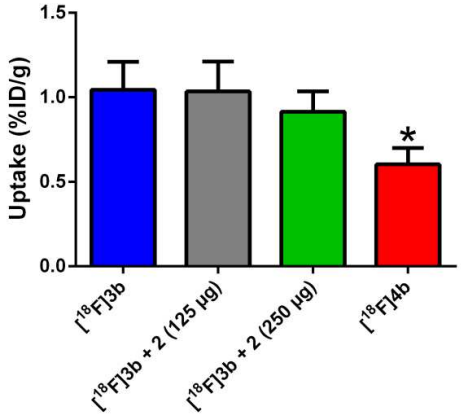

Figure S4. Tumor uptake values as determined from the analysis of PET imaging data. There is no significant difference between tumor uptake of **[^{18}F]3b**(n=14) at 45-60 min post-injection in comparison with tumor uptake of animals injected with **[^{18}F]3b** together with the NPY analog **2** (125 µg/mouse or 250 µg/mouse, each n = 2, $P > 0.05$), whereas there is a significant difference between animals injected with **[^{18}F]3b** and animals injected with the non-binding NPY analog **[^{18}F]4b**(n = 4, *$P < 0.01$, unpaired *t*-test), suggesting specific Y1-receptor-mediated binding of the glycopeptide**[^{18}F]3b**.