Second Generation $Al^{18}F$-labeled D-Amino Acid Peptide For CXCR4 Targeted Molecular Imaging

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1. Supplementary Data

1.1. LC-HRMS of DV constructs

The construct's mass was analyzed in a Dione Ultimate 3000 UPLC system (Thermo Fisher Scientific, Sunnyvale, USA), which was coupled in series to a UV detector, a three inch thick NaI (Tl) radioactivity detector and a ultra-high resolution time-of-flight mass spectrometer set on 'positive mode' with electron spray ionization (MaXis Impact, Bruker, Bremen, Germany). A gradient elution has been run on a 40°C heated Waters Acquity UPLC BEH C18 column (1.7 µm 2.1 x 50 mm) at a flow rate of 0.6 mL/min in a gradient using Milli-Q water, as solvent A, and acetonitrile, as solvent B, with both containing 0.1% formic acid. The multistep gradient consisted of the following steps: 0–2 min: 95% A; 2–8 min: from 95% → 5% A; 8–10 min: 5% A; 10–12 min: 95% → 5% A. The run was monitored with UV set at 220 nm and the average neutral molecular ion mass was calculated with the Compass Isotope Pattern software (Version 3.2, Bruker Daltonik, Bremen, Germany). The deconvolution of the measured mass data was conducted in the software program DataAnalysis (Bruker Daltonik). A summary about the mass data of the different DV constructs has been added in **Table S1**. The purity of every compound was above 95%.

Table S.1 Theoretical and observed masses of DV constructs

Compound	Chemical Formula	Theoretical mass [Da]	Observed mass [Da]
[^{nat}Ga]PentixaFor	$C_{60}H_{77}GaN_{14}O_{14}$	1288.06	1288.72±0.01
DV1	$C_{108}H_{169}N_{33}O_{27}S_2$	2425.85	2425.73±0.00
DV3	$C_{52}H_{79}N_{17}O_{14}$	1166.30	1166.28±0.00
DV1-k-(DV3)	$C_{166}H_{258}N_{52}O_{41}S_2$	3702.32	3702.34±0.01
AlF-NOTA-DV1-k-DV3	$C_{178}H_{275}N_{55}O_{46}S_2AlF$	4031.55	4031.48±0.02
DV1(c11a)-k-(DV3)	$C_{166}H_{258}N_{52}O_{41}S$	3670.26	3670.27±0.01
DV1(c12a)-k-(DV3)	$C_{166}H_{258}N_{52}O_{41}S$	3670.26	3670.25±0.01
DV1(c11ac12a)-k-(DV3)	$C_{166}H_{258}N_{52}O_{41}$	3638.20	3638.22±0.01
DV1(c11s)-k-(DV3)	$C_{166}H_{258}N_{52}O_{42}S$	3686.26	3686.24±0.01
DV1(c12s)-k-(DV3)	$C_{166}H_{258}N_{52}O_{42}S$	3686.26	3686.28±0.01
DV1(c11sc12s)-k-(DV3)	$C_{166}H_{258}N_{52}O_{43}$	3670.20	3670.25±0.01
2xDV1(c11sc12s)	$C_{228}H_{360}N_{70}O_{59}$	5025.72	5025.87±0.01
NOTA-2xDV1(c11sc12s)	$C_{240}H_{379}N_{73}O_{64}$	5311.01	5311.13±0.02
AlF-NOTA-2xDV1(c11sc12s)	$AlFC_{240}H_{377}N_{73}O_{64}$	5355.00	5355.26 ± 0.02
3xDV1(c11sc12s)	$C_{342}H_{539}N_{105}O_{88}$	7529.69	7529.8038±0.04

1.2. Octanol/Water Partition Coefficient of [^{18}F]AlF-NOTA-2xDV1(c11sc12s)

70 kBq of [^{18}F]AlF-NOTA-2xDV1(c11sc12s) was added onto a mixture of 2 mL 1-octanol and 2 mL PBS (0.01 M, pH 7.4) and vortexed for 2 min. The mixture was subsequently centrifuged for 10 min. 200 µL of each layer was extracted and counted in the gamma counter. The octanol/water partition coefficient was calculated by taking the log10 of the ratio between the activity measured in the n-octanol phase with the one in the PBS phase. The ratio was corrected for the differences in mass and density of the two phases.

