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Supporting information

Radiosynthesis of high affinity fluorine-18 labeled GnRH peptide analogues: *in vitro* studies and *in vivo* assessment of brain uptake in rats

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LIST OF ABBREVIATIONS

FBA = 4-fluorobenzoic acid/4-fluorobenzoyl amide

GENERAL

Materials and methods. All reagents and solvents, unless otherwise specified, were obtained from Sigma-Aldrich or VWR (Oslo, Norway), and were used without further purification. O-(7 Azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HATU), Fmoc-rink amide MBHA resin and Fmoc-D-Lys(Boc)-OH were obtained from Anaspec Inc (Freemont, CA, US). Fmoc-Ser(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Arg(Pbf)-OH were purchased from IRIS biotech (Marktredwitz, Germany). *N*-Boc-pyroglutamic acid (Boc-Pyr-OH) was purchased from Bachem (Bubendorf, Switzerland).

2-Chlorotrityl chloride resin was obtained from Novabiochem (Darmstadt, Germany). *N*-Succinimidyl 4-fluorobenzoate (SFB) was a kind gift from ABX GmBH (Radeberg, Germany). Buserelin acetate was obtained from Sigma-Aldrich (St. Louis, MO).

Mobile phases for both preparative and analytical HPLC were: solvent A, water/0.1% TFA and solvent B, acetonitrile/0.1% TFA. UV detection was at 214 and 254 nm.

Analytical reversed-phase HPLC and LC-MS spectra were recorded on a Thermo Finnigan Surveyor MSQ Plus instrument using electrospray ionization (ESI) operated in positive mode coupled to Finnigan Surveyor PDA chromatography system (Instrument Teknikk AS, Norway). Gradient elution (10-40% B over 5 min) was at 0.6 mL/min flow (Method 1) rate on a Phenomenex Luna C18(2) column (3 μm, 20 × 2 mm) (Teknolab, Norway). Preparative reversed-phase HPLC was performed on a Shimadzu LC-8A (Bergman, Norway) system using a Phenomenex Luna C18(2) column (Teknolab, Norway) (250 mm × 21.2 mm, 5 μm), flow rate 10 mL/min, with gradients 5-30% solvent B (P-Method 1) or 10-40% solvent B (P-Method 2) over 60 min. Mass spectrometry of purified peptides **5**, **6**, **7** and **8** was performed using an ABI 4700 matrix-assisted laser desorption-ionization time of flight/time of flight

(MALDI TOF/TOF) spectrometer (Applied Biosystems, Foster City, CA, USA). Analytical reversed-phase Radio-HPLC was performed on a Beckman Gold 166 system using a Phenomenex Jupiter Proteo (250 × 4.6 mm, 4 μm, Phenomenex, Torrance, CA), flow 1.5 mL/min, with solvent B isocratic 9% for 2 min, then linear gradient to 81% over 30 min (R-Method 1). UV detection at 220 nm was combined with a γ-detector (Bioscan flow-count, Bioscan, Washington DC, USA). Semi-preparative reversed-phase HPLC purifications were performed on a Phenomenex Jupiter C-18 column (250×10 mm, 10 μm, Phenomenex, Torrance, CA), flow 3 mL/min, with solvent B isocratic 9% for 2 min, then linear gradient to 81% over 30 min. Solvents were in both instances, A: 0.05% TFA in water (v/v); solvent B: acetonitrile (RP-Method 1).

Chemistry. Assembly of the resin bound amino acid sequence was performed on solid phase using Fmoc-chemistry. Coupling reactions were monitored by the Kaiser test, except for proline where the Chloranil test was used.[1,2] After cleavage from the solid supports and filtration, the solutions were concentrated under reduced pressure and the residue was washed with diethyl ether. Crude products were purified by preparative HPLC and isolated from collected fractions by lyophilizing. Intermediates and final products were characterized by LC-MS.

Synthesis. *Peptide* **1** (*D-Lys*^{6}*-GnRH*). The peptide Pyr-HWSYkLRPG amide was synthesized on an Fmoc-rink amide MBHA resin (0.05-0.1 mmol peptide loading). Simultaneous removal of the peptide from the resin and removal of side chain protecting groups was carried out in trifluoroacetic acid (TFA) containing triisopropylsilane (TIPS) and water (95:2.5:2.5 v/v/v). Crude product was purified by preparative HPLC (P-Method 1). ESI-MS: found m/z = 1253.7 calcd $(M+H)^+$ = 1253.7.

Peptide ***2*** *(D-Lys*^{6}*(Ahx)-GnRH).* Synthesis of **2** by coupling of 6-Boc-aminohexanoic acid *N*-succinimidyl ester (6-Boc-Ahx-OSu) with **1** followed by removal of the Boc-

protecting group was performed via a known protocol.[17] Crude product was purified by preparative HPLC (P-Method 1). ESI-MS: found m/z = 1366.8, calcd $(M+H)^+$= 1366.7.

