SUPPLEMENTARY INFORMATION

An Ultra-High Affinity Small Organic Ligand of Fibroblast Activation Protein for Tumor Targeting Applications

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Author Contributions: J.Millul, D.N. and S.C. designed and planned the study. J.Millul performed most of the experiments with the help of the other authors as follows. S.D.P., L.N. and A.G. produced human and murine recombinant FAP. A.G., E.S., I.B., E.J.D. and F.S. contributed to the *in vitro* characterization of novel FAP ligands. J.Millul and A.E. generated

hFAP expressing tumor cell lines and performed FACS experiments. J.Millul and S.C. performed confocal microscopy analysis. J.M. and A.Z. designed and performed the *ex vivo* tumor and organ penetration experiment. J.Millul and S.C. performed the IVIS experiments. G.B., J. Mock and S.C. performed the biodistribution studies with radiolabeled compounds. J.Millul and C.P. performed the *in vitro* UniCAR-T killing experiments. J.Millul prepared the figures. J.Millul, D.N. and S.C. wrote the manuscript.

Competing Interest Statement: D.N. is a co-founder and shareholder of Philogen (www.philogen.com), a Swiss-Italian Biotech company that operates in the field of ligand-based pharmacodelivery. J.M., G.B., A.Z., S.D.P., L.N., A.G., E.S., I.B., E.J.D., F.S. and S.C. are employees of Philochem AG, daughter company of Philogen acting as discovery unit of the group.

Classification: BIOLOGICAL SCIENCES/Medical Sciences

Keywords: Tumor Targeting, Small Molecule Therapeutics, Fibroblast Activation Protein, Theranostics

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Abbreviations

AA	Antibiotic Antimycotic
AAZ	Acetazolamide
AMC	7-amido-4-methylcoumarin
Asp	Aspartic Acid
Boc	*tert*-Butyloxycarbonyl
Cit	Citrulline
CT	Chlorotrityl
Cys	Cysteine
DIPEA	*N*,*N*-Diisopropylethylamine
DCC	N,N'-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DMAP	4-(Dimethylamino)pyridine
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethylsulfoxide
ESI	Electrospray Ionization
eq	Equivalents
FA	Formic Acid
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
Fmoc	9-Fluorenylmethoxycarbonyl
Glu	Glutamic acid
Gly	Glycine
HATU	*O*-(7-azabenzotriazol-1-yl)-tetramethyl-uronium hexafluorophosphate
HBSS	Hank's Balanced Salt Solution
HPLC	High Performance Liquid Chromatography
IC_{50}	Half maximal inhibitory concentration
ID/g	Injected dose / gram
K_D	Dissociation constant

LC/MS	Liquid Chromatography / Mass Spectrometry
Lys	Lysine
m/z	mass-to-charge ratio
MC	Maleimido-caproyl
MeCN	Acetonitrile
MMAE	Monomethyl Auristatin E
MS	Mass Spectroscopy
mQ	Milli-Q
MW	Molecular Weight
PAB	*para*-Aminobenzoyl
PBS	Phosphate-Buffered Saline
ppm	Part per million
Pro	Proline
RP	Reverse Phase
RPMI	Roswell Park Memorial Institute
r.t.	Room Temperature
SD	Standard Deviation
SEM	Standard error of the mean
SPPS	Solid Phase Peptide Synthesis
*t*Bu	*tert*-Butyl
TFA	Trifluoroacetic Acid
THF	Tetrahydrofuran
TIPS	Triisopropylsilane
Trt	Trityl
Val	Valine
w.t.	wild type
Z	Benzyloxycarbonyl

General Remarks and Procedures

Peptide grade *N,N*-dimethylformamide (DMF) for solid phase synthesis was bought from VWR. All other solvents were used as supplied by Merck or Sigma Aldrich in HPLC or analytical grade. H-Cys(Trt)-2-CT-polystyrene resin was purchased from RAPP Polymere (Ernst-Simon-Strasse 9, 72072 Tuebingen, Germany). Maleimidocaproyl-ValCit-*p*-aminobenzylalcohol-MMAE was purchased from Levena Biopharma (No.9 Weidi Road, Qixia District, Nanjing, 210046, China).

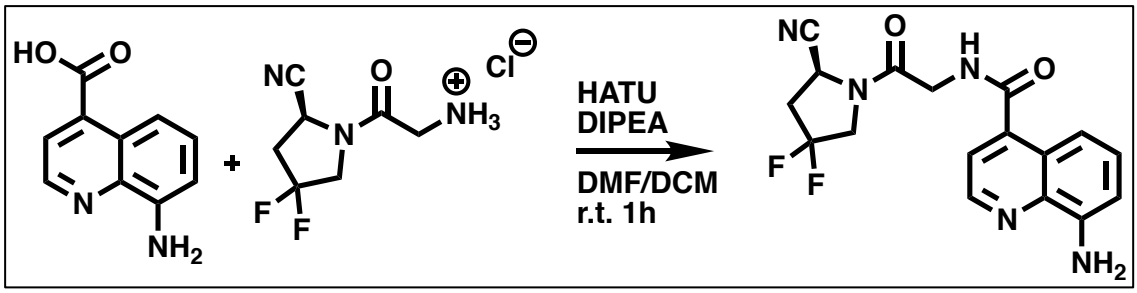
All other reagents were purchased from Sigma Aldrich, Thermo Fisher, Fluorochem, Chematech or Astatech and used as supplied. Yields refer to chromatographically purified and spectroscopically pure compounds, unless noted otherwise.

Liquid-Chromatography/Mass-Spectrometry (LC/MS) spectra presented were recorded on an Agilent 6100 Series Single Quadrupole MS system combined with an Agilent 1200 Series LC, using an InfinityLab Poroshell 120 EC-C18 Column, 2.7 µm, 4.6 × 50 mm at a flow rate of 0.6 ml min^{-1}, 10% MeCN in 0.1% aq. FA to 100% MeCN in 6 min.

Reversed-phase high-pressure liquid chromatography (RP-HPLC) were performed on an Agilent 1200 Series RP-HPLC with PDA UV detector, using a Synergi 4µm, Polar-RP 80Å 10 **×** 150 mm C18 column at a flow rate of 5 ml min^{-1} with linear gradients of solvents A and B (A = Millipore water with 0.1% TFA, B = MeCN with 0.1% TFA).

Size-exclusion chromatography was performed on a Superdex 200 Increase 10/300 GL column on an ÄKTA FPLC (GE Healthcare).

Synthesis of Intermediate A



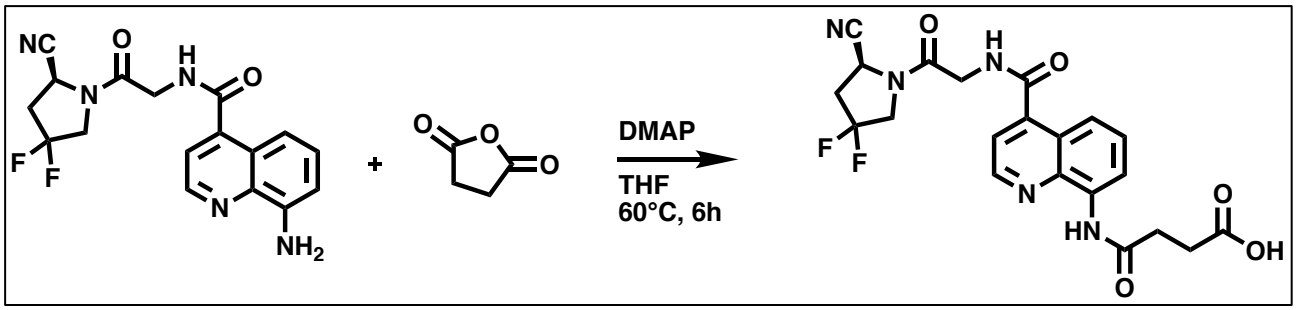
In a 25mL round bottom flask, 8-aminoquinoline-4-carboxylic acid (100 mg, 0.531 mmol, 1 eq), (*S*)-1-(2-aminoacetyl)-4.4-difluoropyrrolidine-2-carbonitrile hydrochloride (132 mg, 0.585 mmol, 1.1 eq) and HATU (202 mg, 0.531 mmol, 1 eq) were suspended in 900 µL of DMF and 4 mL of DCM.

