Supporting Information

for the article

Inhibitor-Decorated Polymer Conjugates Targeting Fibroblast Activation Protein

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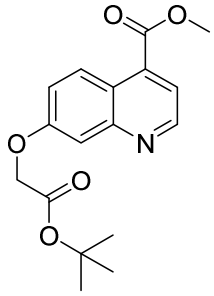
General Information. All chemicals were purchased from Sigma-Aldrich, unless stated otherwise. All inhibitors tested in the biological assays were purified using preparative scale HPLC Jasco PU-975 (flow rate 10 ml/min, water phase containing 0.1 % of TFA; gradient shown for each compound - including R_t) equipped with UV detector UV-975 and with column Waters YMC-PACK ODS-AM C18 Prep Column, 5 µm, 20×250 mm. The purity of compounds was tested on analytical Jasco PU-1580 HPLC (flow rate 1 ml/min, invariable gradient 2-100 % ACN in 30 min, R_t shown for each compound, water phase contained 0.1 % of TFA) with column Watrex C18 Analytical Column, 5 µm, 250×5 mm. The final inhibitors were all at least of 99 % purity. Structure was further confirmed by HRMS at LTQ Orbitrap XL (Thermo Fisher Scientific) and by NMR (Bruker Avance I™ 400 MHz).

SYNTHESIS OF INTERMEDIATE COMPOUNDS

Methyl 7-hydroxyquinoline-4-carboxylate (Compound 5). 150 ml of MeOH was cooled down on ice and 2.57 ml of $SOCl_2$ (35.4 mmol, 1.5 eq) was added dropwise. To this solution 4.46 g of 7-hydroxyquinoline-4-carboxylic acid (23.6 mmol, 1.0 eq; prepared as described previously[1] with 47 % yield over two steps) were added in one portion and the reaction mixture was refluxed. After 16 h the reaction mixture was left to cool down to RT and part of the product precipitated from the solution. The product was filtered of and the filtrate was evaporated to 10 ml of volume. The product was filtered again, however was not as pure as the first portion of precipitate and was therefore triturated in 20 ml of methanol and cooled down to -40 °C. Then the re-triturated product was filtered and the precipitates were combined. 3.72 g obtained (isolated yield = 75 %). ^{1}H NMR (400 MHz, d_6-DMSO) δ 9.10 (d, J = 5.2 Hz, 1H), 8.54 (d, J = 9.4 Hz, 1H), 7.93 (d, J = 5.2 Hz, 1H), 7.61 (d, J = 2.4 Hz, 1H), 7.49 (dd, J = 9.4, 2.5 Hz, 1H), 4.02 (s, 3H). ^{13}C NMR

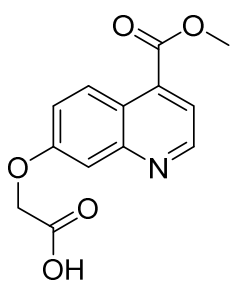
(101 MHz, $CDCl_3$) δ 169.9, 167.9, 147.6, 147.1, 133.3, 129.1, 125.2, 123.8, 123.7, 108.4, 58.9. HRMS (ESI^-): m/z for $C_{11}H_{10}O_3N$ $[M-H]^-$ calc. 204.06552, found 204.06553.

Methyl 6-(2-(tert-butoxy)-2-oxoethoxy)-1-naphthoate (Compound 6).



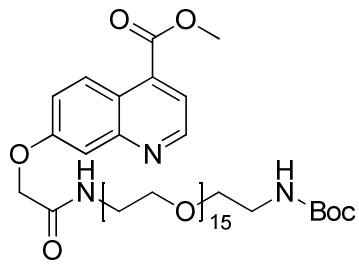
3.72 g of compound **5** (18.3 mmol, 1.0 eq) was suspended in 120 ml of dry DMF and cooled down to -80 °C. 510 mg of NaH (22.0 mmol, 1.1 eq) were added slowly to the stirring solution. The reaction mixture was then left to heat up to RT (change in color from yellow to orange) and 3.52 ml of *t*-butyl 2-bromoacetate (23.8 mmol, 1.3 eq) were added dropwise (quickly). After 3 h the TLC analysis (He:EtOAc - 2:1, product R_f = 0.37) revealed not full conversion and therefore the reaction mixture was cooled down again to – 80 °C and both NaH and *t*-butyl 2-bromoacetate were added (same amounts). After three more hours the conversion was complete (TLC analysis) and the reaction mixture was evaporated and the crude product purified on column chromatography (He:EtOAc - 5:2; TLC - He:EtOAc - 2:1, product R_f = 0.38). 4.53 g of product obtained (isolated yield = 78 %). ^1H NMR (401 MHz, $CDCl_3$) δ 8.86 (d, *J* = 4.5 Hz, 1H), 8.67 – 8.62 (m, 1H), 7.70 (d, *J* = 4.5 Hz, 1H), 7.34 (dd, *J* = 8.7, 2.2 Hz, 1H), 7.33 (s, 1H), 4.63 (s, 2H), 3.95 (s, 3H), 1.45 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.3, 166.6, 158.8, 150.2, 150.2, 134.5, 127.1, 121.2, 120.7, 120.4, 108.7, 82.7, 65.5, 52.7, 28.1. HRMS (ESI^+): m/z for $C_{17}H_{20}O_5N$ $[M+H]^+$ calc. 318.13360, found 318.13367.