*Peptide **3** (D-Lys^6-desGly^{10}-GnRH-ethylamide)*. Fmoc-Pro-OH (0.2 mmol, 67 mg) was dissolved in dichloromethane (5 mL) and added to a pre-swollen 2-chlorotrityl chloride resin (0.1 mmol) in a nitrogen bubbler apparatus followed by *N*,*N*-diisopropylethylamine (DIPEA) (0.4 mmol, 136 μL). After 16 h the reagents were removed by filtration and the resin washed with 3 × 7 mL of DCM/MeOH/DIPEA (17:2:1 v/v/v) and 3 × DCM. The amino acid sequence Pyr-HWSYkLR was then assembled on the Pro-2-chlorotrityl resin and cleaved from the resin fully protected using a mixture of hexafluoroisopropanol (HFIP)-dichloromethane (1:4 v/v) for 2 h. Crude peptide mixture was diluted in hexane (50 mL) and evaporated *in vacuo* to obtain a crystalline solid. The fully protected peptide was activated with (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) (0.12 mmol, 62.5 mg) and reacted with ethylamine hydrochloride (5 mmol, 408 mg) and DIPEA (5.1 mmol, 888 μL) in 1-methyl-2-pyrrolidinone (NMP) (2 mL). Progression of the reaction was monitored using LC-MS. Organic solvents were removed under reduced pressure and removal of side-chain protecting groups was carried out in TFA, TIPS and water (95:2.5:2.5 v/v/v). After filtration, the solution was concentrated under reduced pressure and the residue was washed with diethyl ether. Crude product was purified by preparative HPLC (P-Method 1) and isolated by lyophilizing. ESI-MS: found m/z = 1224.9, calcd $(M+H)^+$ = 1224.7. Yield 41%.

*Peptide **4** (D-Lys^6(Ahx)-desGly^{10}-GnRH-ethylamide)*. Coupling of 6-Boc-aminohexanoic acid *N*-succinimidyl ester (6-Boc-Ahx-OSu) with **3** was performed analogously to **2**. Crude product was purified by preparative HPLC (P-Method 1). ESI-MS: found m/z = 1337.9, calcd $(M+H)^+$ = 1336.7.

FBA labeled peptide standards **5**, **6**, **7** and **8**. Nonradioactive peptide standards **5**, **6**, **7** and **8** were prepared from **1**, **2**, **3** and **4** by reaction with SFB, respectively. In brief, in a 5 mL V-vial equipped with a stir bar, a solution containing 1 eq. of peptide, 1.2 eq. SFB in 1 mL DMF was allowed to react at room temperature for 1 h after addition of 2 eq. of DIPEA. The reaction mixture was diluted with 4 mL water/0.1% TFA and purified by preparative HPLC (P-Method 2) and isolated by lyophilizing.

5. LC-MS* (ESI): t_R = 3.17 min, purity >95%; found m/z = 1375.3, MALDI found m/z= 1375.4495, calcd $(M+H)^+$ ($C_{66}H_{87}FN_{18}O_{14}$) = 1375.6706.

6. LC-MS* (ESI): t_R = 3.21 min, purity >95%; found m/z = 1489.1 $(M+H)^+$, MALDI found m/z = 1488.4905, calcd $(M+H)^+$ ($C_{72}H_{98}FN_{19}O_{15}$) = 1487.7474.

7. LC-MS* (ESI): t_R = 3.42 min, purity >95%; found m/z = 1346.9, MALDI found m/z= 1346.4662, calcd $(M+H)^+$ ($C_{66}H_{88}FN_{17}O_{13}$) = 1346.6874.

8. LC-MS* (ESI): t_R = 3.45 min, purity >95%; found m/z = 1460.2, MALDI found m/z= 1459.5197, calcd $(M+H)^+$ ($C_{72}H_{99}FN_{18}O_{14}$) = 1459.7645.

*Method 1

RADIOCHEMISTRY

[^{18}F]Fluoride was produced from the $^{18}O(p,n)^{18}F$ nuclear reaction on enriched [^{18}O]H_2O (Medical Isotopes, Inc.) using a Siemens CTI RDS 111 negative ion cyclotron

Synthesis of N-Succinimidyl 4-[^{18}F]fluorobenzoate ([^{18}F]SFB). [^{18}F]SFB was prepared manually according to previously described methods.[3]

Peptide labeling with [^{18}F]SFB, purification and formulation. 370 MBq – 11000 MBq [^{18}F]SFB in acetonitrile was added to a stirred solution containing either 1 mg of peptide **1**, **2**, **3** or **4** and 10 µL DIPEA in 150 µL DMF. The mixture was heated to 45°C for 30 min, diluted with solvent A (1.5 mL) and purified by semi-preparative HPLC (C18) (RP-Method 1). The

fraction containing the radioactive material was diluted with water (15 mL) and passed through a Waters C18 Sep-Pak light cartridge preactivated with ethanol (2 mL) and water (5 mL). The cartridge-bound ^{18}F-peptide was washed with water (5 mL) and eluted with 1 mL absolute ethanol into a vial. The ethanol was evaporated under a stream of helium at 40 °C and the ^{18}F-peptide was reconstituted in isotonic PBS adjusted to pH 7.4 for use in *in vitro* and animal experiments. The identities of [^{18}F]**5**, [^{18}F]**6,** [^{18}F]**7** and [^{18}F]**8** were confirmed by coelution with the corresponding nonradioactive reference compounds using analytical Radio-HPLC (R-Method 1).

