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Inhibitor-Decorated Polymer Conjugates Targeting Fibroblast Activation Protein

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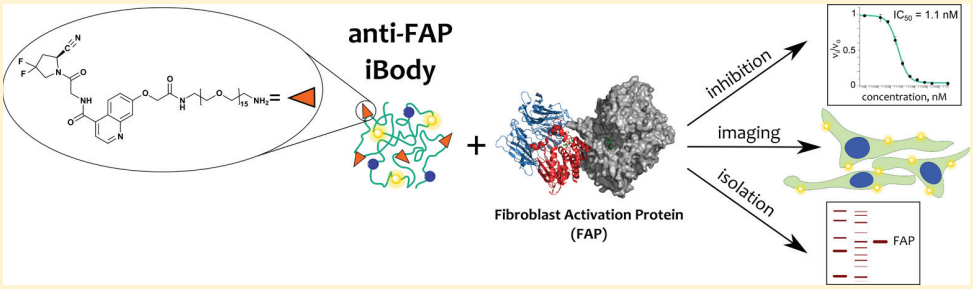
Supporting Information



ABSTRACT: Proteases are directly involved in cancer pathogenesis. Expression of fibroblast activation protein (FAP) is upregulated in stromal fibroblasts in more than 90% of epithelial cancers and is associated with tumor progression. FAP expression is minimal or absent in most normal adult tissues, suggesting its promise as a target for the diagnosis or treatment of various cancers. Here, we report preparation of a polymer conjugate (an iBody) containing a FAP-specific inhibitor as the targeting ligand. The iBody inhibits both human and mouse FAP with low nanomolar inhibition constants but does not inhibit close FAP homologues dipeptidyl peptidase IV, dipeptidyl peptidase 9, and prolyl oligopeptidase. We demonstrate the applicability of this iBody for the isolation of FAP from cell lysates and blood serum as well as for its detection by ELISA, Western blot, flow cytometry, and confocal microscopy. Our results show the iBody is a useful tool for FAP targeting in vitro and potentially also for specific anticancer drug delivery.

INTRODUCTION

Proteases in tumor and stromal cells play an important role in cancer progression by promoting tumor cell invasion and metastasis as well as facilitating neovascularization.[1−3] Because of their pathogenic role and differential expression in tumor tissue, some proteases, such as matrix metalloproteinases, hold promise as therapeutic and diagnostic targets. However, several large-scale clinical trials testing low-molecular-weight matrix metalloproteinase inhibitors failed to show improvement in clinical outcomes. This was most likely due to the imperfect specificity of the tested compounds, on-target side effects caused by interference with the physiological functions of the proteases, and the incompletely understood involvement of the targeted proteolytic enzymes as well as their substitutability by other proteases in disease progression.[4,5]

Since its discovery in the late 1980s, fibroblast activation protein (FAP; seprase, surface expressed protease) has been considered an interesting potential target for cancer therapeutics and diagnostics.[6] FAP is expressed in stromal fibroblasts in more than 90% of epithelial cancers,[6] and its expression is also increased in stromal cells in multiple myeloma[7] and glioblastoma.[8] In addition, FAP is expressed in malignant cells in glioblastoma[8] and pancreatic,[9,10] breast,[11] colorectal,[12] cervical,[13] and oral squamous cell[14] carcinomas. Although the effects of FAP are tumor specific and in certain cancers FAP may even act as a tumor suppressor,[15] it has been established in several cases that high FAP expression contributes to the invasiveness and increased proliferation of the tumor cells.[14,16,17] Moreover, FAP in the bioptic material may be a prognostic marker of aggressive tumor progression, especially when expressed by cancer cells (recently reviewed in ref 18).

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Recent studies also demonstrated that FAP expression is increased in various nonmalignant disease states accompanied by extracellular matrix remodeling such as in idiopathic pulmonary fibrosis,[19] liver cirrhosis,[20] rheumatoid arthritis,[21] myocardial infarction,[22] and advanced atherosclerotic plaques.[23]

With the exception of pancreatic alpha cells,[24] mesenchymal bone marrow cells,[25] and endometrial stroma during the proliferative phase,[26] FAP expression is minimal or absent in the majority of normal adult tissues.[6,27,28] Its soluble form devoid of the cytoplasmic and transmembrane regions is physiologically present in blood plasma (known as antiplasmin-cleaving enzyme, APCE). The origin and function of blood plasma FAP are largely unknown.[29] Given the limited expression of FAP in human tissues under physiological conditions, FAP seems to be a promising molecule for targeting cancer stroma as well as some types of transformed cancer cells.

