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Molecular recognition of fibroblast activation protein for diagnostic and therapeutic applications

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ABSTRACT

Fibroblast activation protein (FAP) is a non-classical serine protease expressed predominantly in conditions accompanied by tissue remodeling, particularly cancer. Due to its plasma membrane localization, FAP represents a promising molecular target for tumor imaging and treatment. The unique enzymatic activity of FAP facilitates development of diagnostic and therapeutic tools based on molecular recognition of FAP by substrates and small-molecule inhibitors, in addition to conventional antibody-based strategies.

In this review, we provide background on the pathophysiological role of FAP and discuss its potential for diagnostic and therapeutic applications. Furthermore, we present a detailed analysis of the structural patterns crucial for substrate and inhibitor recognition by the FAP active site and determinants of selectivity over the related proteases dipeptidyl peptidase IV and prolyl endopeptidase. We also review published data on targeting of the tumor microenvironment with FAP antibodies, FAP-targeted prodrugs, activity-based probes and small-molecule inhibitors. We describe use of a recently developed, selective FAP inhibitor with low-nanomolar potency in inhibitor-based targeting strategies including synthetic antibody mimetics based on hydrophilic polymers and inhibitor conjugates for PET imaging.

In conclusion, recent advances in understanding of the molecular structure and function of FAP have significantly contributed to the development of several tools with potential for translation into clinical practice.

Abbreviations: 4-AN, 4-aminonaphtol; ABP, activity-based probe; AFC, 7-amino-4-(trifluoromethyl)coumarin; AKT, protein kinase B; AMC, 7-amino-4-methylcoumarin; APCE, antiplasmin-cleaving enzyme; BM-MSC, multipotent bone marrow stromal cells; BnO, benzyloxy group; BNP, B-type natriuretic peptide; Boc, *tert*-butyloxycarbonyl group; BoroPro, boronic acid derivative of proline; C1qT6, complement C1q tumor necrosis factor-related protein 6; CAF, cancer associated fibroblasts; CAR T cells, chimeric antigen receptor T cells; CCL/CXCL, chemokines belonging to the CC and CXC family respectively; CD, cluster of differentiation; CT, computed tomography; DOTA, tetraazacyclododecane tetraacetic acid; DPP, dipeptidyl peptidase; ECM-1, extracellular matrix protein 1; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ELISA, enzyme-linked immunosorbent assay; EP, endopeptidase; ERK, extracellular-signal-regulated kinase; FAP, fibroblast activation protein; FDG, ^{18}F-fluorodeoxyglucose; FGF21, fibroblast growth factor 21; GLP, glucagon-like peptide; GRF, growth hormone-releasing factor; HATU, hexafluorophosphate azabenzotriazole tetramethyl uronium; HetAr, heteroaromatic moiety; HMRG, hydroxymethyl rhodamine green; HOBt, hydroxybenzotriazole; HPLC, high-performance liquid chromatography; Iba-1, ionized calcium-binding adapter molecule 1; LOX-L1, lysyl oxidase-like-1; MALDI, matrix assisted laser desorption/ionization; MMP, matrix metalloproteinase; Ms, methylsulfonyl group; MS, mass spectrometry; NPY, neuropeptide Y; PET, positron-emission tomography; PI3K, phosphatidylinositol-3-kinase; PREP, prolyl endopeptidase; ProCN, nitrile derivative of proline; Quin, 4-quinolinoyl group; SP, substance P; SPECT, single-photon emission computed tomography; TBTU, tetrafluoroborate benzotriazole tetramethyl uronium; TIMPs, tissue inhibitors of metalloproteinases; Z, benzyloxy carbonyl.

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1. Introduction

1.1. Expression and biological roles of FAP

Fibroblast activation protein α (FAP, EC 3.4.2.1.B28; also known as seprase, prolyl endopeptidase FAP or antiplasmin-cleaving enzyme [APCE]) belongs to the family of non-classical serine proteases. Three independent lines of research led to identification of FAP. Its selective expression predominantly in activated fibroblasts and sarcomas, detected with the F19 monoclonal antibody, led to its designation as FAP [1–3]. Other research group established a surface-expressed protease (seprase) with collagenolytic activity as an important factor contributing to the invasiveness of melanoma cells and found that this protein was identical to FAP [4,5]. Finally, while investigating mechanisms leading to the formation of a more potent form of alpha-2-antiplasmin, Lee et al. identified a soluble form of FAP in plasma as the responsible protease [6,7].

FAP is scarcely expressed in mammalian healthy adult tissues (see Busek et al. [8] for review). Exceptions include pancreatic alpha cells [9], multipotent bone marrow stromal cells (BM-MSC) [10], uterine stroma especially during the proliferative phase, human placenta and some dermal fibroblasts [11–13]. FAP is present in plasma in humans (at approximately 100 ng/ml or 0.6 nmol/l) and in other species (reviewed in Busek et al. [8]), but the source of this soluble form of FAP is unclear.

In contrast to its low expression in the majority of resting stromal cells, FAP is upregulated by a variety of stimuli in several states accompanied by tissue remodeling. For example, FAP expression is increased in healing wounds, keloids, scleroderma, pulmonary fibrosis, liver cirrhosis, arthritis, scar tissue after myocardial infarction, advanced atherosclerotic lesions and strictured regions in Crohn's disease (see Busek et al. [8] and references therein). In these states, FAP is predominantly upregulated in activated mesenchymal cells such as fibroblasts. In mouse models of pulmonary fibrosis, FAP was found to be involved in matrix metalloproteinase (MMP)-mediated extracellular matrix remodeling and collagen clearance, playing an important role in resolving injury-induced scarring [14]. Whether this mechanism represents a general role of FAP in other fibrotic states remains to be established.

FAP is also upregulated in various tumor types and its expression is linked to an immunosuppressive tumor microenvironment [15], higher tumor grade, increased lymph node metastasis and poor overall survival [8,16]. FAP is an important marker of cancer associated fibroblasts (CAF) and appears to contribute to some of their tumor-promoting activities, possibly by modulating the structure and composition of the extracellular matrix [17–19] and affecting the CAF secretome [20]. Interestingly, these effects may also involve indirect mechanisms including modulation of MMPs, MMP inhibitors (TIMPs), and lysyloxidases [21]. Expression of FAP in other types of tumor-associated stromal cells, such as endothelial cells and macrophages, has also been reported (see Busek et al. [8], Pure et al. [16] and references therein). In endothelial cells, FAP was suggested to play a possible role in angiogenesis [22]. Indeed, in a mouse model of pancreatic adenocarcinoma, stromal FAP accelerated tumor development and promoted tumor cell dissemination [23]. FAP is also expressed in cancer cells and increases their tumorigenicity and proliferation in pancreatic adenocarcinoma, esophageal cancer and breast cancer [24–27]. The underlying molecular mechanisms are not well-understood and may differ in individual tumor types. However, one study revealed that FAP knockdown in oral cancer decreased the activity of the PI3K/AKT and Ras-ERK pathways, leading to decreased proliferation, migration and invasion of cancer cells [28].

1.2. FAP structure, enzymatic activity and homologs

FAP is a 170 kDa type II transmembrane protein that is catalytically active as a dimer (Fig. 1). It exclusively cleaves the post-proline peptide bond, while possessing both dipeptidyl peptidase (DPP) and endopeptidase (EP) activity. FAP belongs to the S9B oligopeptidase subfamily, together with dipeptidyl peptidase IV (DPP-IV, EC 3.4.14.5), a serine protease and FAP's closest homolog (52% sequence identity). As opposed to FAP, DPP-IV is expressed in a variety of human tissues under physiological conditions and is connected to a number of physiological processes, including glucose homeostasis and T-cell activation [29]. Other homologs of FAP within the S9B subfamily include the serine proteases dipeptidyl peptidase 8 and 9 (DPP8, DPP9).

Prolyl endopeptidase (PREP, also known as prolyl oligopeptidase [POP]; EC 3.4.21.26) is phylogenetically related to FAP and belongs to the serine protease subfamily S9A. Changes in expression and activity of this enzyme are associated with aging, several neurological pathologies, inflammation and cancer [30–33]. PREP is predominantly a cytosolic protein [34], but a membrane-bound version also has been reported [35]. Similarly to FAP, PREP cleaves the post-proline peptide bond of its substrates; therefore, it must be considered as a potential unintended target of compounds interacting with FAP active site. Compared to FAP, PREP is much less specific towards the other amino acid residues in the vicinity of the cleaved peptide bond. This could be the main reason underlying the poor FAP/PREP specificity of many artificial substrates and inhibitors.

Aertgeerts et al. reported the FAP structure and identified the important residues in the active site based on X-ray crystallographic analysis [36]. In addition to the catalytic triad composed of Ser^{624}, Asp^{702} and His^{734}, the residues Arg^{123}, Glu^{203}, Glu^{204}, Ala^{657} and Tyr^{656} are responsible for substrate binding (Fig. 2B).