2-((4-(methoxycarbonyl)quinolin-7-yl)oxy)acetic acid (Compound 7).

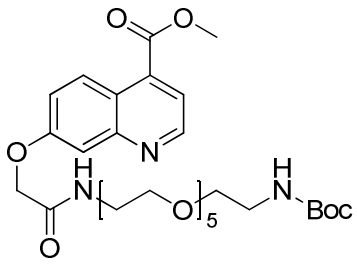


4.2 g of compound **6** (13.2 mmol) were dissolved in 15 ml HPLC grade TFA and the reaction mixture was left stirring for 3 h after which TLC analysis showed no more starting material. The TFA was removed by flow of nitrogen and the reaction mixture was left to dry on high vacuum overnight. The product

solidified upon full drying and was triturated out from Et_2O to give 3.43 g of yellow powder (isolated yield = 99%). Note: the *t*-butyl cannot be cleaved by 20 % TFA in DCM even after prolong reaction time (24 h). ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.99 (d, *J* = 4.5 Hz, 1H), 8.55 (dd, *J* = 9.1, 0.6 Hz, 1H), 7.80 (d, *J* = 4.5 Hz, 1H), 7.44 (m, 2H), 4.91 (s, 2H), 3.98 (s, 3H). The ^{13}C NMR could not be properly measured due to insufficient solubility of the compound in any practical deuterated solvent. HRMS (ESI$^-$): m/z for $C_{13}H_{12}O_5N$ $[M-H]^-$ calc. 262.07100, found 262.007104.

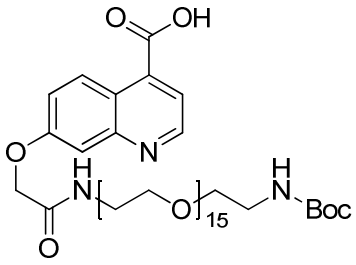


Compound 8a. 25 mg of compound **7** (12 mmol, 1.0 eq) were dissolved in 1 ml of DMF and the carboxylic acid was activated using 39 mg of TBTU (12 mmol, 1.0 eq) in presence of 74 µl of DIEA (43 mmol, 3.5 eq) for 10 minutes. Hard precipitate formed (active hydroxybenzotriazole ester) which could not be dissolved using sonicator. 100 mg of NH_2-PEG_{15}-NH-Boc (12 mmol, 1.0 eq) were added in one portion to the suspension. The reaction mixture cleared in matter of tens of seconds. An immediate analytical HPLC analysis showed complete disappearance of reactants (product R_t = 19.5 min). All volatiles were fully evaporated and the product was dried. Due to possibility of the PEG_{15} to take over the product to the water phase during washing, the mixture was not washed and was used crude in the deprotection step. Analytical HPLC R_t = 19.5 min. HRMS (ESI+) m/z for $C_{50}H_{86}O_{21}N_3$ $[M+H]^+$ calc. 1064.57538, found 1064.57549.

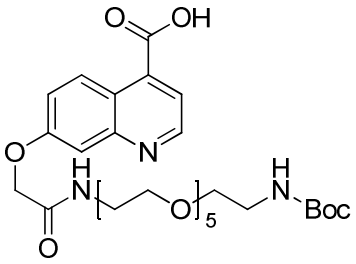


Compound 8b: 142 mg of compound **7** (0.54 mmol, 1.0 eq) were dissolved in 2 ml of DMF along with 175 mg of TBTU (0.54 mmol, 1.0 eq; Iris-Biotech) and 230 µl of DIEA (1.30, 2.4 eq). The

carboxylic acid was left to activate for 5 minutes at RT and the activated ester precipitated from the solution. After addition of 207 mg of NH_2-PEG_5-NH-Boc (0.54 mmo, 1.0 eq; Biomatrik - Shanghai) the solution cleared in matter of 2 minutes. The reaction was monitored by anal. HPLC (product Rt 19.1 min) and was evaporated upon completion (1 h). The crude product was dissolved in EtOAc (30 ml) and was washed 2x with 10 % KHSO4, 2x saturated NaHCO3 and 1x with brine. The water phases were each then backwashed 2x with 15 ml of EtOAc to decrease the losses (the PEG solubilizes the compound in water phase). 242 mg of yellowish gel-like compound were isolated (yield = 72 %). Analytical HPLC Rt = 19.1 min. HRMS (ESI+) m/z for C30H46O11N3 $[M+H]^+$ calc. 624.31269, found 624.31287.