FAP is a type II transmembrane protein belonging to the S9B oligopeptidase subfamily of serine proteases. It consists of 760 amino acids: 6 form the N-terminal cytoplasmic tail, 20 the transmembrane part, and the remaining 734 amino acids are part of a large extracellular C-terminal domain.[30] FAP requires dimerization of two 97 kDa subunits for its catalytic activity[31] and cleaves off dipeptides from the N-terminus of its substrates after a proline residue (N-Xxx-Pro-). In addition, FAP exhibits postproline endopeptidase activity,[32] which is thought to contribute to the remodeling of the extracellular matrix.[33] Nevertheless, it is likely that at least some of the complex effects of FAP in cancer-associated fibroblasts and cancer cells are mediated by nonhydrolytic protein−protein interactions. For example, introduction of FAP endowed normal fibroblasts with an inflammatory phenotype, which was mediated by the activation of FAK−Src−JAK2 signaling pathway by the urokinase-type plasminogen activator receptor (uPAR), a known FAP-interacting membrane protein.[34] Similarly, the suppression of FAP in oral squamous cell carcinoma cells inactivated the PTEN/PI3K/AKT and Ras-ERK pathways and repressed the expression of genes regulating the epithelial−mesenchymal transition, thereby reducing the proliferation and invasiveness of these cells.[14] Thus, the involvement of FAP in the pathogenesis of human malignancies is complex and seems to be cancer-type specific, which may have contributed to the failure of early clinical trials assessing FAP targeting with the rather nonspecific low-molecular-weight inhibitor talabostat[35] or the humanized antibody sibrotuzumab.[36]

Recently, we have described novel biochemical tools called iBodies for the targeting of proteins with known ligands.[37] The iBodies are based on a water-soluble and biocompatible *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer carrier decorated with low-molecular-weight compounds such as enzyme inhibitors used as targeting ligands. The use of iBodies offers several advantages over classical approaches with antibodies. The iBodies are highly modular and versatile; conjugates containing virtually any desired compound can be easily prepared. Synthetic HPMA conjugates are well-characterized compounds for biomedical applications and have long been used as carriers for drug delivery to solid tumors, often making use of the enhanced permeability and retention (EPR) effect.[38−40] Importantly, the molecular weight of the HPMA backbone can be easily adjusted to specifically tailor the pharmacokinetic properties. To prepare a new platform that would allow FAP targeting in cancer, we set out to develop FAP-targeting iBodies.

Dipeptidyl peptidase IV (DPP-IV), the closest homologue of FAP, sharing 52% amino acid sequence identity, is a broadly expressed cell-surface serine protease involved in several physiological processes, including regulation of glucose metabolism[41] and T-cell activation.[42] Besides its physiological roles, DPP-IV was implicated in the pathogenesis of several cancers, acting as a tumor suppressor or promotor depending on the tumor type (recently reviewed in ref 43). The high selectivity of anti-FAP iBodies is critical to avoid interference with DPP-IV and to decrease the risk of undesired side effects. Jansen et al. recently developed low nanomolar FAP inhibitors based on a (4-quinolinoyl)glycyl-2-cyanopyrrolidine scaffold, which showed high selectivity against related proteases, including DPP-IV and prolyl-specific proteases prolyl oligopeptidase (PREP) and dipeptidyl peptidase 9 (DPP9).[44−46]

In this work, we prepared anti-FAP iBody containing a highly specific FAP inhibitor as a targeting ligand and tested its utility using FAP-expressing malignant glioblastoma cells. We demonstrate that this iBody can be used for the specific detection of FAP in various biological matrices by a number of biochemical methods, and we show that it is suitable for the specific targeting and visualization of FAP as well as the inhibition of its enzymatic activity.