DIPEA (371 µL, 2.127 mmol, 4 eq) was added dropwise and the reaction was stirred until completion (checked via LC/MS with the method 90:10 Water/Acetonitrile 0.1% FA to 100% Acetonitrile 0.1% FA in 3min, Positive).

The crude was diluted with DCM, washed with water, dried over Na_2SO_4, filtered and the solvent evaporated under vacuum.

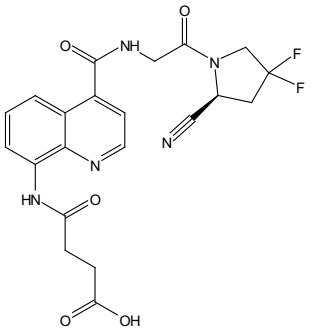
The dried crude was purified via CombiFlesh Nextgen 300+ (parameters: flow 50 ml/min, 40 gr silica column, 100% DCM to 85:15 DCM/MeOH in 5 min) to obtain an amber oil (172 mg, 0.478 mmol, 90% yield). MS (ES^+) *m/z* 360.12 $(M+H)^+$.

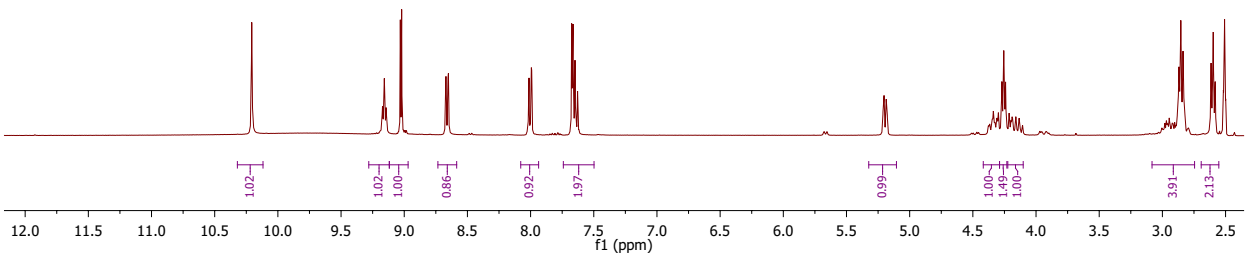
Synthesis of compound 1



In a 25 mL round bottom flask, **Intermediate A** (48 mg, 0.134 mmol, 1 eq), succinic anhydride (669 mg, 6.683 mmol, 50 eq) and DMAP (8 mg, 0.067 mmol, 0.5 eq) were dissolved in 3 mL of THF. The reaction was heated at 60 °C for 6h and checked via LC/MS (method 90:10 Water/Acetonitrile 0.1% FA to 100% Acetonitrile 0.1% FA in 3min, Positive). The reaction was dried under vacuum, diluted with water, extracted with DCM, dried over Na_2SO_4, filtered and concentrated under. The dried crude was purified via CombiFlesh Nextgen 300+ (parameters: flow 30 ml/min, 24 gr silica column, DMC/MeOH 90:10 to 70:30 in 4 minutes) to obtain an amber oil (58 mg, 0.127 mmol, 95% yield). MS (ES^+) *m/z* 460.14 $(M+H)^+$.

1H-NMR (400 MHz, DMSO-d6)

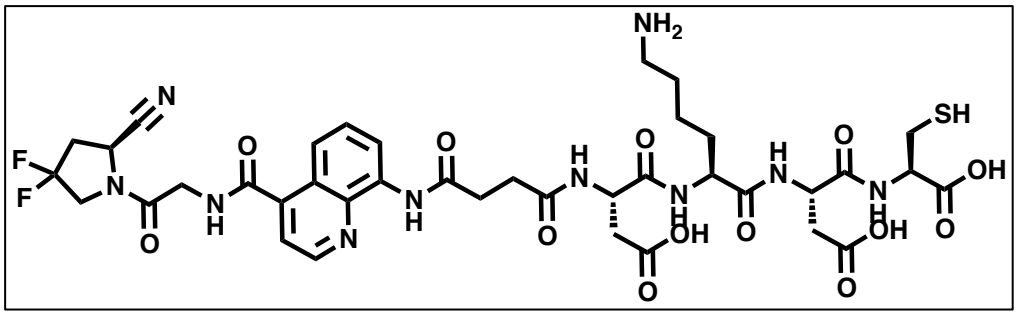




^{1}H NMR (400 MHz, DMSO-d_6) δ 10.21 (s, 1H) **N<u>H</u>Su**, 9.16 (t, *J* = 6.0 Hz, 1H) **N<u>H</u>Gly**, 9.03 (d, *J* = 4.3 Hz, 1H) **C<u>H</u>Qn2**, 8.66 (dd, *J* = 7.8, 1.3 Hz, 1H) **C<u>H</u>Qn7**, 8.00 (dd, *J* = 8.6, 1.3 Hz, 1H) **C<u>H</u>Qn5**, 7.67 (d, *J* = 4.4 Hz, 1H), **C<u>H</u>Qn3**, 7.65 (t, *J* = 8.2 Hz, 1H), **C<u>H</u>Qn6,** 5.19 (dd, *J* = 9.3, 2.7 Hz, 1H) **-C<u>H</u>CN-**, 4.41 – 4.29 (m, 1H) **-NC<u>H</u>2CF2-**, 4.23 – 4.09 (m, 1H) **-NC<u>H</u>2CF2-**, 4.26 (t, *J* = 5.9 Hz, 2H) **C<u>H</u>2Gly**, 3.04 – 2.79 (m, 2H) **-CHCNC<u>H</u>2CF2-**, 2.85 (t, *J* = 7.4 Hz, 3H) **C<u>H</u>2Su**, 2.60 (t, *J* = 7.4 Hz, 2H) **C<u>H</u>2Su**

^{13}C NMR (101 MHz, DMSO-d_6) δ 174.31, 171.25, 168.27, 167.64, 148.91, 143.13, 138.71, 135.27, 129.90, 128.15, 127.44, 124.97, 124.71, 118.32, 117.34, 51.68, 44.72, 41.83, 36.88, 32.02, 29.36

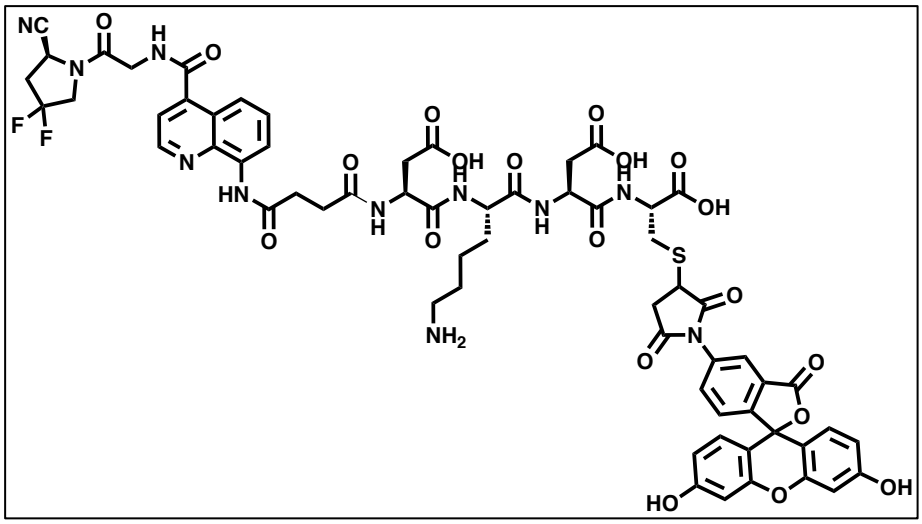
Synthesis of Intermediate B



Commercially available pre-loaded Fmoc-Cys(Trt) on Tentagel resin (500 mg, 0.415 mmol, RAPP Polymere) was swollen in DMF (3 × 5 min × 5 mL), the Fmoc group removed with 20 % piperidine in DMF (1 × 1 min × 5 mL and 2 × 10 min × 5 mL) and the resin washed with DMF (6 × 1 min × 5 mL). The peptide was extended with Fmoc-Asp(tBu)-OH, Fmoc-Lys(NHBoc)-OH and Fmoc-Asp(tBu)-OH in the indicated order. For this purpose, the Fmoc protected amino acid (2.0 eq), HATU (2.0 eq) and DIPEA (4.0 eq) were dissolved in DMF (5 mL) and reacted with the resin for 1 h under gentle agitation. After washing with DMF (6 × 1 min × 5 mL) the Fmoc group was removed with 20 % piperidine in DMF (1 × 1 min × 5 min and 2 × 10 min × 5 mL). Deprotection steps were followed by wash steps with DMF (6 × 1 min × 5 mL) prior to coupling with the next amino acid.