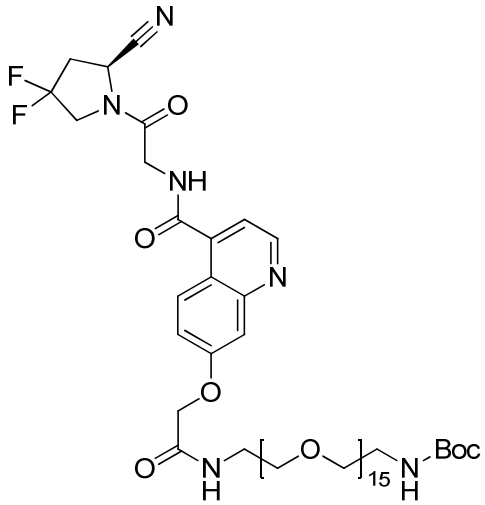


Compound 9a. Crude compound **8a** was dissolved in 1 ml of MeOH and 1 ml of water. 0.5 ml of 5 M NaOH was added. The reaction mixture was left stirring for 2 hours after which all of the compound **8a** disappeared (anal. HPLC analysis). The reaction mixture was evaporated to dryness after which it was dissolved in 1 ml of water and further acidified by 1 M citric acid to pH 2-3. The product was purified by direct preparative scale HPLC (gradient: 15-50 % ACN in 50 minutes, Rt = 38 min). Analytical HPLC Rt = 18.5 min. HRMS (ESI+) m/z for $C_{49}H_{83}O_{21}N_3Na$ $[M+Na]_+$ calc. 1072.54113, found 1072.54163.

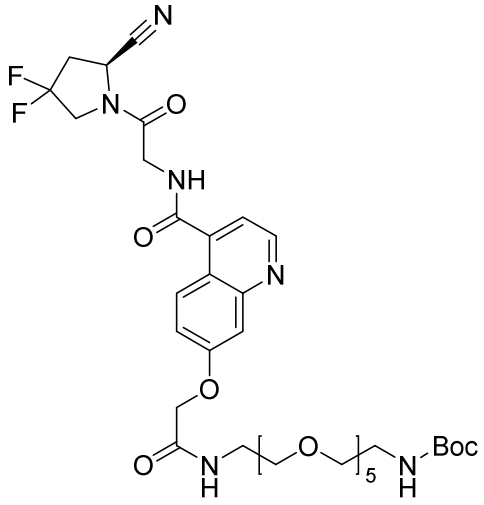


Compound 9b: 240 mg of compound **8b** (0.38 mmol, 1.0 eq) were dissolved in mixture of MeOH and THF (2 ml/2 ml) and 2 ml of 5 M NaOH were added at RT in one portion. The reaction was left stirring for 3 hours after which the whole mixture was

evaporated and dissolved in pure water (10 ml). The insoluble remnants were filtered of using a syringe filter (0.22 µm) and the water phase was acidified using 10% KHSO4 to pH 1. The water phase was then extracted 5x by EtOAc (5x25 ml) and the combined organic phases were dried and evaporated to yield 180 mg of pure product (isolated yield 77%). Analytical HPLC Rt = 16.8 min. HRMS (ESI-) m/z for $C_{29}H_{42}O_{11}N_3$ $[M-H]^-$ calc. 608.28248, found 608.28235.

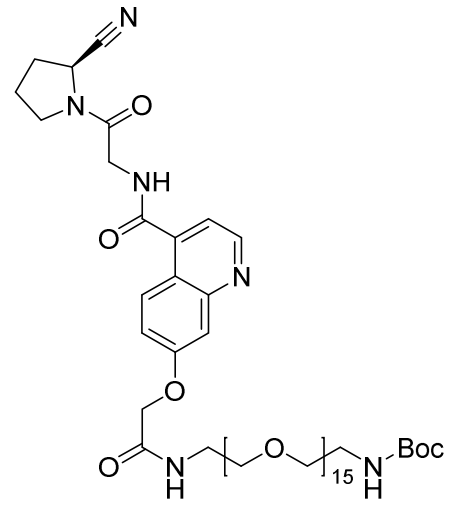


Compound 10a. 197 mg of compound **9a** (188 µmol, 1.0 eq) were dissolved in 2 ml of DMF along with 107 mg of HATU (281 µmol, 1.5 eq) and 114 µl of DIEA (657 µmol, 3.5 eq). After 5 minutes of stirring 39 mg of (S)-2-(2-cyano-4,4-difluoropyrrolidin -1-yl)-2-oxoethanaminium chloride (prepared as described in [2] (206 µmol, 1.1 eq) were added in one portion and the reaction mixture was left stirring overnight. The analytical HPLC showed single purity of well above 90 % and the crude product (after evaporation) was used in next step without any further purification. Analytical HPLC R_t = 18.9 min. HRMS (ESI+) m/z for $C_{56}H_{91}O_{21}N_6F_2$ $[M+H]^+$ calc. 1221.61999, found 1221.62020.