Although the reported structure lacks a ligand in the active site, the substrate binding mode was modeled based on the strong similarity between FAP and its homolog DPP-IV. Comparison with DPP-IV in complex with its substrate showed that the binding pockets of both

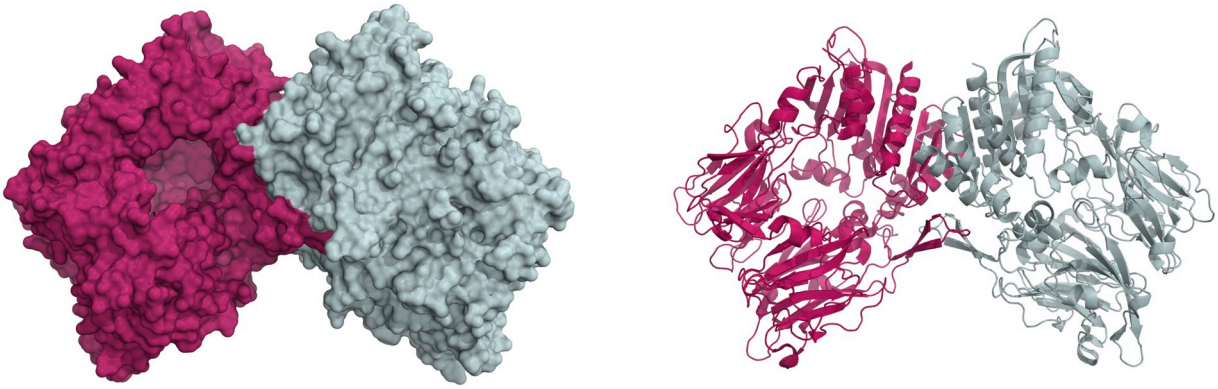


Fig. 1. The crystal structure of the FAP homodimer. Each monomer is pictured in a different color. PDB ID: 1Z68 [36].

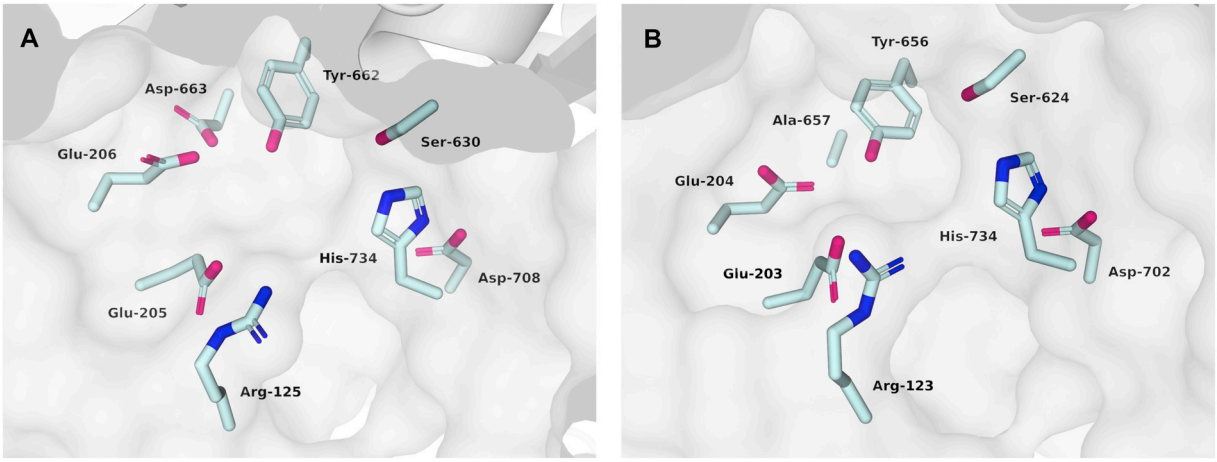


Fig. 2. The active sites of A) DPP-IV (PDB ID: 1NU6 [37]) and B) FAP (PDB ID: 1Z68 [36]). The key residues participating in substrate binding and cleavage are similarly positioned in both proteins.

enzymes are almost identical (Fig. 2) [36].

Aertgeerts et al. also hypothesized the nature of the FAP substrate-binding subsites (see Fig. 3B illustrating the convention used for description of positions in substrates, protein active sites and inhibitors in this review): the flat S1' subsite could accommodate virtually any amino acid, while the S2' subsite lined by Trp^{623} and Tyr^{745} is expected to prefer aliphatic residues. S1 is a defined hydrophobic pocket designed to bind the proline residue and lined by Tyr^{625}, Val^{650}, Trp^{653}, Tyr^{656}, Tyr^{660}, and Val^{705}. According to Aertgeerts et al., large hydrophobic residues can be modeled in the S2 subsite, which is defined by Arg^{123}, Phe^{350}, Phe^{351}, Tyr^{541}, Pro^{544}, Tyr^{625}, and Tyr^{660}.

While the neighboring glutamates (Glu^{203}, Glu^{204}) seem to be crucial for DPP activity by binding the positively charged N-terminus, Ala^{657} is responsible for FAP EP activity, as illustrated by mutagenesis

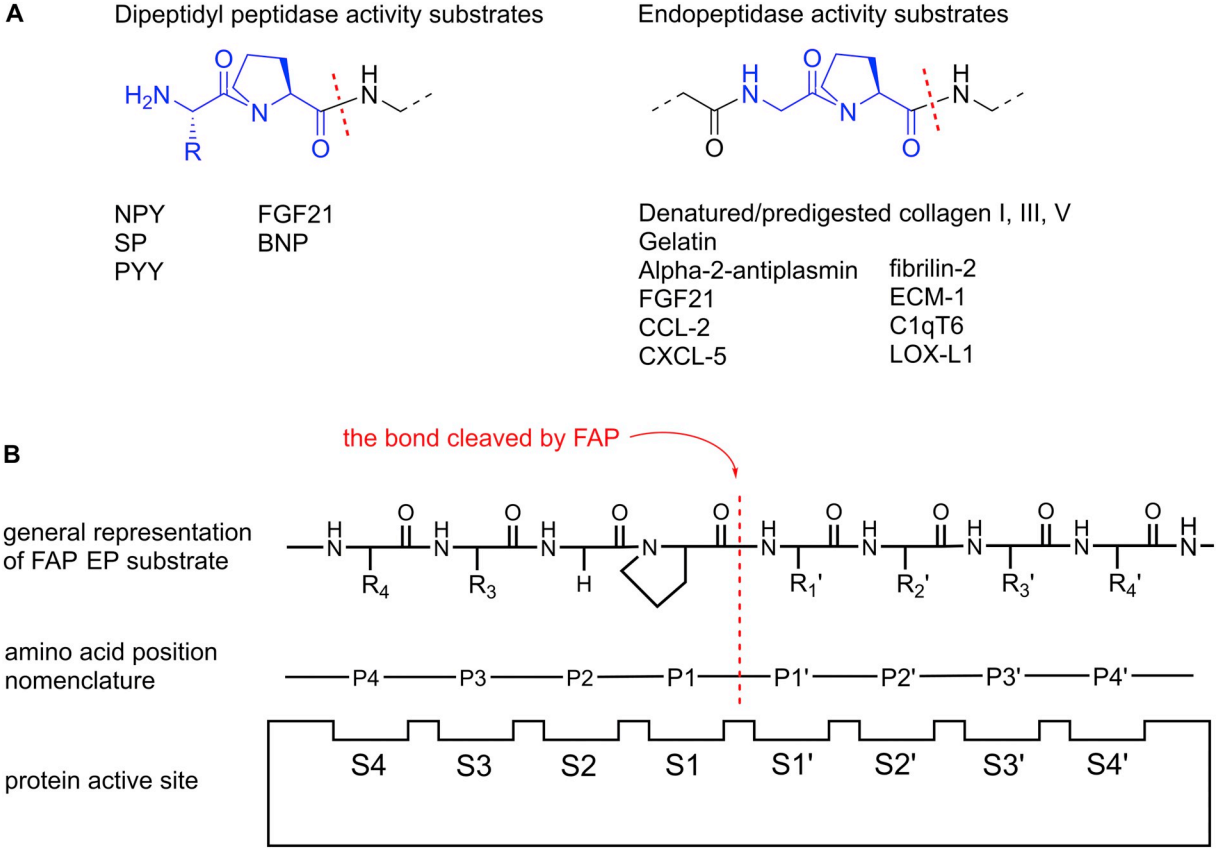


Fig. 3. A) Dipeptidyl peptidase (DPP) and endopeptidase (EP) activity of FAP against its natural substrates. The P2 preferences depend on the type of enzymatic activity. Amino-terminal processing of GLP-1, GLP-2, GIP, PHM and GRF by FAP also has been reported, but only after prolonged incubation [47]. BNP = brain natriuretic peptide; CCL/CXCL = chemokines belonging to the CC and CXC family, respectively; C1qT6 = complement C1q tumor necrosis factor-related protein 6; ECM-1 = extracellular matrix protein 1; FGF21 = fibroblast growth factor 21; GIP = gastric inhibitory polypeptide/glucose-dependent insulinotropic peptide; GLP-1, 2 = glucagon-like peptide-1, 2; GRF = growth hormone-releasing factor; LOX-L1 = lysyl oxidase-like-1; NPY = neuropeptide Y; PHM = peptide histidine-methionine; SP = substance P; PYY = peptide YY. B) Nomenclature of positions of substrates and protease active sites according to Berger and Schechter. This convention is used to describe all substrates and inhibitors in this review.

studies [36,38].

No other experimentally determined FAP structure has been reported to date, and thus we are dependent on computational approaches to predict the binding mode of inhibitors or substrates to the FAP binding cleft.

1.3. Naturally occurring substrates

FAP can cleave a number of peptides and proteins through its DPP and EP activities (Fig. 3A). Nevertheless, the natural, physiologically relevant substrates of FAP are only beginning to be revealed. Similar to DPP-IV-mediated processing of biologically active peptides [39], the functional consequences of FAP cleavage can vary, leading to increased (alpha-2-antiplasmin), decreased (FGF21 [40]) or unchanged (CCL-2, CXCL-5 [21]) bioactivity of the substrate.