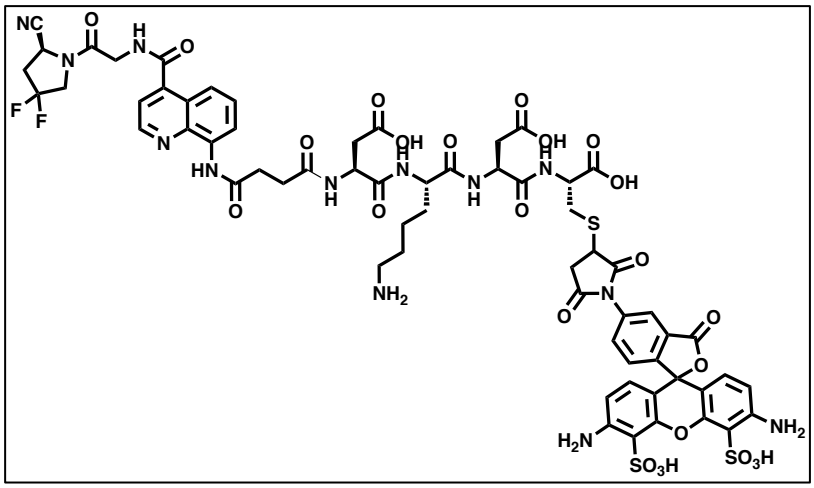
Compound 1 (2 eq) was coupled as described. The peptide was cleaved from the resin with a cleavage cocktail composed by TFA (15%), m-Cresol (3.5%), Thioanisole (3.5%), Triiso-propylsilane (14%), mQ water (4%) and DCM (60%). The crude product was purified by reversed-phase HPLC (95:5 Water/Acetonitrile with 0.1%TFA to 20:80 Water/Acetonitrile with 0.1% TFA in 20 min) and lyophilized, to obtain a white solid (15 mg; 16.3 µmol, 4% yield). MS(ES+) m/z 921.29 $(M+H)^+$.

Synthesis of compound 2



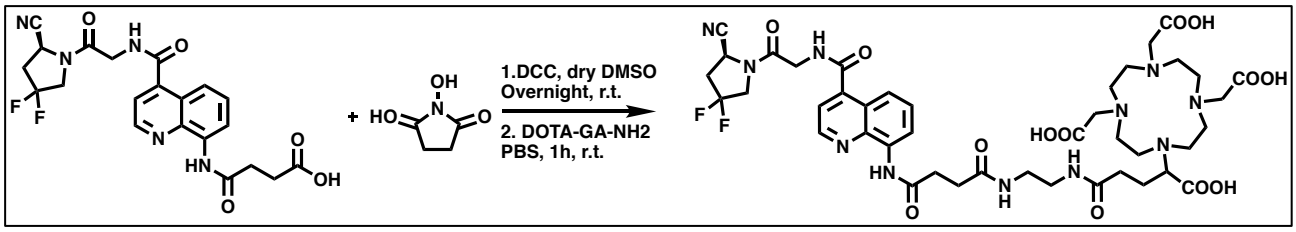
Intermediate B (2 mg, 2.17 µmol, 1.0 eq) was dissolved in PBS pH 7.4 (800 µL). Fluorescein-5-maleimide (1.8 mg, 4.34 µmol, 2.0 eq) was added as dry DMSO solution (200 µL). The reaction was stirred for 3 h at room temperature, protected from the light. The crude material was purified by reversed-phase HPLC (95:5 Water/Acetonitrile with 0.1%TFA to 20:80 Water/Acetonitrile with 0.1% TFA in 20 min) and lyophilized, to obtain a yellow solid (420 nmol, 19.3% yield). MS(ES+) m/z 1348.36 $(M+1H)^{1+}$

Synthesis of compound 3



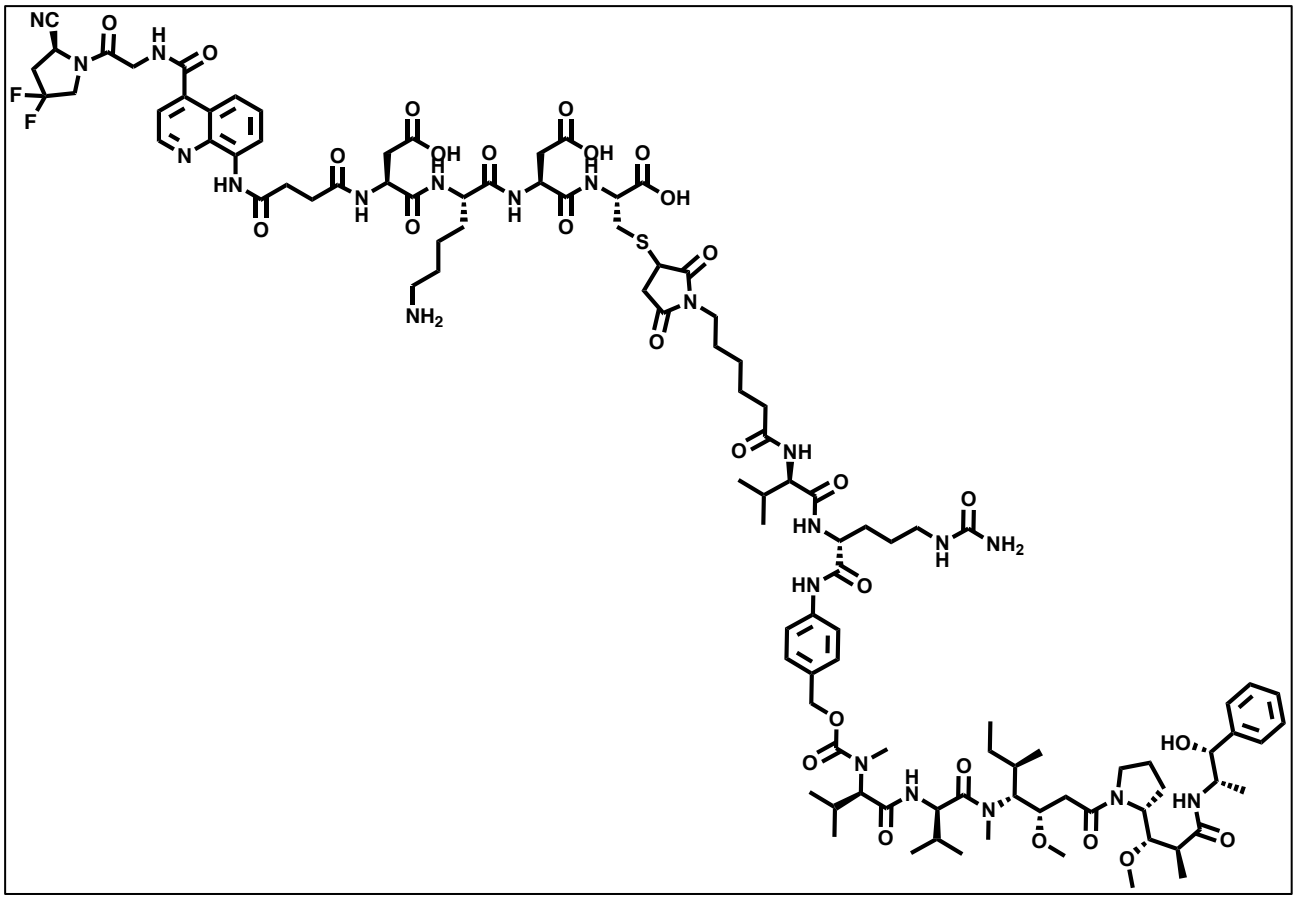
Intermediate B (293 µg, 0.32 µmol, 1.0 eq) was dissolved in PBS pH 7.4 (300 µL). Alexa-488-C5-Maleimide (200 µg, 0.29 µmol, 0.9 eq) was added as dry DMSO solution (1 mg / mL). The reaction was stirred for 3 h at room temperature, protected from the light. The crude material was purified by reversed-phase HPLC (95:5 Water/Acetonitrile with 0.1%TFA to 20:80 Water/Acetonitrile with 0.1% TFA in 20 min) and lyophilized, to obtain an orange solid (70 nmol, 21.9% yield). MS(ES+) m/z 1619.57 $(M+1H)^{1+}$