The octanol/water partition coefficient of [^{18}F]AlF-NOTA-2xDV1(c11sc12s) resulted in -2.71±0.17. The experiment has been conducted in triplicates and is written as mean ± standard deviation (SD).

1.3. [^{nat}F]AlF labeling of NOTA-2xDV1(c11sc12s)

$AlCl_3$ (490 µL, 5.1 mM in 0.1 M sodium acetate, pH 4.1, 10 eq.) and NaF (10 µL, 10 mg/mL in 0.1 M sodium acetate, pH 4.1, 10 eq.) were added for 5 min at room temperature. Then the precursor NOTA-2xDV1(c11sc12s) (1.0 mg in 500 µL absolute ethanol, 1 eq.) (M_r 3987.5 g/mol) was allowed to react for 30 min at 95 °C. After cooling down, the solution was diluted by adding 20 mL HPCE water (Sigma Aldrich, Saint Louis, MO, USA) and was loaded onto a Sep-Pak Plus Light C18 cartridge (Waters, Milford, MA, USA) preconditioned with 5mL ethanol absolute followed by 10mL HPCE water.

[^{nat}F]AlF-NOTA-2xDV1(c11sc12s) was subsequently eluted from the cartridge using 0.5 mL acetonitrile and 1 mL water and was passed through a Captiva PTFE + GF 0.45 µm filter (Agilent Technologies, Santa Clara, CA, USA). The final solution was frozen at -20 °C and lyophilized overnight. Theoretical mass of $C_{240}H_{377}N_{73}O_{64}AlF$ 5355.00 Da; found 5355.00±0.01 Da.

1.4. Radio-HPLC of QC and *in vitro* stability assessment

The *in vitro* stability was assessed by incubating 550 kBq of the radiotracer (77 nM) for 2h at 37°C in human serum (H4522 human male AB plasma, USA origin, sterile-filtered, Sigma Aldrich, Saint Louis, MO, USA) and for 4.5h at room temperature in formulation buffer, consisting of PBS supplemented with 0.58% sodium ascorbate.

The *in vitro* stability of the radiotracer showed 95% or 96% intactness in human serum or formulation buffer, respectively. The resulting radio-HPLC runs of the quality control (QC), the stability in human serum or formulation are shown in **Figure S1**.

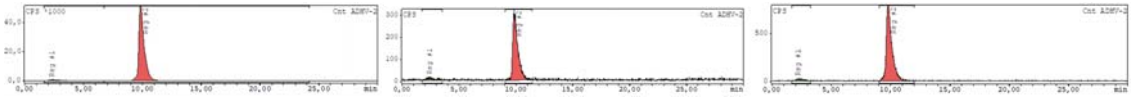

Figure S1: QC (left) and stability assays of [^{18}F]AlF-NOTA-2xDV1(c11sc12s) in formulation buffer (middle) and human serum (right)

1.5. Western blot detection of CXCR4, ACKR3 and CCR5 in mouse liver

Mouse whole liver was homogenized using an R50D homogenizer (CAT) for 20 min at 4°C in 500µL radioimmunoprecipitation assay buffer (RIPA) containing 25 mM Tris HCl, 150 mM NaCl, 1% NP40, 1 mM EDTA, 1 mM PMSF, 2 mM NaF and Protease Inhibitor Cocktail (Roche Applied Science, Basel, Switzerland). After incubating the homogenate for 30 min on ice, it was sonicated and later centrifuged for 30min at 5000 x g at 4°C. The supernatant was then diluted with 200 µL NP-40 and 200µL SDS and upconcentrated using Amicon® Ultra 4 mL centrifugal filters (Merck, Darmstadt, Germany). The remaining supernatant was dissolved in sample buffer (4x) containing DTT (5 mM) and warmed to 40°C for 35 min. The samples were then separated on a 4 to 12% Criterion XT Bis–Tris SDS gel (Bio-Rad, Hercules, CA, USA) using XT MES (Bio-Rad, Hercules, CA, USA). Once transferred onto a PVDF membrane, the surface was blocked with 3% BSA, which was supplemented with 2.5% non-fat dried milk powder for the detection of unphosphorylated proteins. After three washing cycles using TBST, the antibodies were added in a 1:1500 ratio in 3% BSA and were incubated overnight at 4°C. The added antibodies included unphosphorylated CXCR4, pS342.CCR5, and unphosphorylated ACKR3, which were purchased 7TM Antibodies (Jena, Germany). The secondary antibody was added in a 1:2000 ratio in a 3% BSA TBST solution and let incubating for two hours at room temperature. After adding SuperSignal West Femto (Thermo Fisher Scientific, Waltham, MA, USA) to the membrane, the chemiluminescence was detected with the ChemiDoc MP system (Bio-Rad, Hercules, CA, USA) and

Image Lab software v5.0 (Bio-Rad, Hercules, CA, USA). The results from the Western blot assessing the expression of CXCR4, CCR5 and ACKR3 in murine whole liver has been added in **Figure S2**.