Initial studies identified denatured and partially digested collagen I and III as FAP substrates [41,42]. Recently, the importance of collagen I cleavage has been supported by studies demonstrating increased collagen accumulation in FAP knockout mice in a model of lung injury [14] and increased macrophage adhesion to FAP-treated collagen I [18]. These studies thus suggest a role for FAP activity in modulation of fibrotic extracellular matrix and infiltration of immune cells. Recent reports of the cleavage of fibroblast growth factor 21 (FGF21), an important regulator of energy metabolism and insulin sensitivity, point to possible involvement of FAP in metabolic regulations [40,43]. Pharmacological inhibition of FAP was initially reported to enhance levels of FGF21 and offer several metabolic benefits in obese mice [44], but a more detailed follow-up study using specific inhibitors and FAP knockout animals showed only modest improvement in acute oral and intraperitoneal glucose tolerance [45].

Alpha-2-antiplasmin is another endogenous protein cleaved by FAP. The resulting 452-residue version with Asn at the N-terminus (Asn-alpha-2-antiplasmin) is more readily incorporated into fibrin clots and makes them more resistant to plasmin-mediated dissolution *in vitro* [6]. The potential physiological relevance of these results was recently supported by a report demonstrating a weak, but statistically significant, correlation between plasma FAP levels and alpha-2-antiplasmin incorporation into fibrin clots [46].

Among the DPP-IV substrates, neuropeptide Y (NPY), peptide YY (PYY), substance P (SP), and B-type natriuretic peptide (BNP) are cleaved by FAP, with NPY cleavage being the most efficient [47,48]. Recently, unbiased proteomic approaches in immortalized primary mouse embryonic fibroblasts transfected with enzymatically active and inactive FAP were used to uncover novel substrates of FAP. The identified substrates include collagens I, III and V; the extracellular matrix proteins fibrillin-2 and extracellular matrix protein 1 (ECM-1); the chemokine CXCL-5; complement C1q tumor necrosis factor-related protein 6 (C1qT6); and lysyl oxidase-like-1 (LOX-L1) [21].

2. Molecular recognition of FAP

2.1. Anti-FAP antibodies and antibody-based probes

Several anti-FAP antibody-based approaches have been developed to detect and quantify FAP in tissues and plasma and to target FAP-expressing cells. Use of a commercially available ELISA for FAP quantification has been reported in a number of studies [26,49–51]. Various well-established antibodies against human FAP/seprase are available for immunohistochemical detection, but the possibility of different reactivity with FAP originating from the stroma (typically detected with the F19 mouse monoclonal antibody) and FAP/seprase in cancer cells (characteristically detected with the D8 and D28 rat monoclonal antibodies, see Jacob et al. [52] for review) remains an unanswered question. Only a few antibodies that recognize and/or cross-react with FAP orthologues, such as mouse FAP, have been characterized.

Radiolabeled anti-FAP antibodies and their derivatives have been applied to noninvasive imaging of FAP expression in inflammatory diseases and cancer. In line with the pathogenic role of FAP-expressing cells in the progression of rheumatoid arthritis [53], accumulation of anti-FAP antibodies detected by PET and SPECT in a mouse model of rheumatoid arthritis correlated with inflammation severity. In addition, such imaging techniques could be used to noninvasively monitor response to therapy [54,55]. SPECT with radiolabeled anti-FAP antibodies was shown to detect small metastatic lesions in cancer patients [56], and several studies demonstrated selective accumulation of FAP-targeting antibodies and their derivatives in mouse models of cancer [57–60].

Despite their selective accumulation in tumor tissue, naked anti-FAP antibodies seem to have only a modest therapeutic effect. Polyclonal anti-FAP antibodies that inhibited FAP enzymatic activity slowed tumor growth in an animal model of colorectal cancer [61], but did not halt its progression. In a study using a modified vaccinia virus to constitutively express intratumoral anti-FAP antibodies, improved tumor regression after oncolytic virotherapy was observed in a mouse model [62]. In contrast, a humanized version of the F19 antibody (sibrotuzumab) had no effect on tumor regression in a phase II clinical trial [63]. Most current approaches using anti-FAP antibodies thus exploit their ability to accumulate in FAP-rich areas to selectively deliver cytotoxic compounds [57,64], radioisotopes [65] and immunocytokines [66–68] into the tumor microenvironment. Constructs locally stimulating T cells [69,70], apoptosis-inducing antibody-based fusion proteins [71], and several anti-FAP immunoliposomes [72] also have been developed (see Busek et al. [8] for detailed review and references).

Monoclonal anti-FAP antibodies also have guided the engineering of FAP-directed CAR T cells. Several CARs have been prepared and tested in animal models with mostly encouraging results [11,73–76], although one study reported severe side effects [77] (see Busek et al. [8] for detailed review). Similar to other FAP-targeting approaches, the utility of FAP CAR T cells needs to be confirmed in clinical trials.

2.2. Substrate specificity

Based on the high homology of FAP and DPP-IV, especially around the active site, and the considerable overlap in FAP and PREP substrate specificity, this review focuses on FAP/DPP-IV and FAP/PREP specificities.

FAP/DPP-IV substrate specificities have been analyzed using artificial peptide substrates [36,38,78], as well as by scanning of dipeptide libraries [78,79] and alpha-2-antiplasmin-derived libraries [80,81]. FAP and PREP substrate specificities are much less well-explored than FAP/DPP-IV, although a FAP/PREP comparison was performed using a combinatorial library [82].

2.2.1. FAP/DPP-IV specificity

FAP exhibits both DPP activity (*i.e.*, cleaving dipeptides from a protein's N-terminus) and EP activity (Fig. 3). This feature is the main difference between FAP and DPP-IV, which possesses only DPP activity and does not function as an EP. The DPP activity of FAP is rather promiscuous – FAP cleaves a variety of different H-P2-Pro N-terminated substrates [36,78,79], albeit approximately 100-fold less effectively than DPP-IV [78].

Nevertheless, the DPP activities of FAP and DPP-IV differ only slightly in their P2 preferences. While FAP slightly prioritizes Ile, Pro and Arg residues, DPP-IV cleaves most substrates comparably [78].

On the other hand, FAP's dominant EP activity is highly specific for the X-Gly-Pro pattern (where X stands for a peptide or an acyl terminus). This principle has been documented several times [78–82] and is in accord with all the available data. Based on this specificity, various artificial substrates and inhibitors have been developed, and many have shown potential in cancer diagnosis and treatment approaches (see Section 2.3.).

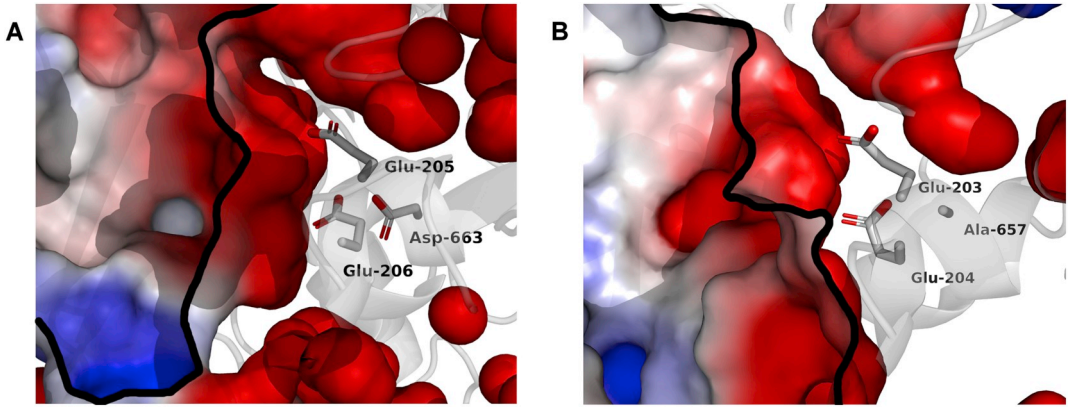


Fig. 4. The electrostatic surface of the binding cavities of A) DPP-IV (PDB ID: 1NU6 [37]) and B) FAP (PDB ID: 1Z68 [36]). Red and blue colors indicate the negatively and positively charged surface, respectively. Black lines indicate the interface between the solvent-accessible area (left part) and the interior of the protein (right part). Asp^{663} in DPP-IV pushes the adjacent Glu^{206} into the solvent-accessible area, while Ala^{657} in FAP allows the analogous Glu^{204} to hide one of its oxygens.

2.2.2. Structural origins of the differences between FAP and DPP-IV enzyme activity

The crystal structure of FAP enabled exploration of the origins of the differences in FAP and DPP-IV enzyme activity [36]. Using a site-directed mutagenesis approach, it has been found that Ala^{657} in the FAP binding pocket is crucial for EP activity. DPP-IV, which lacks EP activity, possesses Asp^{663} in the corresponding position. The D663A mutation in DPP-IV increased its ability to cleave the EP substrate *Z*-Gly-Pro-AMC by two orders of magnitude, while the reverse mutation in FAP had the opposite effect [36,38]. Structural analysis revealed that the substrate binding pocket of DPP-IV is more negatively charged due to Asp^{663}. This residue is positioned just next to Glu^{206}, which is pushed by Asp^{663} into the cavity so both acid oxygens are exposed. FAP possesses Ala^{657} instead of Asp^{663}, and the adjacent Glu^{204} in FAP (analogous to Glu^{206} in DPP-IV) has a single oxygen that is solvent-accessible and thus available for interactions (Fig. 4) [36].

Other amino acids in the binding pocket have negligible effects on EP/DPP enzyme activity. The surrounding residues are more likely responsible for specific binding of the proline moiety to the active site and orientation of the post-proline peptide bond for cleavage [38].

Interestingly, the electrostatics of the protein surfaces offer an additional difference: the surface of DPP-IV bears more negatively charged residues than that of FAP. This negative charge in DPP-IV is present predominantly in the proximity of the active site (Fig. 5). This could result in significant differences in interactions between proteins themselves and the positively charged free N-terminus of substrates.