RESULTS

Expression of Recombinant Human and Mouse FAP and DPP-IV. To test the selectivity of the compounds described in this study, we prepared recombinant human and mouse FAP and DPP-IV bearing cleavable N-terminal purification tags (SF-tag or Avi-tag) (Figure 1a). All proteins were expressed in *Drosophila S2* cells and purified via affinity chromatography according to previously published protocols (using Streptavidin Mutein matrix for Avi-tagged constructs[48] or Strep-Tactin resin for SF-tagged versions[49]). Originally, all four proteins were prepared with an Avi-tag; however, the Avi-hFAP and Avi-mFAP expression yields were not sufficient for biochemical characterization and subsequent experiments. Therefore, we replaced the Avi-tag with the recently described SF-tag, which comprises two Strep-tags and a Flag-tag.[49] The resulting constructs, SF-hFAP and SF-mFAP, were expressed in larger quantities compared to their Avi-tagged counterparts and could be obtained in quantity and purity sufficient for their biochemical characterization, with overall yields of 0.7 and 0.2 mg, respectively (per 1 L of conditioned medium) (Figure 1b). Similarly, we obtained 0.1 and 0.2 mg of Avi-hDPP-IV and Avi-mDPP-IV, respectively. The kinetic properties of the SF-tagged and Avi-tagged proteins were virtually identical (data not shown).

Design, Synthesis, and Characterization of Anti-FAP iBody 1. To select the most suitable targeting ligand for FAP in terms of potency and selectivity, we prepared a small panel of FAP inhibitors (compounds **1**−**4**; Scheme 1 and Figure 2a) and assessed their structure−activity relationships. Most importantly, we investigated the appropriate linkage of the targeting ligand to the polymer backbone. The compounds are based on the previously published structure of a FAP inhibitor with a (4-quinolinoyl)glycyl-2-cyanopyrrolidine scaffold[44,45] and contain a PEG linker for attachment to the HPMA copolymer. We then determined IC_{50} values of compounds **1**−**4** for SF-hFAP using a FAP activity inhibition assay. Compounds **1**−**3** exhibited comparable inhibition constants (0.23, 0.28, and 0.37 nM), whereas compound **4** was substantially less potent (4.8 nM) (Figure 2a). For further experiments, we chose compound **1** to

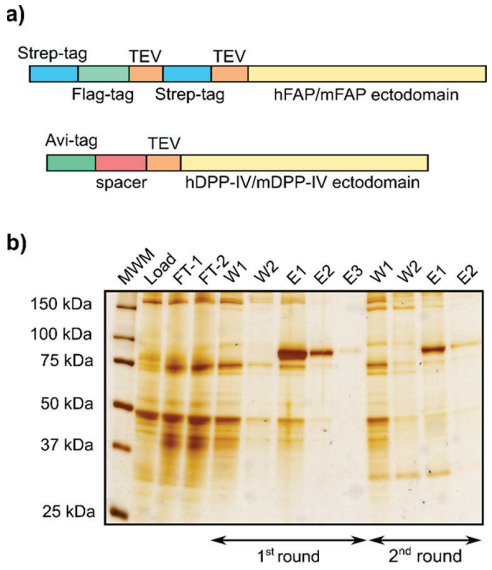


Figure 1. Design and purification of recombinant FAP and DPP-IV. (a) Schematic structures of the recombinant human and mouse FAP and DPP-IV proteins expressed in *Drosophila S2* cells. The extracellular parts of human and mouse FAP containing Strep-tag II and Flag-tag were purified via Strep-Tactin affinity chromatography. The extracellular parts of human and mouse DPP-IV containing Avi-tag were purified via streptavidin mutein affinity purification. (b) A silver-stained SDS-PAGE gel showing a typical two-round affinity purification of recombinant SF-hFAP protein expressed in *Drosophila S2* cells. Load, concentrated medium; FT, flow-through; W1−W2, wash fractions; E1−E3, elution fractions. Ten microliters were loaded onto the gel, except for Load, FT-1, and FT-2 (0.5 μL).

avoid steric problems after conjugation (long linker) and ensure the best inhibitory properties (difluoro substitution at C4 of the proline derivative).