Synthesis of compound 4



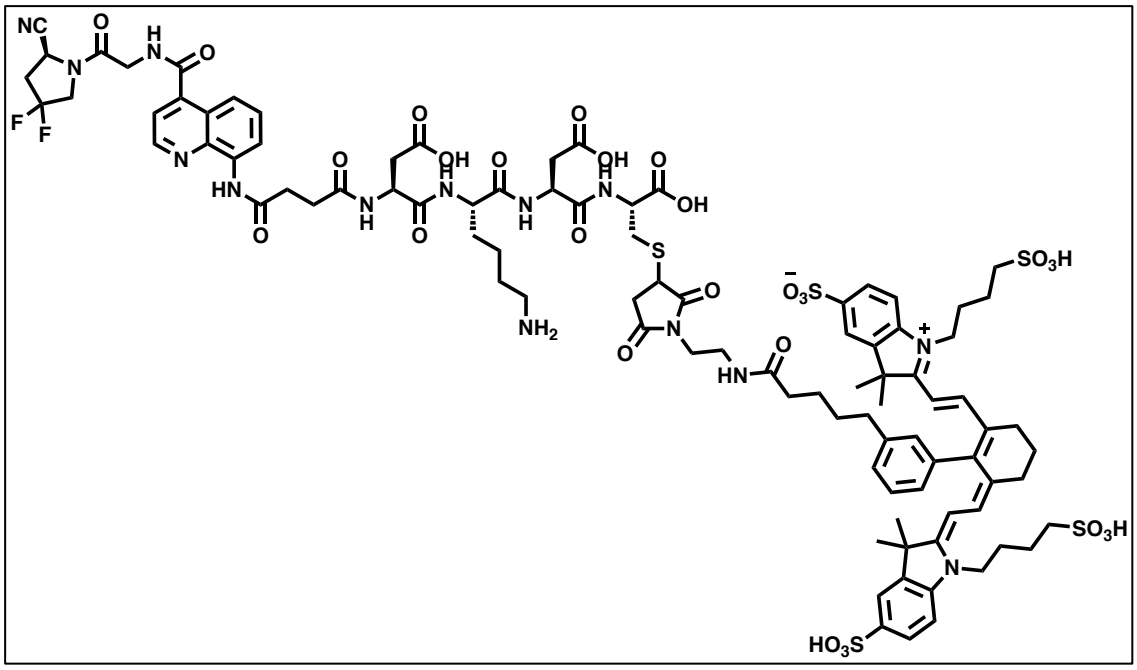
Compound **1** (15 mg, 32.67 µmol, 1 eq), N-hydroxysuccinimide (4.5 mg, 39.2 µmol, 1.2 eq) and DCC (8.8 mg, 42.4 µmol, 1.3 eq) were dissolved in 400µL of dry DMSO and the reaction was stirred overnight at room temperature, protected from the light. The reaction was filtered and at the DMSO solution were added 100 µL of PBS solution containing DOTA-GA-NH2 (20 mg, 38.4 µmol, 1.2 eq) and the resulting solution was stirred at room temperature for 1h. The crude material was purified by reversed-phase HPLC (95:5 Water/Acetonitrile with 0.1%TFA to 20:80 Water/Acetonitrile with 0.1% TFA in 20 min) and lyophilized, to obtain a white powder (3 mg; 3.92 µmol; 10% yield). MS(ES+) m/z 960.39 $(M+2H)^{2+}$

Synthesis of compound 5



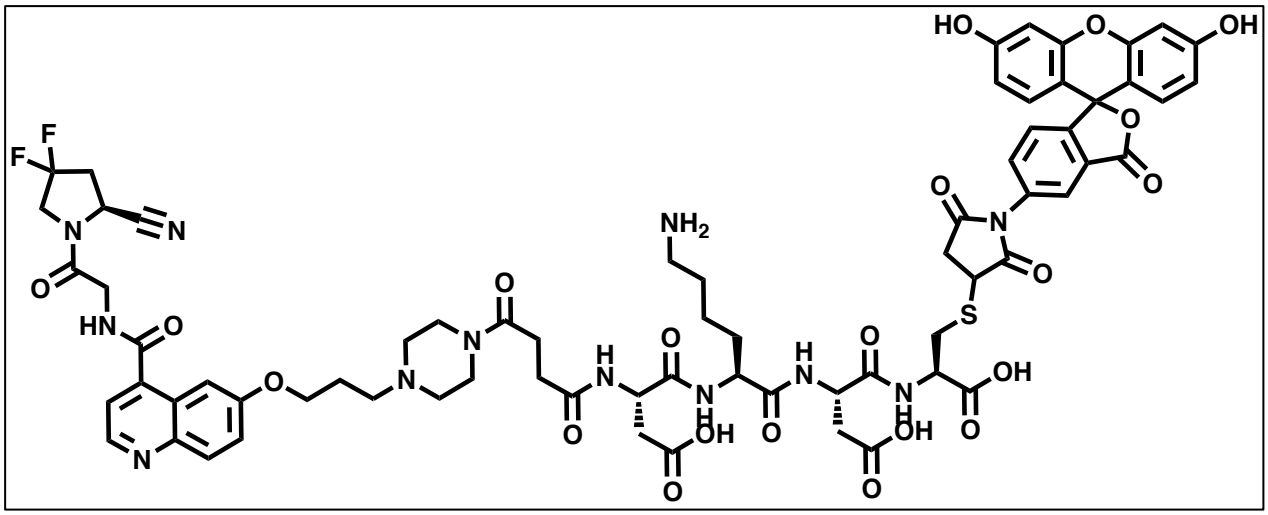
Intermediate B (2 mg, 1.83 µmol, 1.0 eq) was dissolved in PBS pH 7.4 (800 µL). MC-ValCit-MMAE (2.5 mg, 1.8 µmol, 1.0 eq) was added as dry DMF solution (250 µL). The reaction was stirred for 3 h at room temperature and monitored by LC/MS. The crude material was purified by reversed-phase HPLC (95:5 Water/Acetonitrile with 0.1%TFA to 20:80 Water/Acetonitrile with 0.1% TFA in 20 min) and lyophilized, to obtain a white powder (2.43 mg, 1.1 µmol, 60% yield). MS(ES+) m/z 2210.1 $(M+H)^+$

Synthesis of compound 6



Intermediate B (160 µg, 0.174 µmol, 1.0 eq) was dissolved in PBS pH 7.4 (800 µL). IRDye750 (200µg, 0.174 µmol, 1.0 eq) was added as dry DMF solution (200 µL). The reaction was stirred for 3 h. The crude material was purified by reversed-phase HPLC (95:5 Water/Acetonitrile with 0.1%TFA to 20:80 Water/Acetonitrile with 0.1% TFA in 20 min) and lyophilized, to obtain a green solid (0.08 µmol, 50% yield). MS(ES+) m/z 1036.3 $(M+2H)^{2+}$

Synthesis of compound 7



(S)-N-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)-6-(3-(piperazin-1-yl) propoxy)quinoline-4-carboxamide was synthetized as previously described in the literature. Commercially available pre-loaded Fmoc-Cys(Trt) on Tentagel resin (500 mg, 0.415 mmol, RAPP Polymere) was swollen in DMF (3 × 5 min × 5 mL), the Fmoc group removed with 20 % piperidine in DMF (1 × 1 min × 5 mL and 2 × 10 min × 5 mL) and the resin washed with DMF (6 × 1 min × 5 mL). The peptide was extended with Fmoc-Asp(tBu)-OH, Fmoc-Lys(NHBoc)-OH and Fmoc-Asp(tBu)-OH in the indicated order. For this purpose, the Fmoc protected amino acid (2.0 eq), HATU (2.0 eq) and DIPEA (4.0 eq) were dissolved in DMF (5 mL) and reacted with the resin for 1 h under gentle agitation. After washing with DMF (6 × 1 min × 5 mL) the Fmoc group was removed with 20 % piperidine in DMF (1 × 1 min × 5 min and 2 × 10 min × 5 mL). Deprotection steps were followed by wash steps with DMF (6 × 1 min × 5 mL) prior to coupling with the next amino acid.