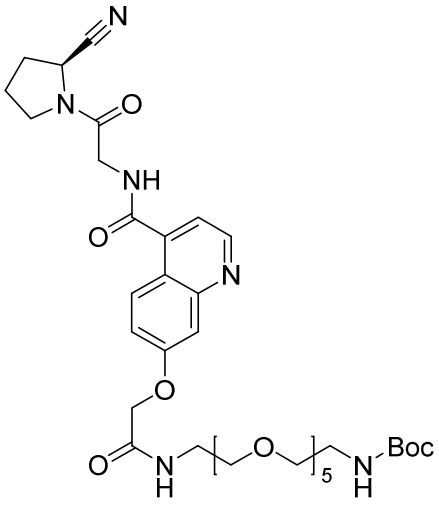


Compound 10b: 15 mg of compound **9b** (24.6 µmol, 1.0 eq) were dissolved in 0.5 ml of DMF along with 7.4 mg of TSTU (24.6 µmol, 1.0 eq) and 15 µl of DIEA (86.1 µmol, 3.5 eq). The reaction mixture was left stirring for 3 hours after which 18 mg of (S)-2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethanaminium chloride (prepared as described in [2], 49.2 µmol, 2.0 eq)

were added in one portion. After 16 hours all volatiles were evaporated and the purity was checked by anal. HPLC (~90 %). The product was used in deprotection step without further purification. **Analytical HPLC** R_t = 18.0 min. **HRMS** (ESI+) m/z for $C_{36}H_{51}O_{11}N_6F_2$ $[M+H]^+$ calc. 781.35784, found 781.35777.



Compound 10c: 83 mg of compound **9a** (79 µmol, 1.0 eq) were dissolved in 1 ml of DMF along with 24 mg of TSTU (79 µmol, 1.0 eq) and 48 µl of DIEA (277 µmol, 3.5 eq) and the activation was left to proceed for 3 hours. 21 mg of (S)-1-(2-aminoacetyl)pyrrolidine-2-carbonitrile (79 µmol, 1.0 eq) were added in one portion and the reaction mixture was left stirring overnight. The reaction was monitored by LC-MS and analytical HPLC (product R_t = 18.6 min) and showed full conversion. The product was used without purification in the next step (the only major impurity was N-hydroxysuccinimide and little bit of free DIEA that was not fully evaporated). **Analytical HPLC** R_t = 18.6 min. **HRMS** (ESI+) m/z for $C_{56}H_{93}O_{21}N_6$ $[M+H]^+$ calc. 1185.63938, found 1185.63957.



Compound 10d: 48 mg of compound **9b** (79 µmol, 1.0 eq) were dissolved in 1 ml of DMF along with 24 mg of TSTU (79 µmol, 1.0 eq; Iris-Biotech) and 50 µl of DIEA (276 µmol, 3.5 eq) and the reaction mixture was left stirring for 3 h. The NHS ester was not isolated but 21 mg

of (S)-1-(2-aminoacetyl)pyrrolidine-2-carbonitrile (79 µmol, 1.0 eq, prepared as described in [2] with exception for deprotection of Boc groups where Tos-OH*H_2O was used instead of TFA) were added directly to the reaction mixture. After 16 hours all volatiles were evaporated and the product was purified using preparative scale HPLC (gradient: 20-60 ACN in 50 minutes, R_t = 34 min) 30 mg of oily yellowish substance obtained upon dry freezing (isolated yield = 51%). Note: The reaction is much cleaner with TSTU than it is with TBTU, however the conversion is not full (±75%). To reach full conversion HATU must be used. **Analytical HPLC** R_t = 17.6 min. **HRMS** (ESI+) m/z for $C_{36}H_{52}O_{11}N_6$ $[M+H]^+$ calc. 744.36941, found 744.36950.

SYNTHESIS OF HPMA COPOLYMERS (IBODIES)

Generally, the HPMA copolymer conjugates (iBodies **1** and **2**) were prepared by reaction of the copolymer precursor containing thiazolidine-2-thione reactive groups (TT) along the polymer chain (poly(HPMA-*co*-Ma-*β*-Ala-TT)) with a combination of fluorophore (ATTO488-amine), affinity anchor (biotin-NH_2), and targeting ligand (absent in negative control iBody **2** lacking the targeting ligand) according to a procedure described previously [3]. The synthesis of iBodies and their polymer precursors is described in more detail below as well as their characterization.