Compound **1**, together with an ATTO488 fluorophore and the affinity anchor biotin, were conjugated to the HPMA copolymer carrier, yielding an HPMA copolymer conjugate targeting FAP (iBody **1**: M_n = 110600 g/mol, M_w = 149900 g/mol, $Đ$ = 1.36; Figure 2b). As a negative control, a corresponding conjugate lacking the FAP inhibitor was prepared (iBody **2**: M_n = 80900 g/mol, M_w = 131000 g/mol, $Đ$ = 1.62). Attachment of compound **1** to the copolymer chain led to an increase in the IC_{50} value [IC_{50}(iBody **1**) = 1.1 nM] (Figure 2c). Importantly, using a DPP-IV activity assay, we determined that iBody **1** is highly selective for FAP with IC_{50} for the FAP homologue DPP-IV more than 4 orders of magnitude higher (IC_{50} > 10 μM) (Figure 2c). We also determined the IC_{50} values for iBody **1** toward mouse FAP and mouse DPP-IV and observed similar selectivity for FAP (IC_{50} = 3.0 nM and IC_{50} > 10 μM, respectively). In addition, iBody **1** did not inhibit recombinant prolyl oligopeptidase (PREP) and dipeptidyl peptidase 9 (DPP9) (Figure 2c).

Use of Anti-FAP iBody 1 for Detection and Visualization of FAP. We used surface plasmon resonance (SPR) to evaluate the interaction between iBody **1** and FAP (Figure 3a). iBody **1** was immobilized to a neutravidin layer via biotin, and four concentrations of recombinant SF-hFAP were loaded. The SPR analysis indicated a relatively high association rate (k_{on} = 3860 M^{-1} s^{-1}) and remarkably low dissociation rate (k_{off} < 2 × 10^{-5} s^{-1}), which was under the detection limit of our SPR instrument. The resulting dissociation constant (K_d < 6 nM) is comparable with the IC_{50} value obtained from the FAP activity assay. Concordantly, we confirmed that inhibition of FAP enzymatic activity persists even when FAP-containing cell lysates preincubated with effective concentrations of anti-FAP iBody were diluted to decrease the iBody concentration prior to the enzymatic assay. In contrast, the inhibition by the low-molecular-weight (4-quinolinoyl)glycyl-2-cyanopyrrolidine-based FAP inhibitors was reversible in the same experimental setup (data not shown).

We tested the anti-FAP conjugate in several biochemical applications. Using iBody **1** immobilized to streptavidin agarose via biotin, we pulled down FAP from a cell lysate of U251 cells stably transfected with human FAP (U251_FAP+; see the "Biochemical Methods" section in the Supporting Information for the discussion of the cell line denomination) (Figure 3b). As negative controls, we used iBody **2**, which lacks the FAP inhibitor, and blank streptavidin agarose (NC-SA) to show potential nonspecific binding to HPMA copolymer backbone and/or the streptavidin agarose resin (Figure 3b). The presence of isolated FAP was verified by LC-MS/MS, which detected FAP protein in the iBody **1** elution sample. Using the same setup, we also successfully isolated FAP protein from human blood plasma (verified by LC-MS/MS; data not shown), further confirming the functionality of anti-FAP conjugates in complex biological samples.