2.2.3. Substrate specificity profiling of FAP

Several peptide libraries, mostly with a systematically varied amino acid at one specific position, have been analyzed to study FAP substrate specificity. Each library peptide is conjugated to a fluorogenic moiety such as 7-amino-4-(trifluoromethyl)coumarin (AFC) or 7-amino-4-methylcoumarin (AMC) [38,78,80], allowing cleavage to be followed spectrophotometrically. In addition to spectrophotometry-based methods, HPLC-MS and MALDI analyses of reaction mixtures after enzymatic cleavage have been used for FAP substrate profiling [79,81].

After the P1 and P2 specificities of the enzyme had been repeatedly confirmed, efforts focused on FAP's preferences for other specific substrate patterns near the cleavage site (P8-P4') [81,82].

Although no specific motif has been found, a few structural preferences have been identified [80,81]. In studies based on alpha-2-antiplasmin-derived libraries, the amino acids in P4-P2' and P8-P4' were systematically varied. FAP slightly preferred uncharged residues in positions P4 and P3 and strongly preferred positively charged residues in P7. Substrates with Ala, Asn, Ser, and Tyr in P1' were cleaved more favorably than those with other residues. Trp was preferred in the P2' position [81]. Other differences in peptide cleavage were minor.

Nevertheless, substrate specificity profiling studies based on the variation of one amino acid in the chain may be biased, as we cannot conclude the influence of the rest of the peptide.

2.2.4. FAP/PREP specificity

To establish selective substrates for FAP and PREP, Jambunathan et al. [82] performed a less biased substrate specificity profiling of the enzymes based on a combinatorial peptide library. They scanned a combinatorial library of 3375 internally quenched fluorogenic probes containing a varied tripeptide between two glycine spacers. When aligned to the FAP substrate specificity, this analysis can be considered

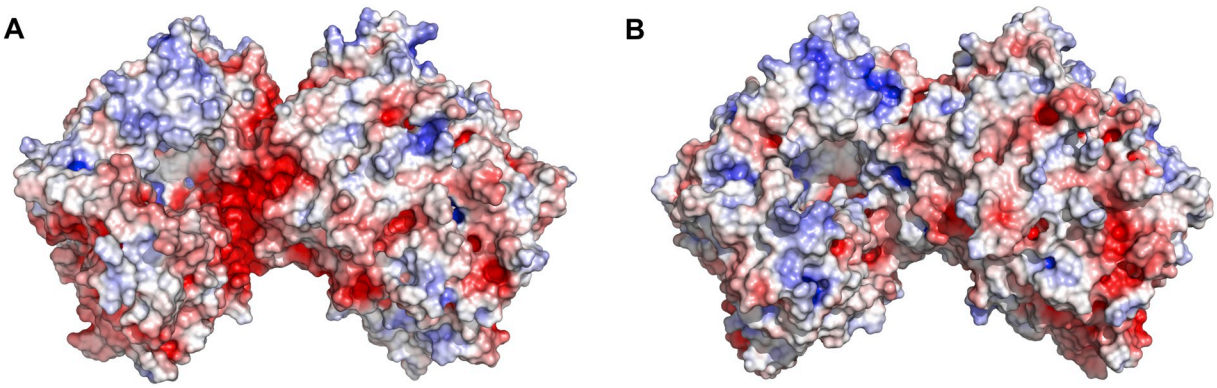


Fig. 5. The electrostatic surface of A) DPP-IV (PDB ID: 1NU6 [37]) and B) FAP (PDB ID: 1Z68 [36]). Red and blue colors indicate the negatively and positively charged surface, respectively.

as a variation of the amino acids in positions P1, P1', and P2'.

In the P1' and P2' positions, FAP could accommodate virtually any amino acid, except for proline in P1'. FAP and PREP both appeared promiscuous regarding the P1' and P2' substrate positions. The peptides cleaved solely by FAP showed a higher ratio of polar residues in P1' and P2', while most peptide sequences cleaved exclusively by PREP accommodated a non-glycine residue in P2 or both P2 and P3.

According to a recent study by Decker et al. [83], FAP/PREP specificity can be increased by substituting the P2 glycine with D-alanine. However, this substitution is accompanied by a significant decrease in FAP catalytic efficiency. This study also revealed the great impact of the P1' moiety on FAP/PREP specificity, which was used in the development of highly selective FAP-targeted activity-based probes (ABP) (see Section 2.3.1.).

2.3. The FAP enzyme activity targeting

2.3.1. Fluorogenic substrates and probes

Insights into FAP substrate specificity enabled the design of probes based on release of a fluorescent moiety *via* specific cleavage by FAP. Several fluorogenic substrates for FAP detection in fluids and tissues have been developed in recent years, for both research and potential diagnostic purposes. The fluorescent cleavage product of such substrates enables quantification of FAP activity and thus quantity in solution and/or tissues. FAP-mediated fluorescence can be achieved by liberating the fluorophore amine moiety or by separating the fluorescent dye from the quencher (Fig. 6).

Multiple fluorogenic probes have been applied to FAP detection (Fig. 7). Hydroxymethyl rhodamine green (HMRG) dye can be present in spirocyclic (non-fluorescent) and acyclic (fluorescent) forms depending on the pH of the solution. The acylated derivative of HMRG prefers the cyclic form at physiological pH. After enzymatic cleavage, the liberated HMRG adopts the acyclic form, which is highly fluorescent and thus allows measurement of the enzymatic activity. For FAP activity quantification, the Ac-Gly-Pro moiety was conjugated to HMRG (Ac-Gly-Pro-HMRG, Fig. 7) [84].

Similarly, in the case of acylated 7-amino-4-methylcoumarin (AMC)-based probes, the fluorescence is quenched and recovered after enzymatic cleavage. Such probes have been developed for FAP activity quantification in body fluids and organs (3144-AMC, Fig. 7) [85]. The same fluorogenic tag was used in a recently developed assay for specific FAP or PREP quantification in plasma samples. This approach utilizes the substrate *Z*-Gly-Pro-AMC (Fig. 7), which lacks FAP/PREP specificity, together with a selective FAP or PREP inhibitor [86].

The probes GP and aP, developed by Brainbridge et al. [49] (Fig. 7), were designed for FAP detection in plasma. Enzyme-mediated cleavage of a bridge peptide leads to fluorophore-quencher dissociation and generation of a fluorescent signal. By optimizing the P2 amino acid in the peptide sequence, the researchers achieved a high selectivity for FAP over PREP. However, the D-Ala residue responsible for this specificity resulted in significantly decreased FAP cleavage efficiency compared to the variant with Gly in P2.

Recently, Decker et al. [83] focused on the FAP/PREP selectivity issue and kinetic characterization of an activity-based probe series structurally derived from 3144-AMC. Although 3144-AMC exhibits satisfactory FAP/PREP specificity thanks to D-Ala in the P2 position, the cleavage efficiency is 40-fold lower than that of the analogous probe with Gly in P2 (UAMC1063-AMC[1]) [83]. Their study found that FAP/PREP selectivity can be strongly influenced by the P1' structure. Replacement of the fluorogenic moiety AMC with 4-aminonaphtol (4-AN) decreased the FAP cleavage efficiency 4-fold, while no cleavage by PREP was observed (UAMC1063–4-AN, Fig. 8, Table 1). The main disadvantage of this approach is that it cannot be used in established spectrophotometry-based assays because of overlap between the absorbance and fluorescence spectra of 4-AN [83].

Different substrate-cleavage-based approaches using FAP as a cancer tissue imaging platform for *in vivo* applications were developed in parallel. All are based on the fact that FAP is predominantly expressed in pathological tissues. Originally, FAP enzyme activity was targeted, but recently, inhibition-based probes have been developed (see Section 2.4.4.). Near-infrared fluorescent probes [88,89], combined prodrug-probes [90,91], and a ferritin nanocage-based optical probe [92] have successfully visualized FAP-expressing tumor tissues in mice.

Ji et al. [93] took a different approach by developing nanocarriers comprising FAP-cleavable peptides. These particles are able to accommodate a drug or fluorescent dye and thus work as a drug delivery platform as well as a cancer-tissue imaging tool.

2.3.2. Prodrugs and drug delivery strategies exploiting FAP enzymatic activity

The unique substrate specificity and expression pattern of FAP make it attractive for the design of prodrugs in which a cytotoxic agent or potent anti-tumor drug is inactivated *via* conjugation with a FAP-cleavable peptide. After prodrug administration, the drug or toxin is released *in situ* by FAP cleavage at the site of FAP expression. This strategy is designed to improve the therapeutic window by reducing the toxicity for healthy tissues.

The therapeutic potential of most proposed FAP-targeted prodrugs has been studied *in vivo* using mouse models with xenografts of human tumors (Table 2) [94–104]. All approaches tested to date resulted in significant tumor growth inhibition and greatly decreased side effects of the therapy. In most cases, parameters tracking the potential negative effects of the treatment (body-weight loss, histopathological analyses, cardiac function, hematological toxicity, marker enzyme activities) did not differ from the negative control (untreated mice). Similar results were achieved with the cleavable peptide nanocarriers for drug delivery developed by Ji et al. [93]

A few other prodrugs were recently synthesized and shown to be cleaved by FAP *in vitro* or *in vivo*, but their therapeutic potential has yet to be established [90,91,105–108].

2.4. FAP inhibitors

2.4.1. The path to selective FAP inhibitors with low-nanomolar potency

In the last decade, a selective FAP inhibitor with low-nanomolar potency was developed (UAMC-1110, Fig. 16), and we summarize the pathway to this highly optimized compound. We review the various strategies employed and their impact on increasing the affinity and selectivity of the inhibitor structure. As most of the inhibitors discussed can be considered peptidomimetics, we extrapolated the Berger and Schechter nomenclature (Fig. 3B) to describe these compounds. The inhibition potencies are represented as IC_{50}, K_i or both, depending on the data reported.