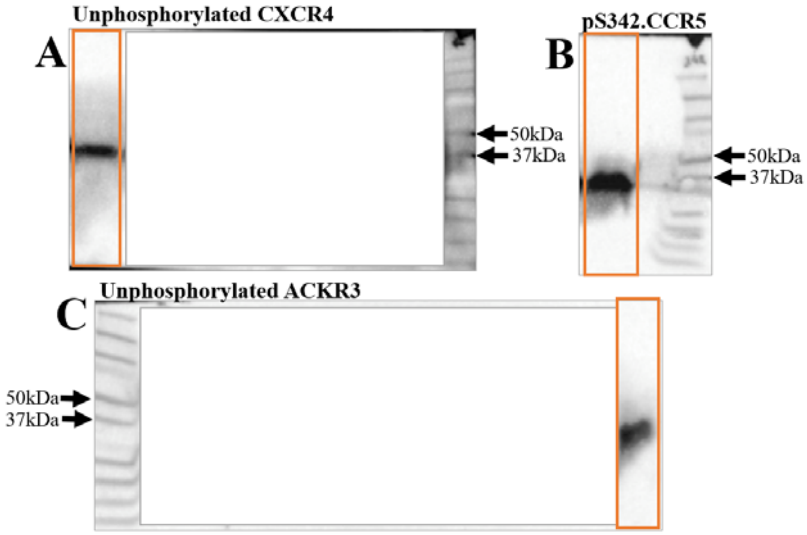

Figure S2: Western Blots of mouse whole liver homogenate performed with unphosphorylated CXCR4 (A), pS342.CCR5 (B) and unphosphorylated ACKR3 (C). The specific receptor lane has been highlighted in orange.

1.6. Radiometabolite study in mice and rhesus macaque

The radiotracer's stability in the plasma or urine of mice or the rhesus macaque have been added in **Figure S3-S5**, respectively.

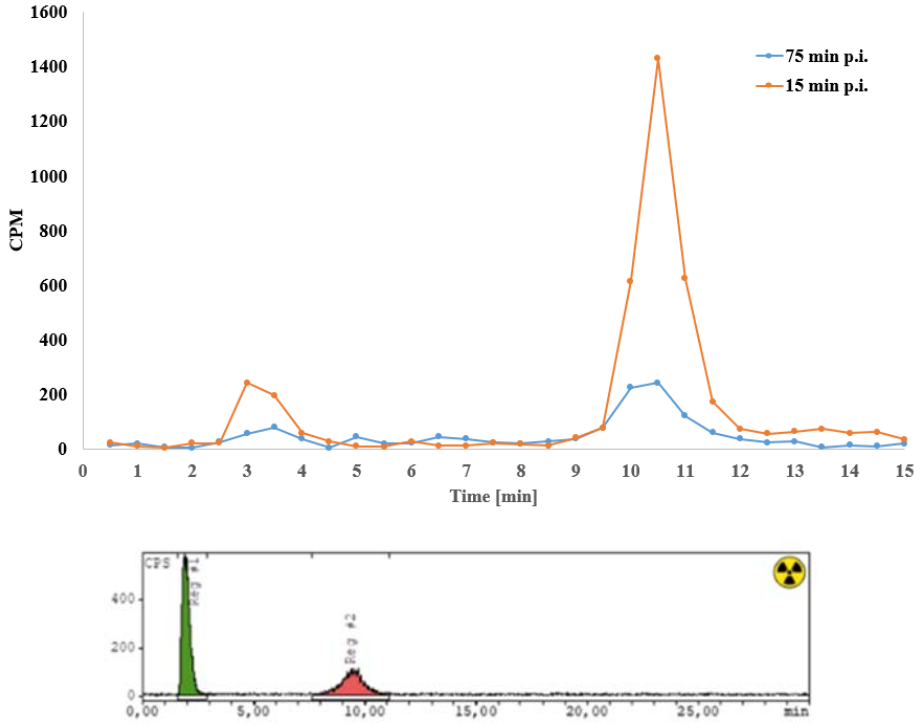

Figure S3: In vivo metabolite study of [^{18}F]AlF-NOTA-2xDV1(c11sc12s) in plasma 15 min or 75 min p.i. (top) and urine (bottom) of NMRI mice