Radiolysis experiments. The radiolytic decomposition of nonradioactive peptide standard **5** was investigated by incubation in 0.9% saline containing 37 GBq [^{18}F]fluoride. As a control, peptide **5** was incubated in saline without adding radioactivity. Aliquots were collected after 15 min and percentage of intact **5** was determined by HPLC.

Octanol/Water Partition Coefficient. Approximately 10 kBq of ^{18}F-peptide in 50 μL PBS was diluted in 450 μL of PBS pH 7.4 and added to 500 μL of *n*-octanol in an Eppendorf tube (n = 4). After vortexing for 3 min, the tubes were centrifuged (10 300 rpm; 6 min), and 100 μL aliquots of the PBS and *n*-octanol phases were carefully transferred to separate tubes and the radioactivity counted in a γ-counter.

BIOLOGY

Rodents*. All animal studies were conducted in compliance with a protocol approved by the University of California, Davis, Animal Use and Care Committee.*

Membrane preparation and radioligand binding assays. Cell membrane preparations of cells expressing the human GnRHR were obtained from Merck Millipore (Oslo, Norway). Rat

GnRHR expressing cell membranes were generated from HEK293 cells grown in Dulbecco's modified Eagle's medium with 10% Fetal Bovine Serum, penicillin (100 U/mL) and streptomycin (100µg/mL) and transiently transfected with the rat GnRH receptor (kindly provided by Dr. Eidne) using LipofectAMINE 2000 (Invitrogen), according to the manufacturer's protocol.[4] 48 h later, membranes were prepared and radioligand binding was performed as previously described.[5] Radioligand binding studies were performed with 0.08-0.20 nM [^{125}I]triptorelin ([^{125}I]LHRH D-Trp^{6}), PerkinElmer, Boston, USA) using increasing concentration of the indicated peptide. K_d of [^{125}I] triptorelin was 0.3 nM for human GnRHR and 0.07 for rat GnRHR (determined by saturation binding of [^{125}I]triptorelin), which was used to determine the K_i of the peptides from the calculated IC_{50}.

Stability in rat serum. Approximately 5 MBq of ^{18}F-peptides in PBS (50 μL) was added to freshly collected rat serum (0.4 mL) and samples incubated at 37 °C in an Eppendorf tubes. After 1 and 2 h, ice-cold ethanol (400 μL) was added and the mixtures was centrifuged at 13 400 rpm for 10 min. The resulting supernatant was diluted with Solvent A and analyzed by radio-HPLC (R-Method 1).

***In vivo* PET experiments**. Male Sprague-Dawley rats (8 weeks) were obtained from Charles River laboratories (USA) and were provided with food and water ad libitum. All procedures were carried out under isoflurane anesthesia (2% isoflurane in 0.8 L/min oxygen). An Inveon small-animal PET scanner (Siemens Medical Solutions USA, Knoxville, TN) was used for imaging. A heat lamp provided a constant body temperature during image acquisition. Anesthetized rats were injected via a tail vein with 4-30 MBq of ^{18}F-peptides under isoflurane anesthesia. Dynamic PET scans (Time framing 6×30, 6×60, 7×180, 6×300, 6×600 s) were performed continuously from time 0 to 120 min. Data was reconstructed with attenuation correction using the OSEM3D/MAP algorithm. For the receptor blocking experiment, [^{18}F]**7**

was administered 10 min after injection of 100 μg (74 nmol) of buserelin acetate. Small animal CT was performed following the PET scans using an Inveon CT (Siemens Medical Solutions USA, Knoxville, TN). CT scans were acquired over a full rotation with 1° steps with a tube voltage of 80 kVp and a current of 500 µA. Images were reconstructed with a 98 µm pixel size using the system's reconstruction software. PET and CT images were co-registered using three [^{18}F]fluoride filled capillaries as fiducial markers. Regions of interest (ROIs) were drawn based on CT images. Time-activity-curves (TAC) were generated for framed PET data.

Biodistribution. Immediately following small-animal PET and CT scans animals were euthanized. Blood and major organs and tissues were collected, wet-weighed, and their radioactivity counted on a Wizard 1470 γ-counter (Perkin-Elmer, Waltham, MA). Data are reported as the percentage of injected dose per gram (% ID/g).

***In vivo* blood and urine stability**. Urine and blood samples were collected and analyzed by analytical radio-HPLC. Urine samples were collected 2 h after injection and blood (approx. 1 mL) was collected after 10 and 20 min. Full blood was centrifuged and the resulting serum was collected. Ice-cold ethanol (1 mL) was added to aliquots of either urine or the serum and centrifuged at 13 400 rpm for 10 min. The supernatant was diluted with Solvent A and analyzed by radio-HPLC. A quantifiable signal from blood could not be obtained later than 10 min post-injection.

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