Information about the substrate specificity of FAP enabled rational design of specific small molecules targeting the active site of the enzyme. Hu et al. [109] first reported a small-molecule FAP inhibitor in 2005. They performed a structure-activity relationship (SAR) study of the pseudodipeptide X-boroPro, where X stands for the P2 amino acid with either substituted or unsubstituted amine. The N-terminal substituents studied were mainly aliphatic cyclic alkyls, and these did not further improve inhibitor binding (Fig. 9).

Despite the fact that FAP prefers glycine in the P2 position according to subsequent SAR studies, the pseudodipeptide Val-boroPro displayed favorable affinity, which can be explained based on the substrate specificity of FAP DPP activity. As FAP has this type of

[1] The compound was derived from the known inhibitor UAMC1063 [87]. For the purpose of this review, the code UAMC1063-X refers to the compound with an identical structure bearing a cleavable amide bond instead of the nitrile warhead.

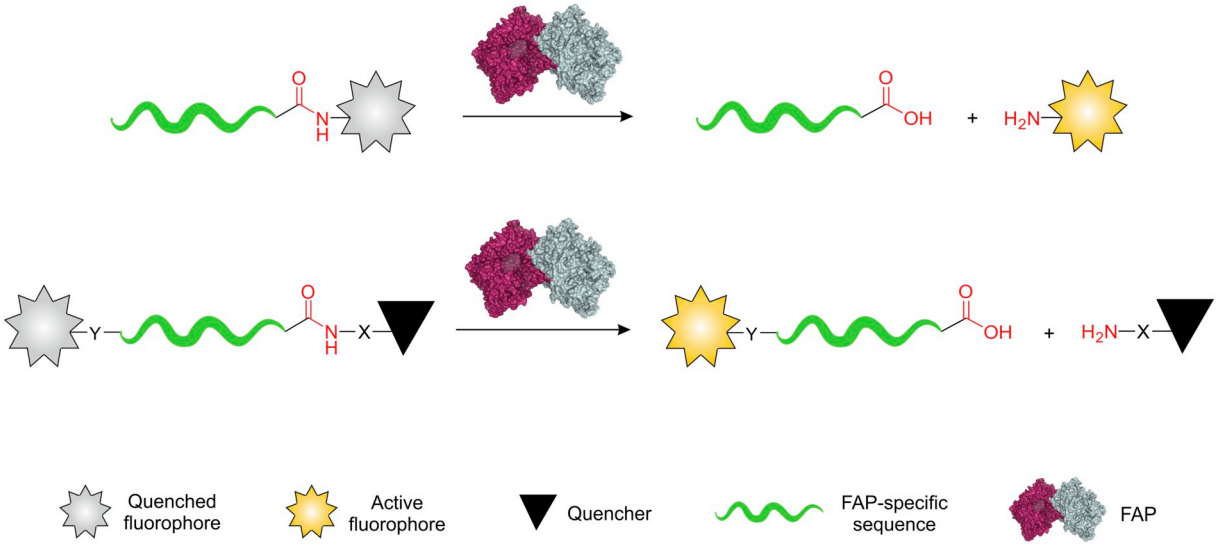


Fig. 6. General scheme of fluorogenic substrate-based probes. The fluorescence quenched by the chemical environment of the fluorophore is recovered following enzymatic cleavage of the FAP-specific sequence.

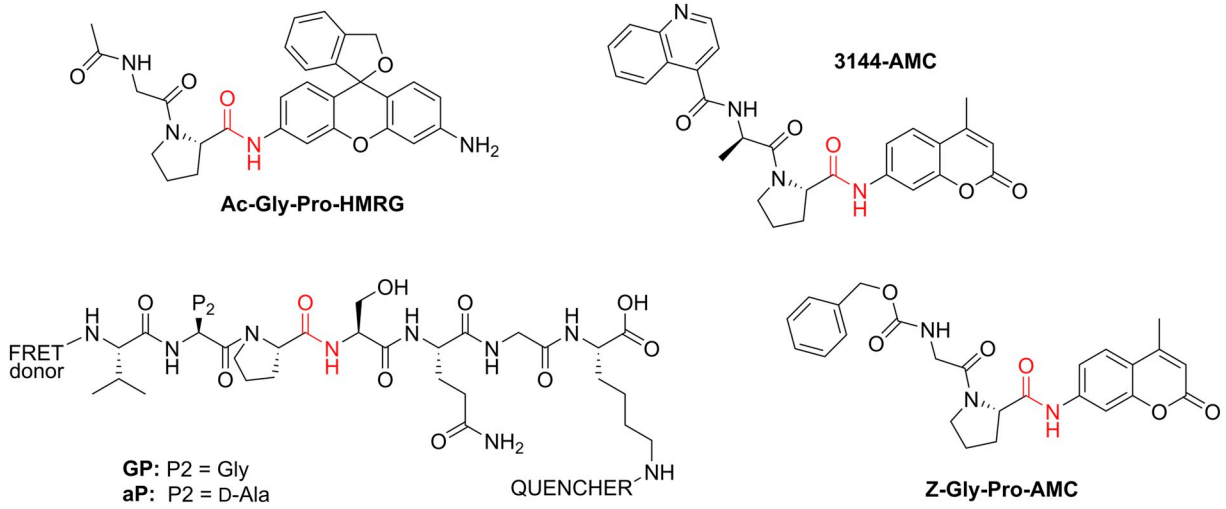


Fig. 7. Fluorogenic substrates developed for FAP detection in fluids and tissues. HMRG = hydroxymethyl rhodamine green; AMC = 7-amino-4-methylcoumarin; Z = benzyloxy carbonyl.

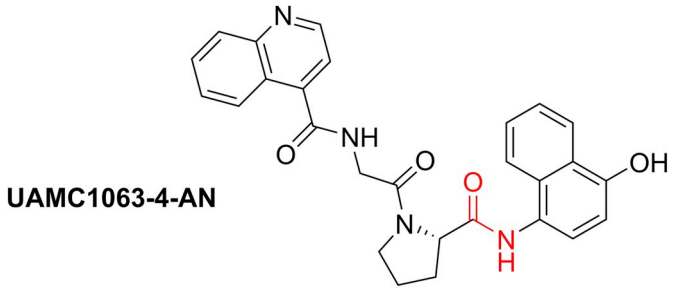


Fig. 8. An activity-based probe highly selective for FAP. The FAP/PREP selectivity was achieved by the nature of P1' moiety. 4-AN = 4-aminonaphtol.

enzyme activity in common with DPP-IV, which accepts Val in the P2 position, it is not surprising that Val-boroPro binds to both enzymes.

Although the resulting lead compound Val-boroPro (i1, PT-100, Talabostat) had unsatisfactory FAP/DPP-IV selectivity, it had a nanomolar IC_{50} value and proceeded to early clinical testing due to its potential anti-tumor therapeutic potency [110] (see Section 2.4.3.).

Edosada et al. [78] performed FAP/DPP-IV substrate specificity profiling and found that the Ac-Gly-Pro pattern was cleaved more effectively by FAP than DPP-IV. This information enabled identification of ligands specific for FAP or DPP-IV based on FAP EP activity and led to the design of the first slightly selective FAP inhibitor Ac-Gly-boroPro i2a (Fig. 10). The same research group established the related chloromethylketone i2b as a weak but irreversible FAP inhibitor [80] and the nitrile i2c as a moderate reversible inhibitor [38] (Fig. 10).

After Edosada et al. [78] achieved modest FAP/DPP-IV and FAP/PREP selectivity with their potent Ac-Gly-boroPro compounds, Tran et al. [111] continued with further structure optimization to identify a more selective compound. Their SAR study focused on the small aliphatic and aromatic P3 substituents of the X-boroPro pseudodipeptide (Fig. 11). According to their affinity data, DPP-IV does not tolerate aliphatic or aromatic N-terminal acyls, except acetyl and γ-lactam groups. These findings largely solved the FAP/DPP-IV inhibitor selectivity issue, but despite the favorable FAP/DPP-IV selectivity of i3, the issue of FAP/PREP selectivity remained unresolved. Although the nature of the N-terminal acyl seems to modulate FAP and PREP inhibition potency, the authors were unable to identify a FAP inhibitor clearly selective over PREP.

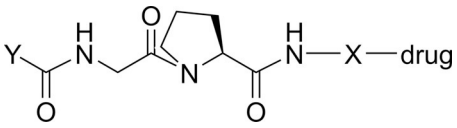
All the submicromolar inhibitors from the series bound PREP more

Table 1
Important kinetic parameters of selected activity-based probes.

Compound	k_{cat}/K_m ($10^6 M^{-1}s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	FAP/PREP SI	ref
3144-AMC	0.040 ± 0.003	ND	ND	15	Keane et al. [84]
GP [a]	0.025	0.14 ± 0.1	5.5 ± 0.5	NR	Brainbridge et al. [49]
aP [a]	0.0038	0.0202 ± 0.0003	5.3 ± 0.3	NR	Brainbridge et al. [49]
Z-Gly-Pro-AMC	0.0202 ± 0.004	ND	ND	0.04	Decker et al. [82]
UAMC1063–4-AN	0.40 ± 0.06	ND	ND	FAP > > PREP	Decker et al. [82]

SI = selectivity index, defined as k_{cat}/K_m(FAP)/k_{cat}/K_m(PREP); ND = not determined; NR = not reported.
[a] Values were obtained under different experimental conditions than for other compounds in the table.