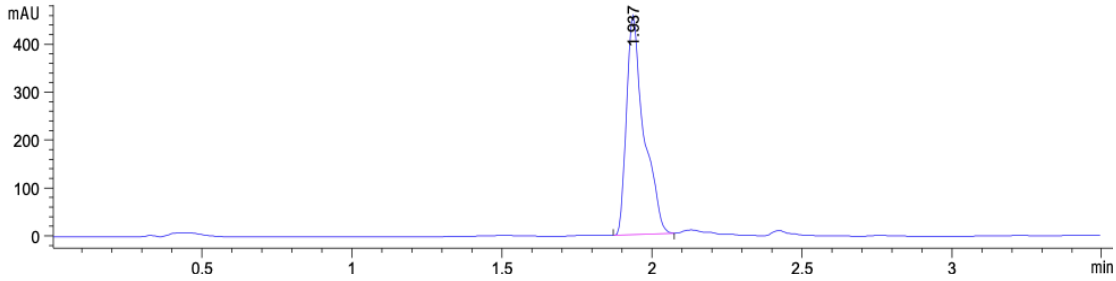
(S)-N-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)-6-(3-(piperazin-1-yl) propoxy)quinoline-4-carboxamide (2 eq) was coupled as described. The peptide was cleaved from the resin with a cleavage cocktail composed by TFA (15%), m-Cresol (3.5%), Thioanisole (3.5%), Triisopropylsilane (14%), mQ water (4%) and DCM (60%). The crude product was purified by reversed-phase HPLC (95:5 Water/Acetonitrile with 0.1%TFA to 20:80 Water/Acetonitrile with 0.1% TFA in 20 min) and lyophilized, to obtain a white solid.

The obtained peptide (2 mg, 1.9 µmol, 1.0 eq) was dissolved in PBS pH 7.4 (800 µL). Fluorescein-5-maleimide (2 mg, 4.82 µmol, 2.5 eq) was added as dry DMSO solution (200 µL). The reaction was stirred for 3 h at room temperature, protected from the light. The crude

material was purified by reversed-phase HPLC (95:5 Water/Acetonitrile with 0.1%TFA to 20:80 Water/Acetonitrile with 0.1% TFA in 20 min) and lyophilized, to obtain a yellow solid (420 nmol, 19.3% yield). MS(ES+) m/z 1474.46 $(M+1H)^{1+}$

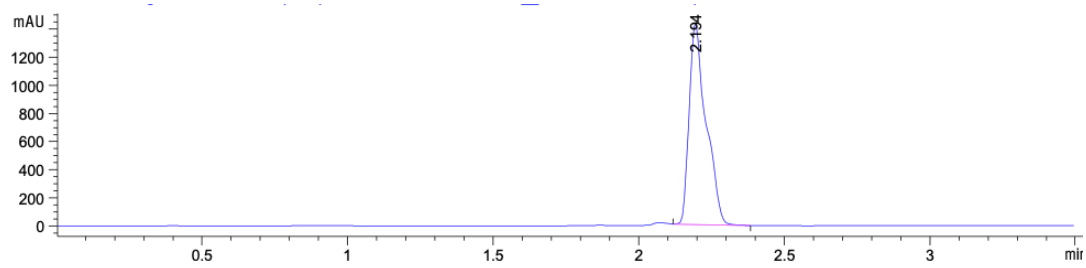
LC/MS and HPLC profiles

Intermediate ***A***



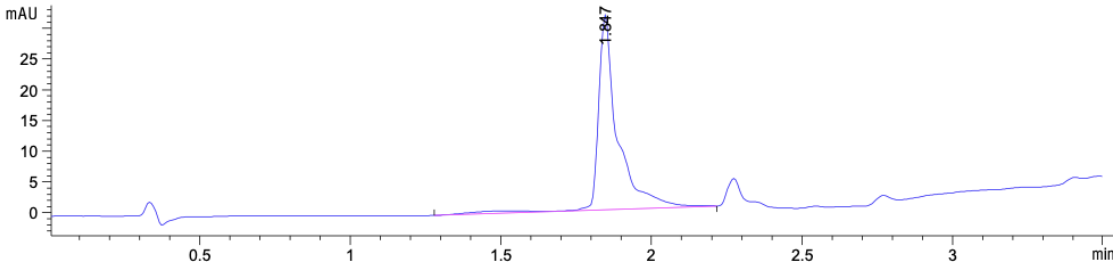
MS (ESI) *m/z* calculated for $[C_{17}H_{15}F_2N_5O_2]^+$: 360.12 $[M+H]^+$, found: 360.20.

Compound ***1***



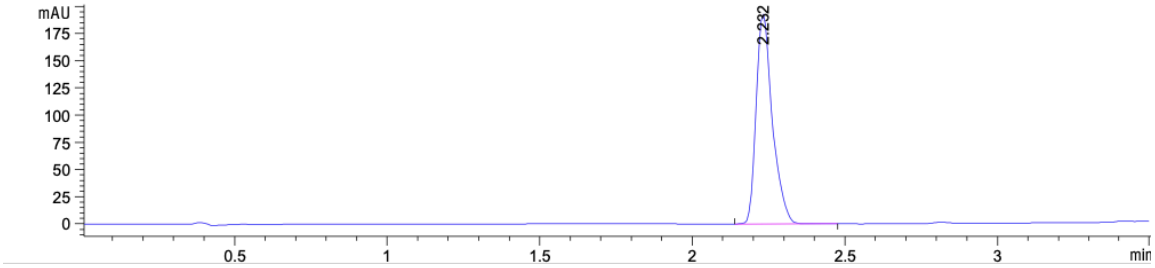
MS (ESI) *m/z* calculated for $[C_{21}H_{19}F_2N_5O_5]^+$: 460.14 $[M+1H]^{1+}$, found: 460.20.

Intermediate ***B***



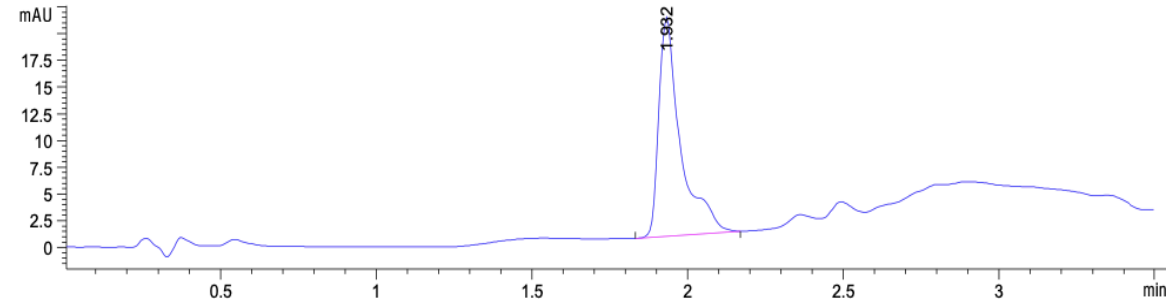
MS (ESI) *m/z* calculated for $[C_{38}H_{46}F_2N_{10}O_{13}S]^+$: 921.29 $[M+H]^+$, found: 921.30.

Compound ***2***



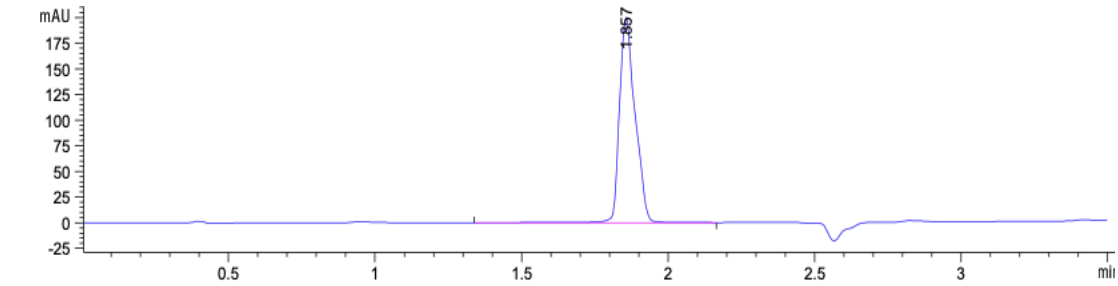
MS (ESI) *m/z* calculated for $[C_{62}H_{59}F_2N_{11}O_{20}S]^+$: 1348.36 $[M+H]^+$, found: 1348.40.