Determination of molecular weights and the composition of the iBodies

The weight-average molecular weights (M_w), number average molecular weights (M_n), and dispersity ($Đ$) of the polymer precursor and conjugates were determined using HPLC Shimadzu system equipped with a UV detector, an Optilab®rEX differential refractometer and multi-angle light scattering DAWN® 8™ (Wyatt Technology, USA) detector and size-exclusion chromatography TSKgel G4000SW column. The M_w, M_n and $Đ$ were calculated using the

Astra V software. The refractive index increment dn/dc = 0.167 ml/g was used for calculation. For these experiments, a 20 % 300 mM sodium acetate, pH 6.5: 80 % methanol (v/v) buffer was used. The flow rate was 0.5 mL/min. Content of TT reactive groups in polymer precursors was determined spectrophotometrically (ε_{302nm} = 10,600 l.mol^{-1}.cm^{-1}, methanol).

Content of the targeting group (compound **1**) was determined by a fluoride selective electrode (2 fluorine atoms per a compound **1** molecule). The iBody sample was precisely weighed (approx. 2 mg, on an analytical microscale) and combusted by Schöniger method (i.e. in a quartz Erlenmeyer flask in an oxygen atmosphere). Fluoride was determined potentiometrically using an ion-selective electrode (Cyberscan Ion 510, Fischer Scientific) with a compact fluoride ion-selective electrode (Elektrochemicke Detektory, Czech Republic). For an ionic strength adjustment, a total ionic strength adjustment buffer (TISAB) buffer (Elektrochemicke Detektory, Czech Republic) was used. An external calibration using standard fluoride solutions (Eutech Instruments, Singapore) was used, in a fluoride concentration range 0 – 1.0 ppm.

Content of ATTO488 was determined using spectrophotometry (ε_{502nm} = 90,000 l.mol^{-1}.cm^{-1}, water); content of biotin was determined using Colorimetric Biotin Assay Kit according to the manufacturer's instructions (Sigma, #MAK171); the results were corrected for the effect of ATTO488 absorbance at 500 nm.

The determined contents of the ligands (knowing the molecular weights of the individual ligands and the polymer) were then converted into "a number of units per iBody".

Synthesis of a copolymer precursor for iBody 1 conjugate

Monomers *N*-(2-hydroxypropyl)methacrylamide (HPMA) and 3-(3-methacrylamido-propanoyl)thiazolidine-2-thione (Ma-*β*-Ala-TT) were synthesized as described earlier [4, 5]. Copolymer precursor poly(HPMA-*co*-Ma-*β*-Ala-TT) was prepared by reversible addition-fragmentation chain transfer (RAFT) copolymerization [6, 7]. 0.623 g of mixture of HPMA (88 %mol, 500 mg), Ma-*β*-Ala-TT (12 %mol, 123 mg dissolved in 0.85 ml dimethyl sulfoxide), 1.36 mg 2-cyanopropan-2-yl ethyl carbonotrithioate and 0.45 mg 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile) was dissolved in 4.8 ml tert-butanol and the solution was introduced into polymerization ampule. The mixture was bubbled with argon for 10 min and ampule was sealed. Copolymerization was carried out at 40 °C for 16 h. Polymer precursor was isolated by precipitation into mixture of acetone:diethyl ether (3:1), filtered off, washed with acetone and diethyl ether and dried in vacuum. Terminating trithiocarbonate group was removed as described by Perrier [8]. The copolymer precursor poly(HPMA-*co*-Ma-*β*-Ala-TT) with molecular weight M_n = 61,700 g/mol, M_w = 66,600 g/mol, dispersity $Đ$ = 1.08 and content of reactive thiazolidine-2-thione groups 11.7 mol% was obtained.

Synthesis of a copolymer precursor for iBody 2 conjugate

Copolymer precursor poly(HPMA-*co*-Ma-*β*-Ala-TT) was prepared by RAFT copolymerization using following composition of copolymerization mixture: HPMA (88 %mol; 1,000 mg), Ma-*β*-Ala-TT (12 %mol, 246 mg dissolved in 1.32 ml dimethyl sulfoxide (DMSO)), 2.34 mg 2-cyano-2-propyl benzodithioate and 0.87 mg 2,2′-azobis(2-methylpropionitrile) was dissolved in 7.5 ml tert-butanol and the solution was introduced into polymerization ampule. The mixture was bubbled with argon for 10 min and ampule was sealed. Copolymerization was carried out at 70 °C for 16 h. Polymer precursor was isolated by precipitation into mixture of acetone:diethyl ether (3:1), filtered off, washed with acetone and diethyl ether and dried in vacuum. Terminating

benzodithioate group was removed as described by Perrier [8]. The polymer precursor with molecular weight M_n = 59,600 g/mol, M_w = 72,700 g/mol, dispersity $Đ$ = 1.22 and the content of reactive thiazolidine-2-thione groups 11.8 mol% was obtained.