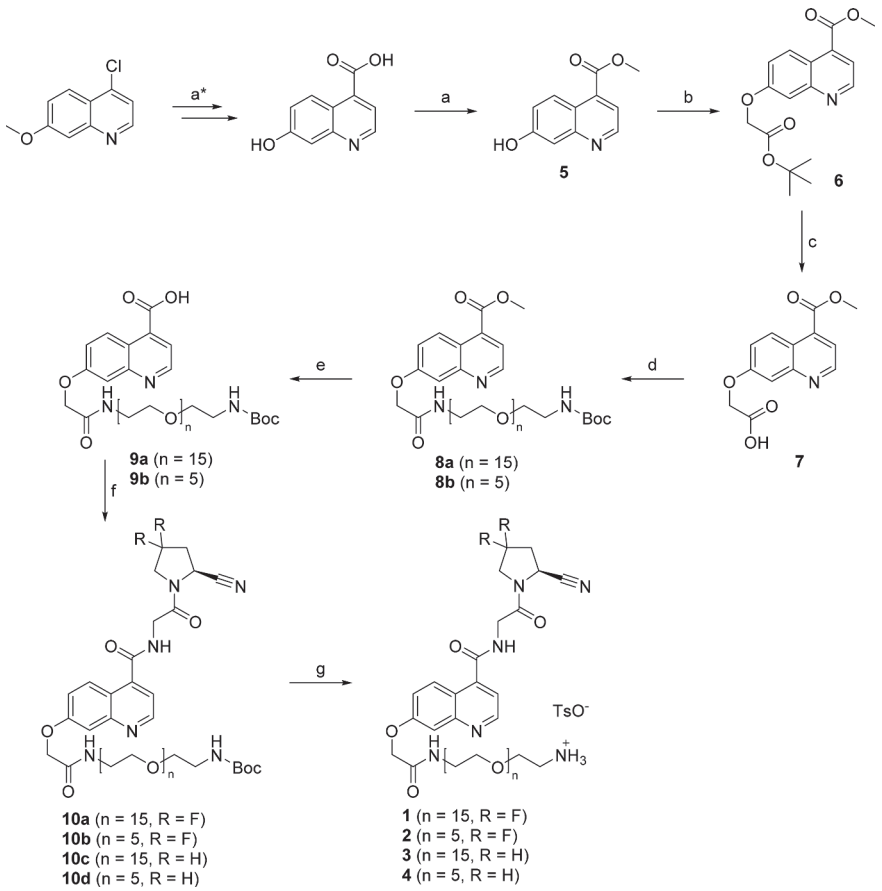
Additionally, we developed sandwich ELISA for FAP quantification employing iBody **1** as a substitute for the detection antibody (Figure 3c). Recombinant SF-hFAP was first captured by the FAP-specific monoclonal antibody F-19 and then detected with iBody **1**, followed by incubation with neutravidin−HRP conjugate. The detection limit of this newly developed ELISA was as low as 0.4 ng/mL of FAP.

iBody **1** (followed by IRDye 800CW streptavidin conjugate) could also be used to visualize FAP on a "semi-native" Western blot (Figure 3d). Both recombinant SF-tagged FAP (SF-hFAP) and endogenous full-length FAP migrated at around 130 kDa, corresponding to FAP dimers; the detection limit was about 50 ng of FAP (Figure 3d).

Application of Anti-FAP iBody 1 for Imaging of FAP-Expressing Cells. We tested the suitability of iBody **1** as a tool for the specific imaging of FAP-positive cells using confocal microscopy (Figure 4a,d) and flow cytometry (Figure 4b,c). Live cells expressing (U251_FAP+) or not expressing (U251_FAP−) FAP were incubated with anti-FAP iBody **1**; iBody **2**, which lacks the FAP inhibitor, was used as a negative control. Confocal microscopy imaging showed that iBody **1** binds only to FAP-expressing cells and not to cells lacking FAP, whereas control iBody **2** did not bind to any of the cells analyzed (Figure 4a). Upon binding to FAP on the cell surface, iBody **1** underwent slow internalization, as evidenced by the accumulation of the signal inside cells after prolonged incubation. Similar results were obtained with cells transfected with mouse FAP (data not shown).

The binding of anti-FAP iBody **1** to mouse FAP was also confirmed by flow cytometry. Anti-FAP iBody **1** strongly stained mouse GL261 glioma cells transfected with mouse FAP, whereas neither the FAP-negative parental cell line GL261 nor Gl261 cells transfected with mouse DPP-IV were stained by the conjugate (Figure 4b). We further analyzed the utility of the compounds in detecting endogenous levels of FAP expression. Using flow cytometry, FAP expression was visualized in cultured human fibroblasts and human glioblastoma U87 cells, which are known to express the protein [50] by anti-FAP

Scheme 1. Synthesis of Compounds 1−4, Specific Inhibitors of FAP Modified with PEG Linkers[a]



[a]Reagents and conditions: (a*) described in ref 47; (a) $SOCl_2$, MeOH, reflux; (b) NaH, *t*-butyl 2-bromoacetate, DMF, −80 °C to RT; (c) TFA; (d) TBTU, DIEA, NH_2-PEG_n-NH-BOC (n = 5, or n = 15), DMF; (e) 5 M NaOH/H_2O, THF/MeOH; (f) (1) TSTU, DIEA, DMF, (2) (*S*)-2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethanaminium chloride or (*S*)-2-(2-cyanopyrrolidin-1-yl)-2-oxoethanaminium chloride; (g) Ts-OH, ACN.

iBody **1** followed by an amplification step with a streptavidin−phycoerythrin conjugate (Figure 4c). Finally, anti-FAP iBody **1** was used to visualize FAP-transfected tumor cells in frozen tissue sections of glioma tumor xenografts (Figure 4d).