Compound ***3***



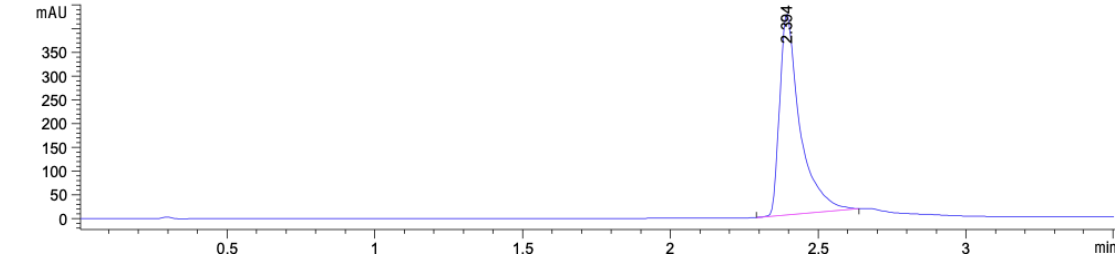
MS (ESI) *m/z* calculated for $[C_{68}H_{72}F_2N_{14}O_{25}S_3]^+$: 1619.39 $[M+H]^+$, found: 1618.30.

Compound ***4***



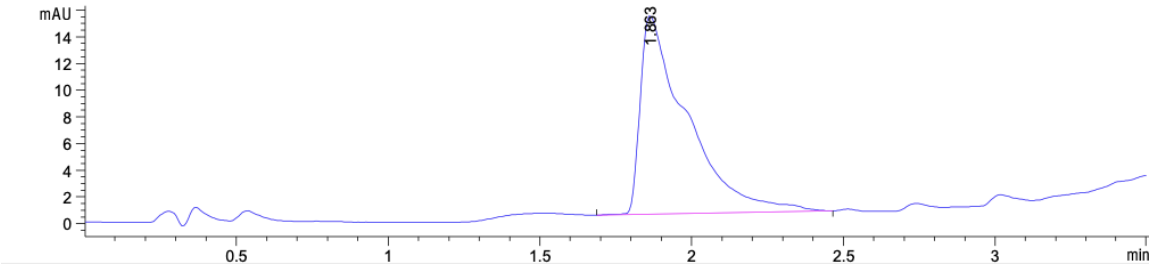
MS (ESI) *m/z* calculated for $[C_{42}H_{55}F_2N_{11}O_{13}]^+$: 960.39 $[M+H]^+$, found: 960.40

Compound ***5***



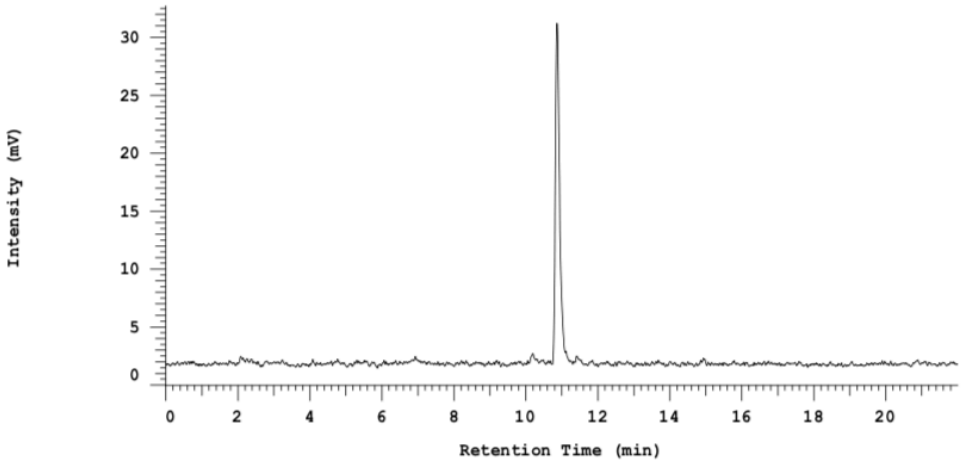
MS (ESI) *m/z* calculated for $[C_{106}H_{151}F_2N_{21}O_{28}S]^+$: 1118.9 $[M+2H]^{2+}$, found: 1119.1

Compound ***6***



MS (ESI) *m/z* calculated for $[C_{93}H_{112}F_2N_{14}O_{28}S_5]^+$: 2071.64 $[M+H]^+$, found: 1034.20 $[M+H]^{2+}$

lutetium-177-OncoFAP



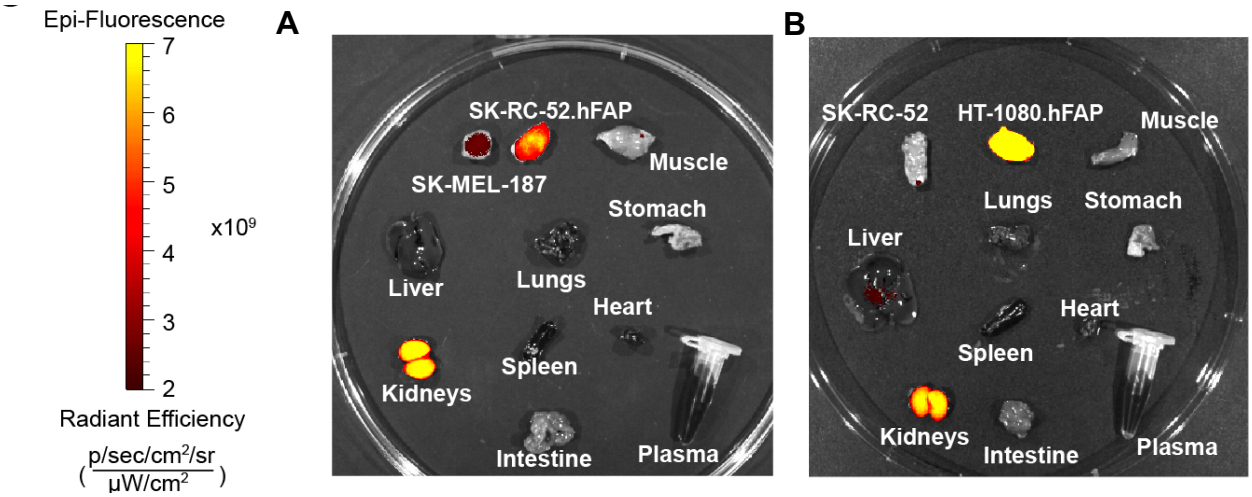
Radio-HPLC profile of lutetium-177-OncoFAP

IVIS Experiment

SK-MEL-187 or SK-RC-52.wt xenografted tumors were implanted into the right flank of female athymic Balb/c AnNRj-Foxn1 mice (6-8 weeks of age) as described above, and allowed to grow to an average volume of 200 mm^3.

SK-RK-52.hFAP or HT-1080.hFAP xenografted tumors were implanted into the left flank of female athymic Balb/c AnNRj-Foxn1 mice (6-8 weeks of age) as described above, and allowed to grow to an average volume of 200 mm^3.

Mice were injected intravenously with compound **4** (150 nmol/Kg, as 30 µM solutions prepared in sterile PBS, pH 7.4). One hour after the intravenous injection, mice were sacrificed by CO_2 asphyxiation. Blood, heart, muscle, lung, kidneys, liver, spleen, stomach, a section of the intestine, SK-MEL-187 tumor, SK-RC-52.hFAP tumor, SK-RC-52.wt tumor and HT-1080.hFAP tumor were collected and fluorescence images acquired on an IVIS Spectrum imaging system (Xenogen, exposure 1s, binning factor 8, excitation at 745 nm, emission filter at 800 nm, f number 2, field of view 13.1).