Table 2
FAP-targeted prodrugs of general structure Y-CO-Gly-Pro-NH-X-drug. Y represents the N-terminal part, while X stands for variable linkage between cleavable bond and drug in the C-terminal part of the prodrug.



Y	Drug	*In vivo* tumor growth inhibition	Ref
Oligopeptide	Mellitin	Yes	LeBleau et al. [94]
BnO-	Doxorubicin [a]	Yes	Huang et al. [95]
Oligopeptide	Thapsigargin	Yes	Brennen et al. [96,97]
BnO-	Arenobufagin	Yes	Deng et al. [98]
BnO-	Epirubicin	Yes	Wang et al. [99]
Phthalocyanine-Thr-Ser-	Epirubicin	Yes	Ke et al. [91]
H_2O_3P-CH_2-CO-Ala-Leu-	Doxorubicin	Yes	Cornillie et al. [100]
BnO-	Doxorubicin [a]	Yes	Huang et al. [102]
$(CH_3)_2N$-CH_2-	Bufalin	Yes	Chai et al. [103]
BnO-	Gemcitabine	Yes	Sun et al. [104]
Pyropheophorbide *a* [b]	Black hole quencher 3 [b]	NR	Lo et al. [90]
BnO-	Epirubicin	NR	Liu et al. [106]
Oligopeptide	Emetine	NR	Akinboye et al. [107]

BnO- = benzyloxy substituent; NR = not reported.
[a] According to the chemical structure of the reported prodrug, epirubicin was used.
[b] Positions of Y and drug were reversed.

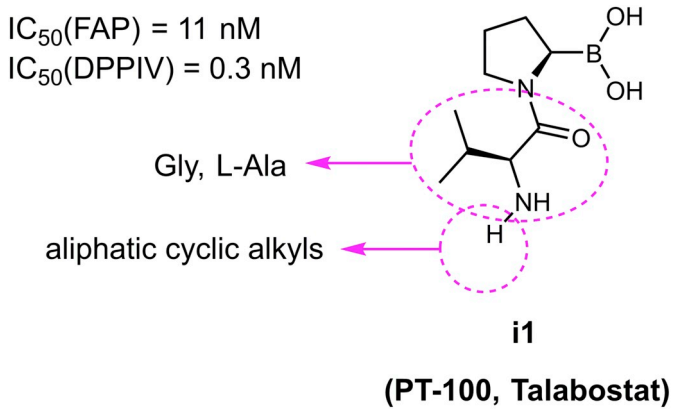


Fig. 9. The boronic acid-based inhibitor described by Hu et al. [109]. The pictured structure is representative of all compounds studied in this SAR study. The dashed ellipses designate the varied parts of the molecule, and the modifications studied are listed on the side.

tightly than FAP, except for two compounds: Ac-D-Ala-boroPro and Ms-Gly-boroPro. Although these two compounds were not among the most potent inhibitors, one was later used as a structural framework for development of boronic acid inhibitors selective for FAP over PREP [79].

Tsai et al. [112] observed FAP inhibition by peptidomimetics with nitrile warheads that were originally intended to inhibit DPP-IV. Later, they conducted an entire SAR study based on nitrile warhead-bearing pseudopeptides intended to inhibit FAP [113]. One possible advantage of nitriles over boronates is their better selectivity. While boronic acid pseudopeptides have poorer FAP/DPP-IV and FAP/PREP selectivity, the mild electrophilicity of the nitrile could help boost the selectivity *in vivo*. Based on their most potent compound targeted to inhibit DPP-IV, Tsai et al. [113] developed a FAP-targeted series with P2 ring constraint, yielding a nitrile FAP inhibitor with an IC_{50} of 79 nM. Double substitution with fluorine atoms in position 3 of the pyrrolidine ring improved the IC_{50} value to 22 nM (Fig. 12). All the promising compounds in this FAP inhibitor structure optimization study displayed excellent FAP/DPP-IV selectivity.

As an inhibitor with a boronic acid warhead (i5) had higher affinity than 1-naphtoyl-Gly-ProCN, Tsai et al. also explored the influence of this warhead on the properties of their lead compound (i4). Although inclusion of boronic acid improved binding to the protein, it led to a significant decrease in inhibitor selectivity. This finding suggests that the nitrile warhead is more relevant for potential biomedical applications. In mice, the three most potent compounds showed very poor oral bioavailability. However, a derivative chlorinated on the indoline moiety was proposed for further testing of its pharmacological potential for i.v. applications [113].

The double fluorine substitution of *N*-1-naphtoyl-Gly-ProCN (i6) improved the nitrile inhibitor binding potency from an IC_{50} value of 670 nM to 110 nM (Fig. 13) [114]. This modification also slightly improved the FAP/PREP selectivity of the original non-fluorinated analogue with an IC_{50} value for PREP inhibition of 3300 nM.

Rybtsova et al. [114] explored diverse P3 substituents of the X-Gly-ProCN pseudodipeptide, as well as substituents in position 3 of the pyrrolidine ring in P1. None of the enzymes studied in this broad SAR analysis tolerated pyrrolidine substitutions larger than fluorine or

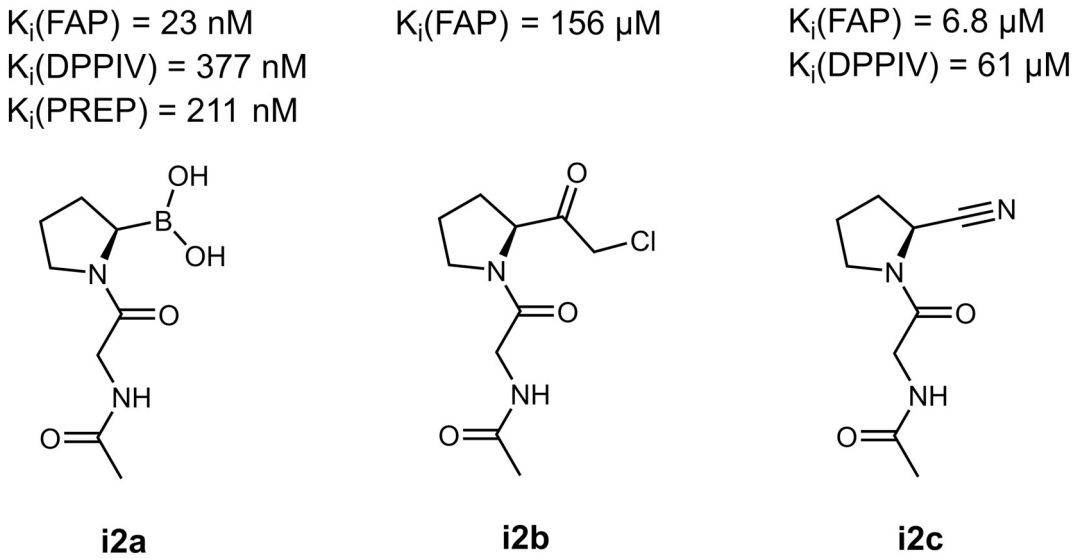


Fig. 10. The Ac-Gly-Pro derived inhibitors with different warheads reported by Edosada et al. [78,80] and Meadows et al. [38].

expansion of the pyrrolidine to piperidine. According to the inhibition potencies, PREP seemed to tolerate the pyrrolidine substitutions more poorly than FAP.

Two other independent SAR studies focused predominantly on FAP/PREP selectivity. Jansen et al. focused on the P3 moiety of the X-Gly-ProCN scaffold and established quinoline as the most favorable for FAP-binding [115]. Poplawski et al. identified the nature of the P2 amino acid in the HetAr-P2-boroPro pseudopeptide as a key factor influencing FAP/PREP selectivity [79]. Both studies established the crucial role of the presence and position of the nitrogen atom in the P3 aromatic acyls.

Jansen et al. observed a 65-fold improvement in affinity for an inhibitor containing a 4-quinolinoyl moiety (i7) compared to its 1-naphtoyl analogue (IC_{50} 670 nM *vs.* 10.3 nM, respectively). The authors explored all potential nitrogen positions in the (iso)-quinolinoyl scaffold and observed dramatic decreases in FAP inhibition potency compared to the 4-quinolinoyl variant (Fig. 14). The study confirmed the key role of the amide linkage between the P2 glycine and P3 aromatic acyl, as any change in this linkage led to a significant decrease in inhibitor affinity.

Once the best P3 aromatic acyl and optimal linkage were identified, possible substitutions on the quinoline rings were explored. FAP tolerates well halogen and methoxy substituents in positions 6 and 7, while similar groups in other positions decrease the inhibitory activity. Interestingly, a methoxy group in position 6 and halogen substituents in position 5 dramatically increased the FAP/PREP selectivity in comparison to the non-substituted analogue. Neither FAP nor PREP seemed to tolerate any substitution on the pyridine ring of the quinoline moiety.

In their SAR study, Poplawski et al. [79] varied the P2 amino acid and P3 acyls of the boronic acid-derived pseudopeptides (Fig. 15). In addition to underscoring the importance of the nitrogen in the P3 aromatic acyl, they identified the P2 amino acid as the moiety defining FAP/PREP selectivity in most cases. However, this trend was not confirmed for analogues with a nitrile warhead. Quin-D-Ala-ProCN bound FAP and PREP with comparable affinities (low-micromolar IC_{50}) [116]. Nevertheless, Poplawski et al. established highly selective FAP (i8) and PREP (i9) boronic-acid based inhibitors.