Collectively, these data suggest the applicability of anti-FAP iBodies in a broad spectrum of methodologies traditionally utilizing antibodies.

DISCUSSION

Multiple proteases are involved in oncogenesis. FAP, along with other proteases, is proposed to participate in the processes of cell adhesion, invasion, migration, and tumor neovascularization.[51] However, in contrast to most other cancer-associated proteases, FAP is expressed very sparsely in healthy adult tissues. In cancerous tissues, FAP is characteristically present in stromal cells as well as in the transformed elements of several malignancies.[51] This makes FAP a promising potential target to exploit for cancer therapeutics and/or diagnostics. Recently, we described the iBody concept for specific targeting of enzymes.[37] In this work, we aimed to prepare anti-FAP iBodies and demonstrate their potential to specifically bind FAP and FAP-expressing cells.

To identify the most potent and specific FAP-targeting ligand, we synthesized and characterized four FAP inhibitors based on the (4-quinolinoyl)glycyl-2-cyanopyrrolidine scaffold, which has high potency and selectivity for FAP.[44] The inhibitors were synthesized with PEG linkers of two different lengths, as we had previously observed that short linkers impaired binding of the protein target to the inhibitor molecule "immobilized" on the polymer backbone. We prepared inhibitors with or without a 4,4-difluoro substitution of the 2-cyanopyrrolidine moiety, as Jansen et al. showed that this substitution leads to more potent FAP binding and improved selectivity with respect to the close FAP homologue prolyl oligopeptidase.[45] Conjugation of compound **1** to the HPMA copolymer resulted in a 5-fold increase in the IC_{50} value. This was somewhat surprising, as we observed a significant drop in IC_{50} value for the iBody targeting GCPII.[37] Nevertheless, anti-FAP iBody **1** is a low nanomolar binder of FAP, which is still more than sufficient for effective in vitro and in vivo targeting. Highly specific discrimination between FAP and its close homologue DPP-IV, an almost ubiquitously expressed multifunctional protease,[52] is essential to prevent off-target effects of anti-FAP iBodies in vivo. We showed that iBody **1** is highly selective for FAP, exhibiting a more than four-order-of-magnitude lower IC_{50} for FAP than for DPP-IV. Therefore, even hundred nanomolar concentrations should lead to specific FAP targeting. We also verified that IC_{50} values of iBody **1** toward other FAP homologues, prolyl oligopeptidase (PREP) and dipeptidyl peptidase 9 (DPP9), are more than three-orders-of-magnitude higher than for FAP itself, meaning that iBody **1**