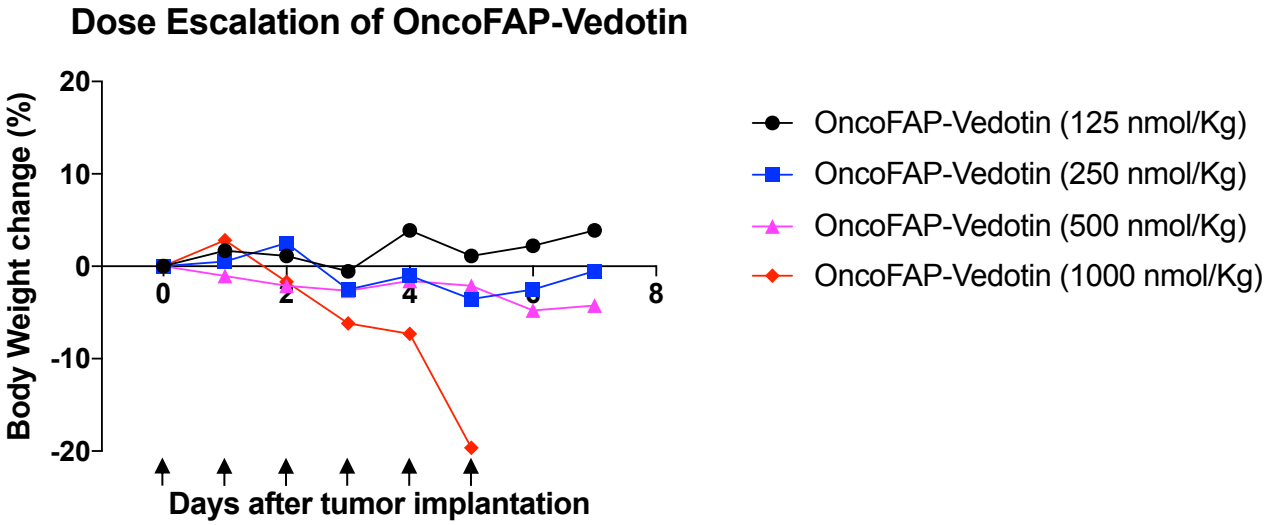


SI 1 (A) Near-infrared fluorescence imaging evaluation of the targeting performance of targeted dye compound **6** (250 nmol/kg) in mice bearing SK-RC-52.hFAP tumors (right flank of the animals). SK-MEL-187 tumors were implanted in the left flank of the animals and used as negative control (no expression of FAP). Images were collected 1 h after the intravenous injection. **(B)** Near-infrared fluorescence imaging evaluation of the targeting performance of targeted dye compound **6** (250 nmol/kg) in mice bearing HT-1080.hFAP tumors (right flank of the animals). SK-RC-52.wt tumors were implanted in the left flank of the animals and used as negative control (no expression of FAP). Images were collected 1 h after the intravenous

injection. OncoFAP-IRDye750 (compound **6**) selectively accumulated in FAP-positive tumors (HT-1080.hFAP and SK-RC-52.hFAP).

Dose escalation study in SK-RC-52.hFAP

A dose escalation study in order to assess the tolerability of OncoFAP-Vedotin has been performed. SK-RC-52.hFAP tumors were implanted into the right flank of female athymic Balb/c AnNRj-Foxn1 mice (6-8 weeks of age) as described in the manuscript, and allowed to grow to an average volume of 100 mm^3. Mice received daily intravenous injections of 125 nmol/kg, 250 nmol/kg, 500 nmol/kg and 1000 nmol/kg of OncoFAP-vedotin (compound **5**). as sterile PBS solution with 2% of DMSO. Animals were weighted daily to assess tolerability of the treatment. Animals were sacrificed when one or more termination criteria indicated by the experimental license were reached (e.g. weight loss > 15%).



SI 2 Body weight change of mice treated with different doses of OncoFAP-Vedotin. At 1000 nmol/kg, OncoFAP-Vedotin was highly toxic. All the other treatments were well tolerated.

Therapy experiment with OncoFAP-Vedotin in combination with L19-IL2 in tumor-bearing mice

Statistical analysis of tumor size (mm^3) differences is performed using Prism 6 software (GraphPad Software, regular two-way ANOVA followed by Bonferroni test).

OncoFAP-Vedotin + L19-IL2 vs L19--IL2

Day 19	$p < 0.05$
From day 20	$p < 0.0001$

OncoFAP-Vedotin + L19-IL2 vs OncoFAP-Vedotin

Day 29	$p < 0.001$
From day 30	$p < 0.0001$

OncoFAP-Vedotin + L19-IL2 vs vehicle

Day 12	$p < 0.05$
From day 13	$p < 0.0001$

OncoFAP-Vedotin vs L19-IL2

Day 15	$p < 0.01$

OncoFAP-Vedotin vs vehicle

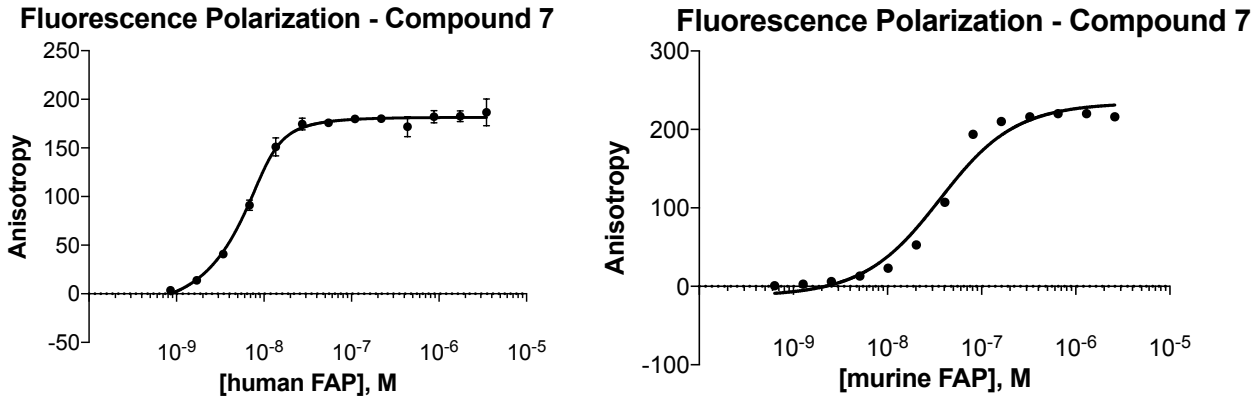
Day 12	$p < 0.05$
From day 13	$p < 0.0001$

L19-IL2 vs vehicle

From day 15	$p < 0.0001$

In vitro affinity measure of FAPI-04

The *in vitro* K_D measurement of FAPI-04 fluorescent derivative is shown below.



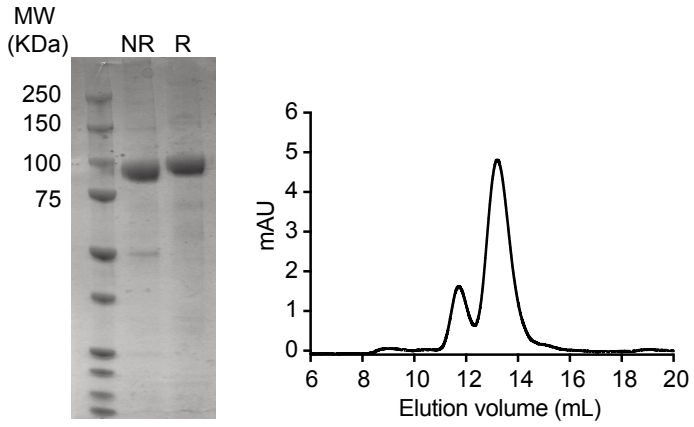
SI 3 Affinity of Compound **7** for human and murine FAP was measured by fluorescence polarization. Compound **7** shows a lower affinity for human and murine FAP ($K_{D,humanFAP}$ = 1.02 nM, $K_{D,murineFAP}$ = 30.9 nM) compared with OncoFAP-Fluorescein, [**Figure 1C**] ($K_{D,humanFAP}$ = 0.68 nM, $K_{D,murineFAP}$ = 11.6 nM).

Protein Sequence of Extracellular Domain Murine FAP and quality control

LRPSRVYKPEGNTKRALTLKDILNGTFSYKTYFPNWISE-
QEYLHQSEDDNIVFYNIETRESYIILSNSTMKSVNATDYGLSPDRQFVYLESDYSKLWRY
SYTATYYIYDLQNGEFVRGYELPRPIQYLCWSPVGSKLAYVYQNNI-
YLKQRPGDPPFQITY-
TGRENRIFNGIPDWVYEEEMLATKYALWWSPDGKFLAYVEFNDSDIPIIAYSYYGDGQY
PRTINIPYPKAGAKNPVVRVFIVDTTYPHHVGPMEVPVPEMIASSDYYFSWLT-
WVSSERVCLQWLKRVQNVSVLSICDFREDWHAWECPKNQEHVEESRTGWAGGFFVS
TPAFSQDATSYYKIFSDKDGYKHIHYIKDTVENAIQITSGKWEAIYIFRVTQDSLFYSS-
NEFEGYPGRRNIYRISIGNSPPSKKCVTCHLRKERCQYYTASFSYKAKYYALVCYGPGL
PISTLHDGRTDQEIQVLEENKELENSLRNIQLPKVEIKKLKDGGLT-
FWYKMILPPQFDRSKKYPLLIQVYGGPCSQSVKSVFAVNWITYLASKEGIVIALVDGRGT
AFQGDKFLHAVYRKLGVYEVEDQLTAVRKFIEMGFIDEERIAIWGWSYGGYVSSLALAS-
GTGLFKCGIAVAPVSSWEYYASIYSERFMGLPTKDDNLEHYKNSTVMARAEYFRNVDY
LLIHGTADDNVHFQNSAQIAKALVNAQVDFQAMWYSDQNHGISSGRSQNHLYTHMTH-
FLKQCFSLSD

mFAP Quality control

SDS-page and size-exclusion chromatography were performed in order to confirm the quality of the protein.