Jansen et al. further improved their previous most potent compound i7 by combining the previous findings about FAP inhibition [116]. Double fluorine substitution on the pyrrolidine ring of i7 yielded FAP inhibitor i10 (UAMC-1110) with an IC_{50} value of 3.2 nM and favorable FAP/PREP selectivity (Fig. 16). Jansen et al. [116] thus established the lead structure for FAP inhibition with low-nanomolar potency and a satisfactory FAP/PREP and FAP/DPP-IV selectivity: UAMC-1110. They performed *in vivo* profiling of four compounds from their series, including UAMC-1110, in rats.

Another study by Jansen et al. [117] assessed non-peptidic FAP inhibitors based on a xanthine scaffold. However, the compounds from this study did not achieve satisfactory FAP/DPP-IV selectivity.

Various enzyme activity and kinetics assays were used to determine the IC_{50} and K_i values discussed in this section, depending on the laboratory performing the experiment. All these approaches were based on the same principle using spectrophotometric determination of

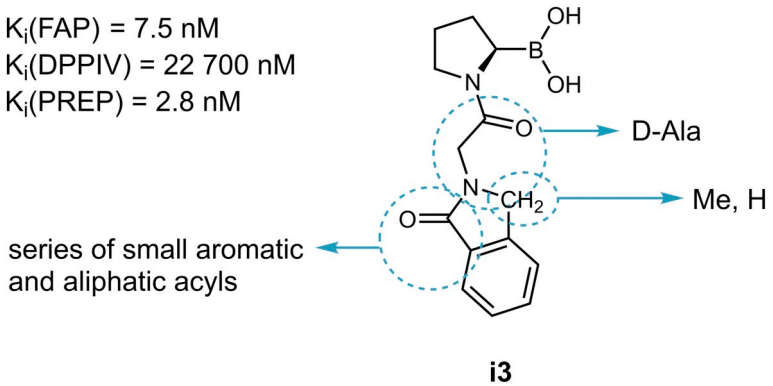


Fig. 11. Boronic acid-based inhibitor developed by Tran et al. [111]. The pictured structure is representative of all compounds studied in this SAR analysis. The dashed ellipses designate the varied parts of the molecule, and the modifications studied are listed on the side.

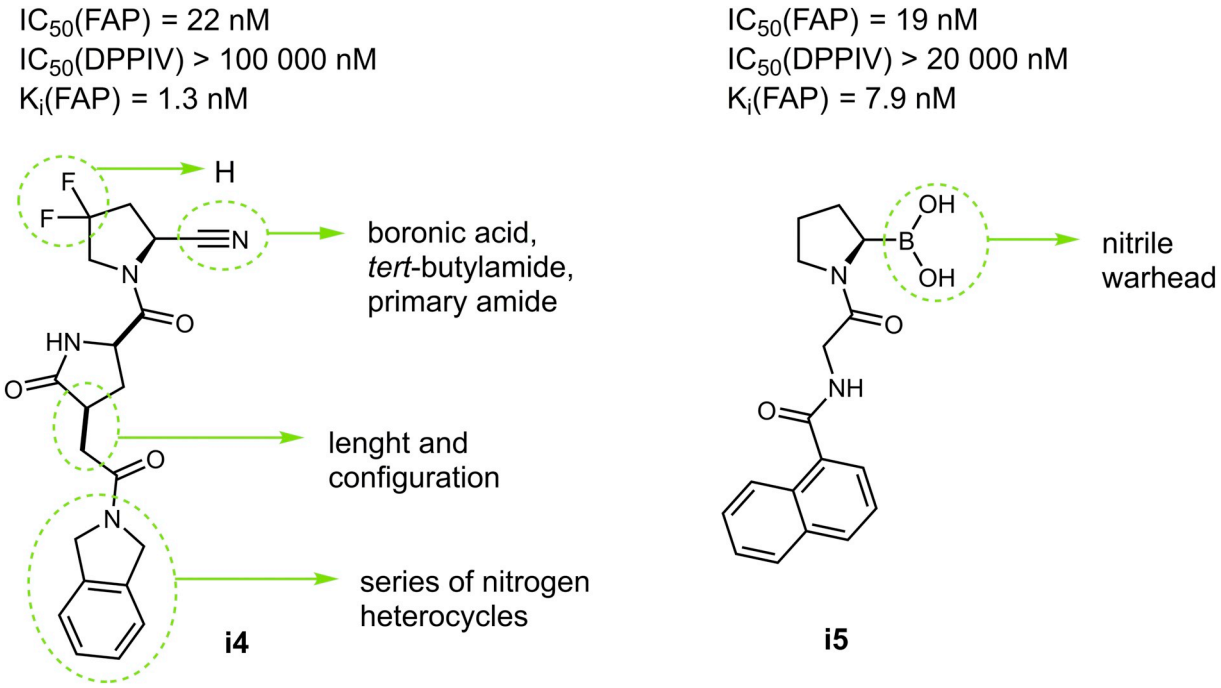


Fig. 12. Potent nitrile and boronate-based inhibitors developed by Tsai et al. [113]. The pictured structures are representative of all compounds studied in this SAR analysis. The dashed ellipses designate the varied parts of the molecule, and the modifications studied are listed on the side.

fluorogenic or chromogenic substrate hydrolysis. The substrates varied among the laboratories and included Ala-Pro-*p*-nitroanilide, *Z*-Gly-Pro-AMC, and Gly-Pro-AFC.

2.4.2. Synthetic strategy for preparation of pseudopeptide inhibitors

One major advantage of pseudopeptide inhibitors is the well-established synthetic strategy. Generally, it involves condensation of commercially available or appropriately modified protected building blocks alternated with deprotection steps (Fig. 17). For FAP inhibitors bearing boronate (A) or nitrile (B) warheads, the synthesis consists of analogous steps: a) peptide coupling of pyrrolidine bearing the (+)-pinanediol-protected boronate or a nitrile with a *N*-protected amino acid, b) acidic deprotection of Boc-protected moieties, c) peptide coupling with a desired carboxylic acid, and eventually d) acidic deprotection of the boronate with phenylboronic acid.

Different reagents were used for peptide coupling (a), including EDC/HOBt [111,113,118], TBTU [114] and HATU [79,115,116]. The second amide creation (c) may be performed under the same conditions, or in some cases, an acyl chloride was used instead of an activated ester [79,109,114–116].

For boronate inhibitors, acidic deprotection (b) is performed with a solution of HCl in an organic solvent [111,118]. For nitrile analogues, milder conditions, such as *p*-toluenesulphonic acid in acetonitrile, are needed because of the possible Ritter reaction [115,116].

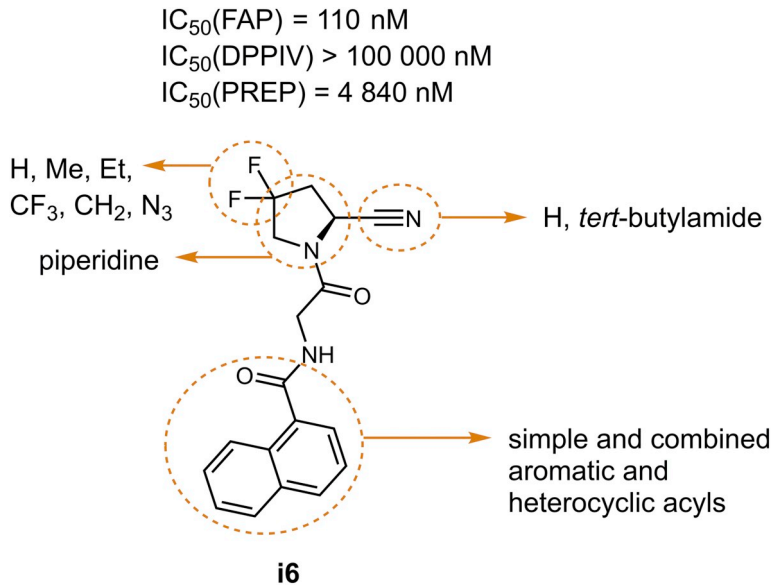


Fig. 13. Nitrile-based inhibitor containing the pattern examined by Ryabtsova et al. [114]. The pictured structure is representative of all compounds studied in this SAR analysis. The dashed ellipses designate the varied parts of the molecule, and the modifications studied are listed on the side.

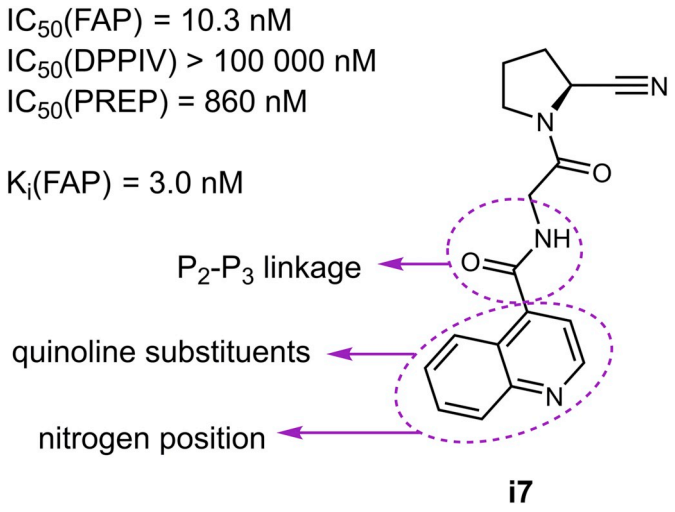


Fig. 14. The most potent inhibitor from the SAR study by Jansen et al. [115]. The pictured structure is representative of all compounds studied in this SAR analysis. The dashed ellipses designate the varied parts of the molecule, and the modifications studied are listed on the side.