BIOCHEMICAL METHODS

Preparation of expression plasmids for FAP and DPP-IV

The plasmid encoding the SF-tag (pMT/BiP/SF-PSMA) was a kind gift from Dr. Cyril Bařinka (Institute of Biotechnology, The Czech Academy of Sciences, Prague, Czech Republic) [9]. The plasmid encoding the Avi-tag (pMT/BiP/Avi-GCPII) was prepared earlier in our laboratory [10]. The DNA sequence encoding human FAP was obtained from Origen in the pCMV6-XL5 vector. The plasmid containing full-length human DPP-IV was prepared earlier in our laboratory [11, 12]. DNA sequences encoding mouse full-length FAP and DPP-IV were obtained from mouse cDNA libraries isolated from mouse uterus and heart, respectively.

DNA encoding the extracellular parts of human and mouse FAP (amino acids 26-760 and 26-761, respectively) was amplified by PCR. The PCR products were cloned *via Bgl*II and *Xho*I restriction sites into cleaved pMT/BiP/SF-PSMA [9], resulting in the plasmids pMT/BiP/SF-hFAP and pMT/BiP/SF-mFAP.

Similarly, DNA encoding the extracellular parts of human and mouse DPP-IV (amino acids 29-766 and 29-760, respectively) was amplified by PCR, and the PCR constructs were cloned *via Bcl*I and *Xho*I (human DPP-IV) or *Bam*HI and *Xho*I (mouse DPP-IV) restriction sites into *Bgl*II/*Xho*I-cleaved pMT/BiP/Avi-GCPII, resulting in the plasmids pMT/BiP/Avi-hDPP-IV and pMT/BiP/Avi-mDPP-IV.

For expression of mouse FAP and DPP-IV in GL261 cells, we prepared plasmids pcDNA4_mFAP and pcDNA4_mDPP-IV. DNA sequences encoding full-length mouse FAP and DPP-IV were amplified by PCR, and the obtained products were subcloned into pcDNA4 plasmid *via Kpn*I and *Xho*I restriction sites.

The sequence of all obtained plasmids was verified by DNA sequencing.

Expression of recombinant FAP and DPP-IV proteins

The expression of all proteins was performed in Drosophila S2 cells according to a previously published protocol [10].

Purification of recombinant FAP and DPP-IV proteins

Purification of recombinant human and mouse SF-FAP and human and mouse Avi-DPP-IV was performed as previously described [9, 10]. Briefly, conditioned medium containing recombinant proteins was concentrated 10-fold using a LabScale TFF System (Millipore) with a Pellicon® XL 50 Cassette, Biomax 100. The recombinant proteins were purified by affinity chromatography on Strep Tactin (*via* Strep-tag in case of FAP) [9] or on Streptavidin Mutein Matrix (*via* Avi-tag in case of DPP-IV) [10].

FAP, DPP-IV, PREP, DPP9 activity assay

IC_{50} values for all inhibitors and iBodies were determined using a fluorescence spectroscopy assay employing a fluorogenic FAP-specific substrate [13], which was prepared by standard Boc-peptide chemistry. The assay was performed in a black 96-well polypropylene plate with a U-bottom (Greiner). In each well, 0.5 ng of SF-FAP in PBS containing 0.001% octaethylene glycol monododecyl ether (Affymetrix) was mixed with the inhibitor/iBody in a final volume of 70 µl. Ten inhibitor concentrations were used to obtain a full inhibition curve. The reactions were pre-

incubated at 37 °C for 5 min and then started by the addition of 30 µl of 500 µM *N*-(quinoline-4-carbonyl)-Ala-Pro-7-amino-4-methylcoumarin. The fluorescence (kinetic mode measurement) was recorded on an Infinite M1000 PRO (TECAN) with an excitation wavelength of 380 nm and emission wavelength of 460 nm. Afterwards, data were processed, and IC_{50} values were obtained using GraFit v.5.0.11 (Erithacus Software Ltd.).

The DPP-IV activity assay was performed analogously: 0.5 ng of DPP-IV in PBS containing 0.001% octaethylene glycol monododecyl ether (Affymetrix) was mixed with inhibitor/iBody and pre-incubated at 37 °C for 5 min. The reactions were started by addition of 30 µl of 166 µM Gly-Pro-AMC (Sigma).

The PREP and DPP9 activity assays were performed in a black 96-well polypropylene plate with a flat bottom (Greiner) according to the standardized protocol provided by the supplier of the recombinant proteins (R&D Systems; catalog numbers: 4308-SE-010 and 5419-SE-010, respectively). 20 ng of PREP and 50 ng of DPP9 were used per one well. The reactions were started by addition of 50 µl of 200 µM Gly-Pro-AMC (Sigma-Aldrich) and 50 µl of 100 µM Z-Gly-Pro-AMC (Bachem) for DPP9 and PREP, respectively.