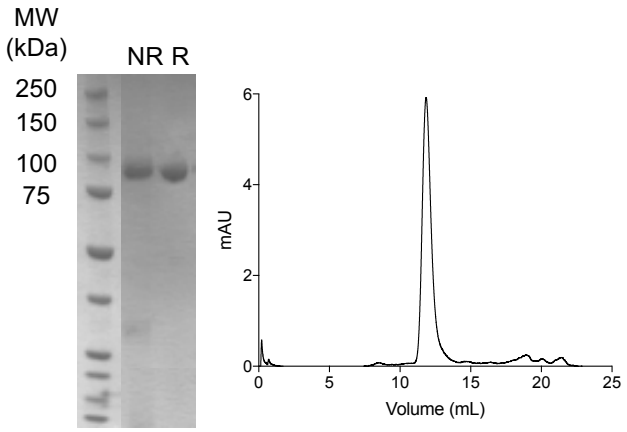
SI 3: The biochemical properties of murine FAP protein were assessed by SDS-PAGE (A), size exclusion chromatography eluting after around 13 mL (elution volume) (B) and by ESI-MS (C). NR, non-reducing conditions; R, reducing conditions.

Protein Sequence of Extracellular Domain Human FAP and quality control

LRPSRVHNSEENTMRALTLKDILNGTFSYKTFFPNWISGQEYLHQSADNNI-
VLYNIETGQSYTILSNRTMKSVNASNYGLSPDRQFVYLESDYSKLWRYSYTATYYIYDLSN
GEFVRGNELPRPIQYLCWSPVGSKLAYVYQNNIYLKQRPGDPPFQITFNGRENKIFNGI-
PDWVYEEEMLATKYALWWSPNGKFLAYAEFNDTDIPVIAYSYYGDEQYPRTINIPYPKAG
AKNPVVRIFIIDTTYPAYVGPQEVPVPAMIASSDYYFSWLTWVTDERVCLQWLKRV-
QNVSVL-
SICDFREDWQTWDCPKTQEHIEESRTGWAGGFFVSTPVFSYDAISYYKIFSDKDGYKHIH
YIKDTVENAIQITSGKWEAINIFRVTQDSLFYSSNEFEEYPGRRNI-
YRISIGSYPPSKKCVTCHLRKERCQYYTASFSDYAKYYALVCYGPGIPISTLHDGRTDQEI
KILEENKELENALKNIQLPKEEIKKLEVDEITLWYKMILPPQFDRSKKYPLLIQVYGG-
PCSQSVRSVFAVNWISYLASKEGMVIALVDGRGTAFQGDKLLYAVYRKLGVYEVEDQITA
VRKFIEMGFIDEKRIAIWGWSYGGYVSSLALASGTGLFKCGIAVAPVSSWEYYASVYTER-
FMGLPTKDDNLEHYKNSTVMARAEYFRNVDYLLIHGTADDNVHFQNSAQIAKALVNAQV
DFQAMWYSDQNHGLSGLSTNHLYTHMTHFLKQCFSLSD

hFAP Quality control

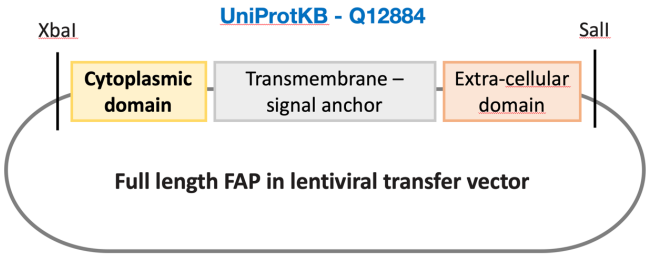
SDS-page and size-exclusion chromatography were performed in order to confirm the quality of the protein.



SI 4: The biochemical properties of human FAP protein were assessed by SDS-PAGE (A), size exclusion chromatography eluting after around 13 mL (elution volume) (B) and by ESI-MS (C). NR, non-reducing conditions; R, reducing conditions.

hFAP Lentiviral transfer vector and amino acid sequence

The strategy to generate the lentiviral transfer vector with hFAP in shown below, together with the amino acid sequence.



(A) **Cytoplasmic domain**
MKTW

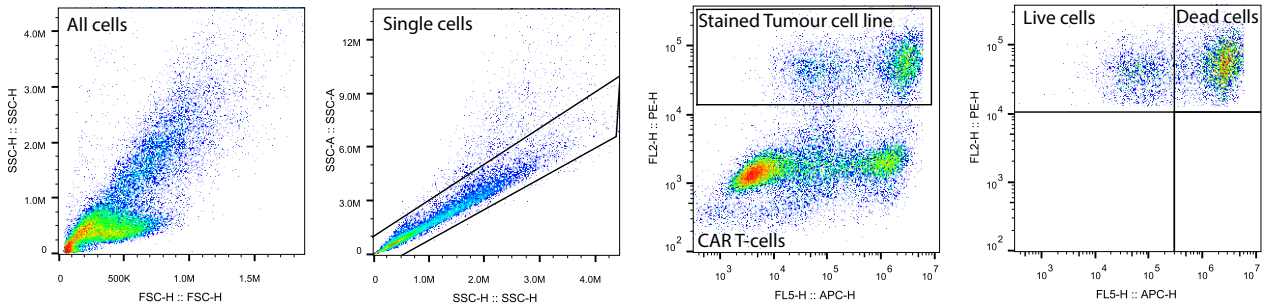
(B) **Transmembrane - signal anchor**
VKIVFGVATSAVLALLVMCIV

(C) **Extra-cellular domain**
LRPSRVHNSEENTMRALTLKDILNGTFSYKTFFPNWISGQEYLHQSADNNIVLYNIETGQSYTILSNRTMKSVNASNYGLSPDRQFVYLESDYSKLWRYSYTATYYIYDLS
NGEFVRGNELPRPIQYLCWSPVGSKLAYVYQNNIYLKQRPGDPPFQITFNGRENKIFNGIPDWVYEEEMLATKYALWWSPNGKFLAYAEFNDTDIPVIAYSYYGDEQY
PRTINIPYPKAGAKNPVVRIFIIDTTYPAYVGPQEVPVPAMIASSDYYFSWLTWVTDERVCLQWLKRVQNVSVLSICDFREDWQTWDCPKTQEHIEESRTGWAGGFFV
STPVFSYDAISYYKIFSDKDGYKHIHYIKDTVENAIQITSGKWEAINIFRVTQDSLFYSSNEFEEYPGRRNIYRISIGSYPPSKKCVTCHLRKERCQYYTASFSDYAKYYALVCY
GPGIPISTLHDGRTDQEIKILEENKELENALKNIQLPKEEIKKLEVDEITLWYKMILPPQFDRSKKYPLLIQVYGGPCSQSVRSVFAVNWISYLASKEGMVIALVDGRGTAF
QGDKLLYAVYRKLGVYEVEDQITAVRKFIEMGFIDEKRIAIWGWSYGGYVSSLALASGTGLFKCGIAVAPVSSWEYYASVYTERFMGLPTKDDNLEHYKNSTVMARAEY
FRNVDYLLIHGTADDNVHFQNSAQIAKALVNAQVDFQAMWYSDQNHGLSGLSTNHLYTHMTHFLKQCFSLSD

SI 5: The graphic representation of the transfer viral vector with hFAP is presented, together with the aminoacidic sequence of the cytoplasmic domain (**A**), the transmembrane – signal anchor (**B**) and the extra-cellular domain (**C**).

UniCAR-T *in Vitro* Killing Assay

The gating strategy to determine the killing rate is shown below.



SI 6: The gating strategies used to determine the UniCAR T-cell induced tumor cell lysis.