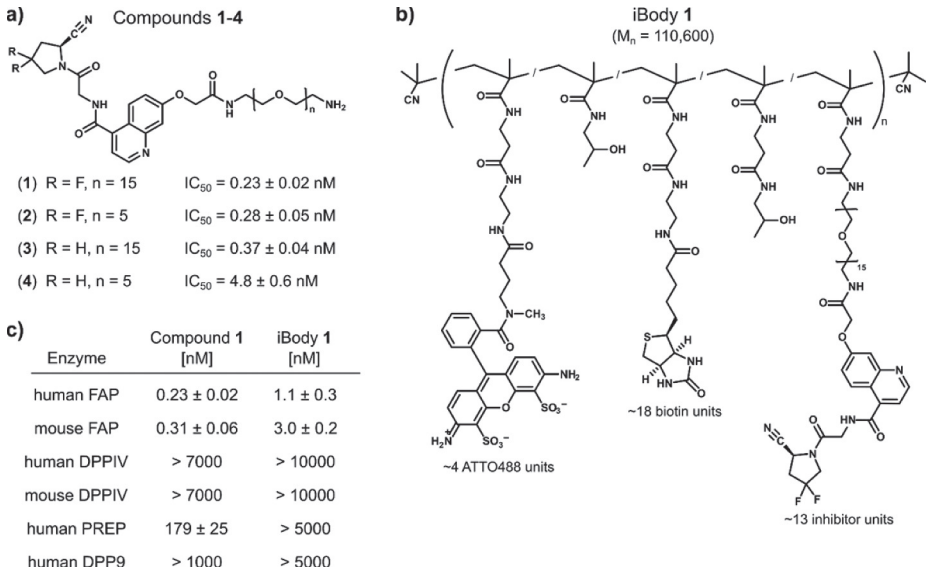


Figure 2. Structures of FAP inhibitors and anti-FAP polymer conjugate iBody **1** and their kinetic characterization. (a) The effect of prolyl moiety modification of the (4-quinolinoyl)glycyl-2-cyanopyrrolidine scaffold and PEG linker length on the kinetic properties of the tested inhibitors. The IC_{50} values for modified inhibitors with or without fluorine substitution and PEG linkers of various lengths are presented as mean ± standard deviation. Measurements were performed in duplicate. (b) Schematic structure of anti-FAP polymer conjugate (iBody **1**) containing an ATTO488 fluorophore, an affinity anchor (biotin), and compound **1**, the FAP-specific inhibitor. (c) Comparison of IC_{50} values of the FAP inhibitor and the anti-FAP iBody toward recombinant FAP, DPP-IV, DPP9, and PREP (IC_{50} values are presented as mean ± standard deviation).

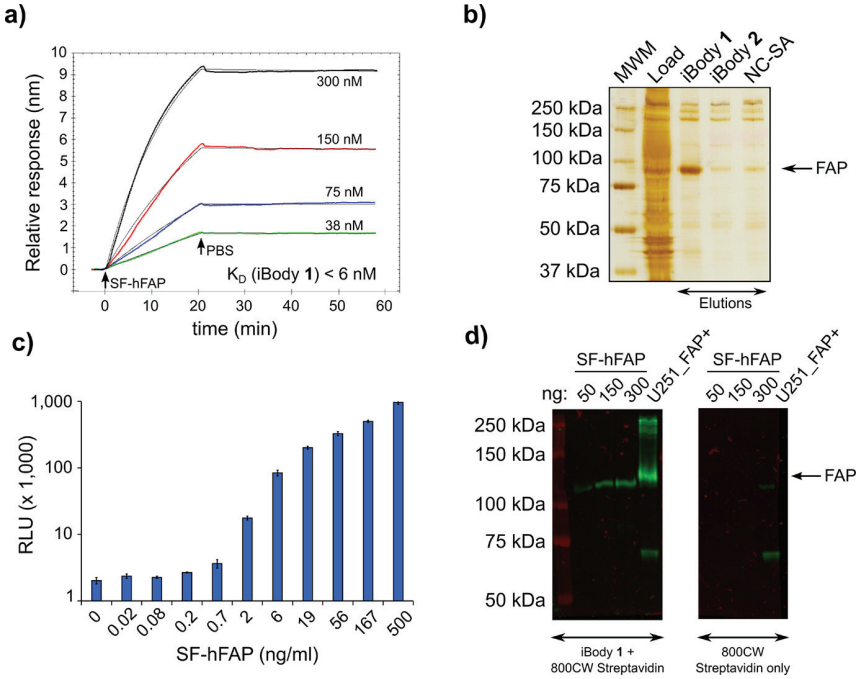


Figure 3. Application of anti-FAP iBody **1** in biochemical methods. (a) SPR analysis of SF-hFAP binding to immobilized iBody **1** (K_D < 6 nM). (b) Affinity isolation of FAP from FAP-transfected U251 (U251_FAP+) cell lysate using iBody **1**. iBody **2** (lacking FAP inhibitor) and blank streptavidin agarose (NC-SA) were used as negative controls. Load = U251_FAP+ cell lysate. (c) Sandwich ELISA for quantification of FAP using iBody **1** and anti-FAP F-19 antibody as a detection "agent" and a capture antibody, respectively. Each sample was measured in triplicate; values are presented as the mean ± standard deviation. (d) Western blot visualization of FAP using iBody **1** followed by an IRDye 800CW streptavidin conjugate. Purified recombinant human FAP (SF-hFAP) and a lysate of U251_FAP+ cells were used. The right section refers to the membrane probed with IRDye 800CW streptavidin conjugate only.

keeps the selectivity for FAP over these predominantly intracellularly localized homologues as well.

In contrast to antibodies, which target surface epitopes, the binding of iBodies relies on a specific interaction between the inhibitor molecule and the active site of the enzyme, which is usually the most conserved part of a protein molecule. Therefore, we expected anti-FAP iBody to bind FAP orthologues with similar affinity. Indeed, we found that iBody

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