Surface plasmon resonance (SPR) analysis

The SPR measurements were performed according to a previously described protocol [3, 14, 15]. The SPR chip was mounted to the prism and treated as described [3, 14, 15]. After EDC/NHS activation of carboxyl terminal group, 20 ng/µl neutravidin solution in 10 mM sodium acetate buffer, pH 5.0, was applied. Finally, PBS containing 0.5 M NaCl was used, followed by 1 M ethanolamine.

A solution of 500 nM iBody **1** in PBS was immobilized on the neutravidin layer. Finally, recombinant FAP protein in PBS was loaded in four different concentrations (300 nM, 150 nM,

75 nM, 38 nM), followed by washing with PBS for 30 min. Kinetic curves of binding were exported and subsequently fitted using the One-To-One interaction model in TraceDrawer v.1.5 (Ridgeview Instruments AB) to obtain k_{on} and k_{off} parameters.

Cell lines and preparation of clones with FAP expression

Human glioma cell lines U-251MG and U-87MG were obtained from the ATCC; the U-251MG (CVCL_0021) cell line was previously sold by the ATCC as U-373MG cell line; however, as shown later by the ATCC and others[16] and verified also by us using the Short Tandem Repeat analysis, the cell line has been identified as U-251MG; human fibroblasts were a gift from Prof. Karel Smetana (First Faculty of Medicine, Charles University, Prague). Cells were cultured under standard conditions in DMEM supplemented with 10% FBS [12]. The mouse glioma cell line Gl261 was obtained from the Division of Cancer Treatment and Diagnosis Tumor Repository at the National Cancer Institute, USA, and was cultured under standard conditions in RPMI 1640 supplemented with 1% Glutamax (Thermo Fisher Scientific) and 10% FBS. To prepare a vector for mammalian cell transfection, the DNA sequence encoding full-length human FAP was cleaved from the pCMV6-XL5_FAP vector using *Not*I and ligated into the pTRE-Tight vector (Clontech), resulting in the plasmid pTRE-Tight_hFAP. U251 cells were transfected with the pTet-On-Advanced plasmid (Clontech) using Lipofectamine 2000 (Invitrogen); stable clones were then transfected with the pTRE-Tight_hFAP plasmid and selected using hygromycin (Clontech). The expression of FAP was induced by adding 1 μg/ml of doxycycline (Clontech) into the culture medium. Gl261 cells were transfected with pcDNA4_mFAP (Clontech), and stable clones were selected using Zeocine (Thermo Fisher Scientific).

Pull-down of FAP from cell lysate

Stably transfected U251_FAP+ cells were grown on 100 mm Petri dishes in the presence of 100 nM doxycycline in DMEM with 10% FBS and 4 mM L-glutamine. After reaching 90-100% confluence, the medium was removed, and cells were rinsed with PBS and subsequently detached from the dish surface with a trypsin/EDTA solution [0.25 % (w/V) trypsin and 0.01 % (w/V) EDTA]. Cells were resuspended and transferred into DMEM medium, centrifuged at 250×g for 2 min, and washed twice with 1 ml PBS. The U251-FAP cell lysate was prepared by 5 min sonication in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4 (TBS), with 1% Tween 20. Cells from 1 Petri dish were lysed in 450 µl of the lysis buffer. The solution was centrifuged at 16,100×g for 10 min, the supernatant was transferred into a new tube and diluted with TBS containing 0.1% Tween 20 (TBST).

Next, 1 ml of 1 µM iBody **1** and iBody **2** in TBST were incubated with 30 µl of Streptavidin Agarose Ultra Performance (Solulink) for 1 h at 4 °C. After twice washing the resin with 1 ml TBS, 900 µl of the U251-FAP cell lysate was added to the resin and incubated for 1.5 h at 4 °C. Then, flow-through fractions were collected, and the resin was washed three times with TBST. Finally, proteins were eluted with 30 µl of reducing SDS sample buffer and heated to 98 °C for 10 min. Proteins were resolved by SDS-PAGE and visualized by silver staining.