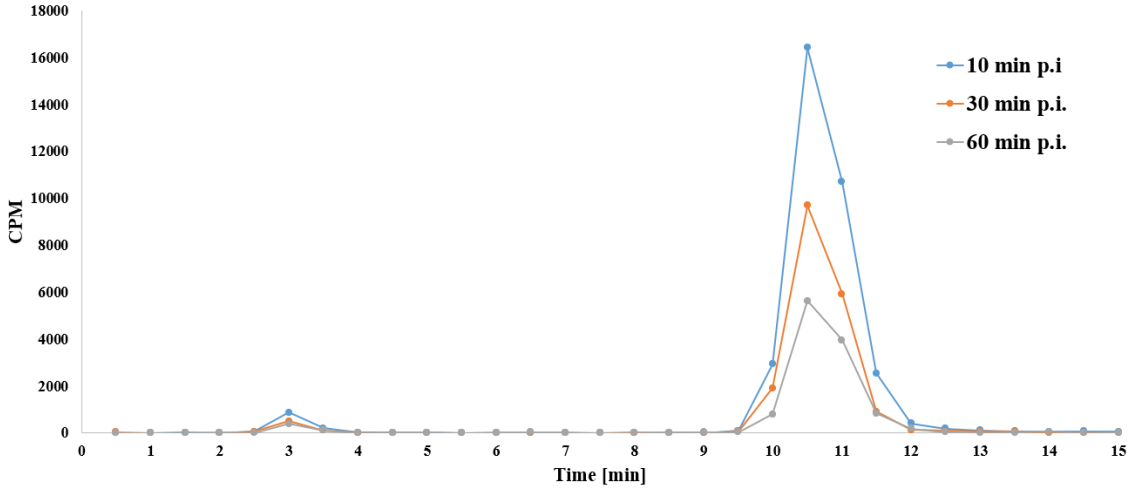

Figure S4: In vivo metabolite study of [^{18}F]AlF-NOTA-2xDV1(c11sc12s) in plasma at 10min (blue), 30 min (yellow) or 60 min (grey) p.i. of a rhesus macaque

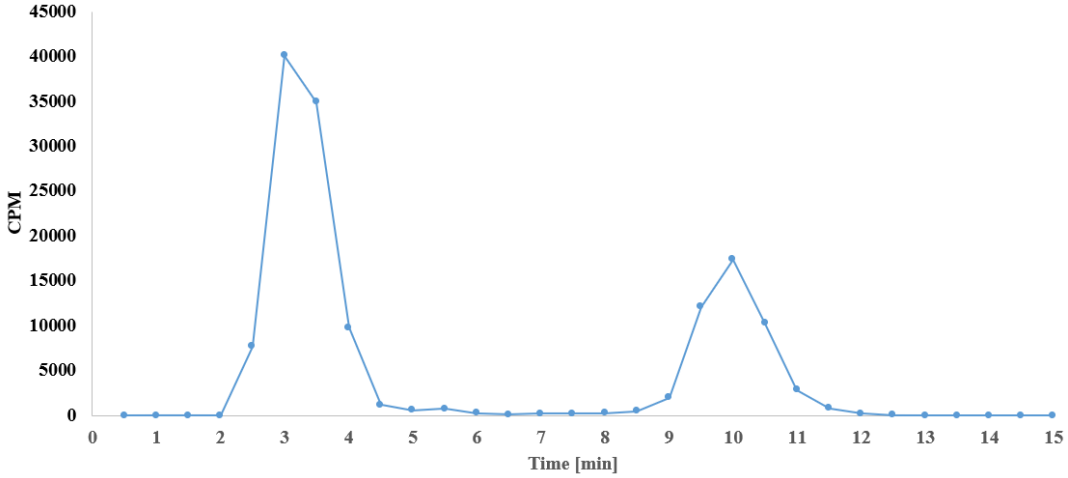

Figure S5: In vivo radiometabolite study of [^{18}F]AlF-NOTA-2xDV1(c11sc12s) in urine 180 min p.i. of a rhesus macaque