Imaging of viable FAP-expressing cells using confocal microscopy

Stably transfected U251-FAP cells were grown in the presence or absence of 100 nM doxycycline in 4-Chamber 35 mm Glass Bottom Dishes (In Vitro Scientific) in DMEM with 10% FBS and 4 mM L-glutamine to approximately 60% confluence. The next day, media were removed, and iBodies diluted in fresh serum-free media were added to each chamber to a final concentration of 200 nM. Cells were incubated for 1 h at 37 °C. Before the end of the incubation, Hoechst Stain Solution H34580 (Sigma) was added for 10 min (0.5 µg/ml final concentration) to

stain cell nuclei. The medium was removed, cells were rinsed twice with 500 µl of PBS, and fresh medium containing serum was added. Confocal images (pinhole 1 Airy unit) were obtained at 37 °C using a Zeiss LSM 780 confocal microscope (Carl Zeiss Microscopy) with an oil-immersion objective (Plan-Apochromat 63x/1.40 Oil DIC M27); the images were captured 1, 3, 6 and 21 h after addition of the iBody **1**. The fluorescent images were taken using 1.1% of the 405 nm diode laser (max. power 30 mW) for excitation with emission collected from 410 to 585 nm (voltage on detector: 750 V) for Hoechst 34580 and 1.8% of the 488 nm argon-ion laser (max. power 25 mW) for excitation with emission collected from 490 to 630 nm (voltage on detector: 720 V) for ATTO488. All images were taken using the same settings. The microscope was operated and images were processed with ZEN 2011 software (Carl Zeiss Microscopy).

Flow cytometry analysis of FAP-expressing cells

Cultured cells were harvested using a trypsin/EDTA solution (Sigma Aldrich), washed with PBS and resuspended in PBS containing 10% FBS. Anti-FAP iBody **1** was added to the cell suspension (10^5 cells in 50 µl) to achieve a final concentration of 100 nM; the cells were incubated for 60 min at 4 °C. After washing with PBS, phycoerythrin-conjugated streptavidin (Biolegend) was added (1:50) and incubated for 30 min at 4 °C. Samples were analyzed using the flow cytometer BD FACSVerse (BD Biosciences) with the PCSuite software for acquisition and FlowJo (TreeStar Inc.) for data evaluation.

Quantitative detection of FAP using ELISA

For detection of native FAP, a 96-well Maxisorb plate (Nunc) was coated with FAP-specific mouse monoclonal antibody in borate buffer (100 ng/well; 1 h at RT). The well surface was blocked with 0.55% (w/v) casein in TBS (Casein Buffer 20X-4X Concentrate, SDT; 12 h at RT). Then, recombinant SF-FAP in TBST was added (ranging from 3 pg – 50 ng/well; 1 h at RT).

After washing the wells with TBS containing 0.05% Tween 20 (TBST′; 3×200 µl), 10 nM iBody **1** in TBST was added to bind FAP (1 h at RT). Afterwards, unbound iBody **1** was washed out with TBST′ (3×200 µl), and NeutrAvidin-HRP conjugate was added (50 ng/well; Thermo Scientific; 30 min at RT). Finally, the wells were washed with TBST′ (5×200 µl) and 4-iodophenol-enhanced luminol chemiluminescence was detected on a Tecan Infinite M1000 PRO spectrophotometric reader. Each sample was measured in triplicate; values are presented as the mean ± standard deviation. The limit of detection was determined as a mean background signal from hexaplicate measurements with five standard deviations added.

Detection of FAP on Western blot

Protein samples were mixed with non-reducing Laemmli sample buffer containing 1% SDS. The samples were not heated. They were resolved by SDS-PAGE and blotted onto a nitrocellulose membrane (1 h, 100 V, cooled). The membrane surface was blocked with 0.55% (w/v) casein in PBS (Casein Buffer 20X-4X Concentrate, SDT; 1 h at RT). FAP was probed with 10 nM iBody **1** in TBST containing 0.055% (w/v) casein (1 h at RT). The membrane was washed with PBST′ (3×5 min) and subsequently incubated with IRDye® 800CW Streptavidin conjugate [LI-COR Biosciences; 1:15,000 in PBS with 0.1% (w/v) Tween 20; 1 h at RT]. The unbound conjugate was washed out with PBST′ (3×5 min), and FAP was visualized using an Odyssey CLx Infrared Imaging System (LI-COR).

Mouse xenotransplantation glioma model

FAP-transfected U251 cells were orthotopically implanted into immunodeficient mice as previously described [12]. The experimental use of animals was approved by The Commission for Animal Welfare of the First Faculty of Medicine of Charles University in Prague and The

Ministry of Education, Youth and Sports of the Czech Republic, according to animal protection laws.

Immunohistochemistry

Immunohistochemistry was performed as previously described with minor modifications [17]. Ten-micrometer frozen sections were fixed with 2% paraformaldehyde for 5 min at RT and subsequently incubated overnight with 100 nM anti-FAP iBody **1** (in TBS with 1% bovine serum albumin) at 4 °C. The slides were washed with PBS and deionized water, mounted in Aqua Polymount (Polysciences, Eppelheim, Germany) and viewed on the Olympus IX 70 microscope equipped with the DP30BW camera. Hoechst 33258 (50 ng/ml, Sigma-Aldrich, St. Louis, USA) was used for nuclear counterstaining. The intensity of FAP staining could be further enhanced using a Streptavidin-Alexa Fluor 488 conjugate (Life Technologies, S32354, 1:750, 30 min at RT).

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