2.4.3. Therapeutic potential of direct FAP inhibition

Low molecular weight inhibitors have been used in multiple studies to determine the pathogenic role of FAP enzymatic activity and explore its possible therapeutic exploitation. However, the relatively low specificity of the inhibitors used represents a major caveat to interpretation of these studies. Thus, the reported immunomodulatory effects of PT-100 (talabostat) and linagliptin may in fact stem from inhibition of other post-proline proteases. Indeed, by inhibiting DPP8 and DPP9 in monocytes and macrophages, PT-100 can activate the immune system by triggering a proinflammatory form of cell death (pyroptosis) [119]. Similarly, a highly specific DPP-IV inhibitor can enhance immune-mediated tumor rejection by increasing the bioavailability of chemokines [120].

In a mouse model of idiopathic pulmonary fibrosis, PT-100 reduced bleomycin-induced lung injury. Treated animals had lower fibrosis, FAP expression, and MMP12 expression in the lungs and higher infiltration of Iba-1 positive cells. In this setting, PT-100 had an anti-fibrotic effect and led to macrophage activation, but this did not translate into improved survival [121]. The use of FAP inhibitors also is of potential interest in the context of metabolic disorders and thrombolytic therapy; however, currently available data are inconclusive [44,45].

Detailed information about studies of FAP inhibitors in the context of cancer is provided in our recent review [8]. In brief, preclinical studies with PT-100 suggested significant antitumor activity in several tumor types [122–125], prompting rapid initiation of clinical trials. Administration of PT-100 was well-tolerated [126], but neither alone nor in combination with other cytostatics resulted in meaningful therapeutic responses in phase II clinical trials [110,127,128]. Other, non-specific FAP inhibitors have only been tested in preclinical models. PT-630 (Glu-BoroPro), a FAP and DPP-IV inhibitor, inhibited angiogenesis, decreased the number of intratumoral myofibroblasts, and slowed cancer cell proliferation and tumor growth [19]. A dual FAP and DPP-IV inhibitor, linagliptin, was recently shown to enhance the effect of an anti-PD-1 immune checkpoint antibody in a syngeneic model of colorectal cancer [129]. Another dual FAP and PREP inhibitor, M83, suppressed the growth of xenotransplants by inhibiting angiogenesis and promoting collagen accumulation [130]. Recently, a detailed preclinical study assessed the highly selective FAP inhibitor UAMC-1110 in a mouse model of pancreatic cancer. During administration, UAMC-1110 decreased macrophage infiltration and promoted collagen accumulation. Withdrawal of the compound resulted in rapid rebound of infiltration by macrophages and increased CD8+ T cells. UAMC-1110 did not slow tumor growth and did not enhance the effect of radiotherapy [131]. Based on the available data, inhibition of FAP enzymatic activity may have some beneficial effects on the tumor microenvironment, but by itself is likely insufficient to halt tumor progression.

2.4.4. Small molecule inhibitors as FAP-targeting moieties

Since the structure of a selective FAP inhibitor with low-nanomolar potency (UAMC-1110) was established between 2010 and 2014, until recently, only few biomedical applications using FAP inhibitors as a targeting moiety have been described.

The first inhibitor-based probe was reported in 2015 by Meletta

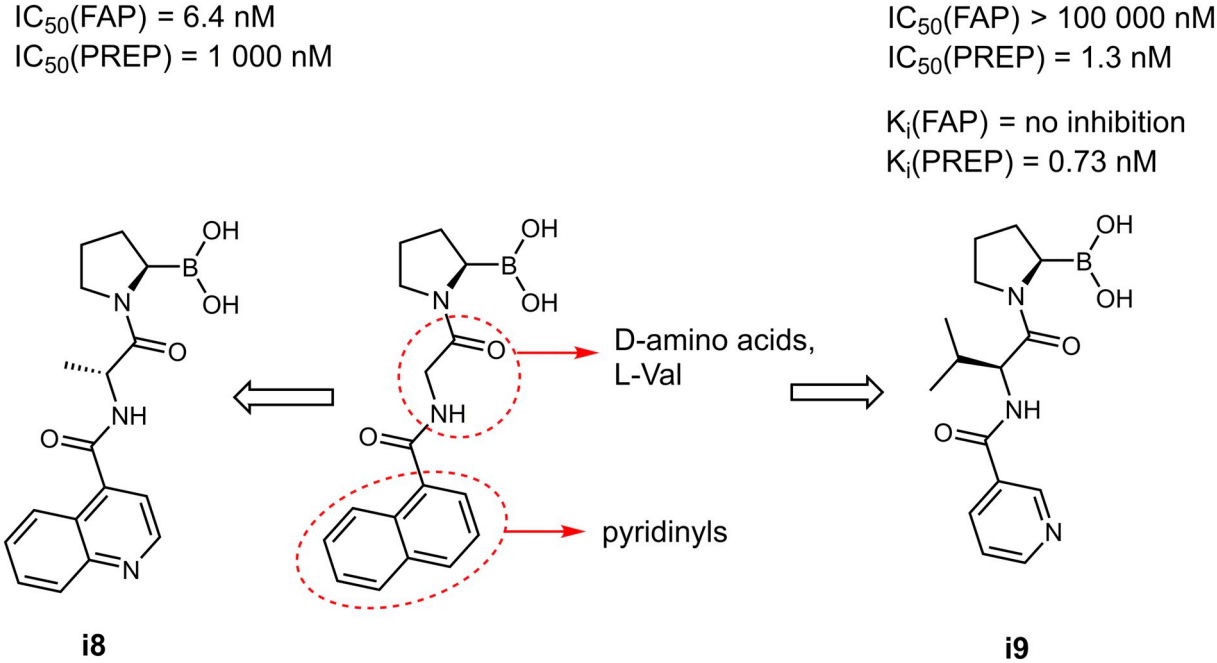


Fig. 15. FAP- and PREP-selective boronate-based inhibitors developed by Poplawski et al. [79]. The structure pictured in the center is representative of all compounds studied in this SAR analysis. The dashed ellipses designate the varied parts of the molecule, and the modifications studied are listed on the side.

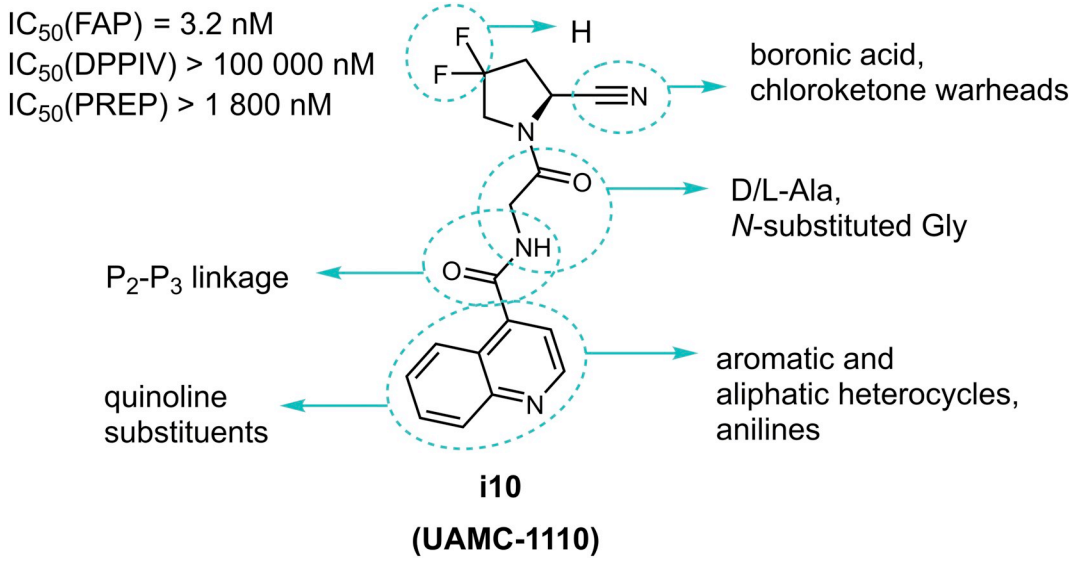


Fig. 16. Lead FAP inhibitor bearing a nitrile warhead developed by Jansen et al. [116]. The pictured structure is representative of all compounds studied in this SAR analysis. The dashed ellipses designate the varied parts of the molecule, and the modifications studied are listed on the side.

et al., who aimed to visualize atherosclerotic plaques (Fig. 18A). Based on *in vitro* studies, this approach was not useful for the intended atherosclerosis imaging, but it appeared highly relevant for tumor tissue imaging [132].

The UAMC-1110 was first used by Dvořáková et al. [133] in 2017 as a protein-targeting moiety in synthetic antibody mimetics called iBodies (Fig. 18B), which serve as a tool for various biochemical methods requiring specific immobilization or visualization of a target protein. Matos et al. [134] then designed bismuth ferrite harmonic nanoparticles decorated with non-fluorinated nitrile inhibitors, which are intended to serve as a FAP-targeted imaging tool using multiphoton microscopy.

The development of a targeted radiotracer by the Haberkorn group at the University of Heidelberg is among the most clinically relevant applications of FAP-targeting. Loktev et al. [135,136], Lindner et al. [137] and Giesel et al. [138] described a series of inhibitor-DOTA conjugates. Based on its pharmacokinetic properties, one conjugate (Fig. 18C) was chosen to be tested in humans with advanced cancers. The compound was used as a chelate with the radioactive isotope ^{68}Ga for PET/CT imaging. The FAP-targeted radiotracer accumulated in tumor tissues in patients with various cancer types and had equal or even improved tumor-to-background contrast ratio compared to FDG. This FAP-targeted PET probe also has been applied to visualize activated fibroblasts post-myocardial infarction [139].

3. Conclusion and future perspectives for FAP-based diagnostic and therapeutic approaches

Although the physiological role of FAP has not yet been clearly established, selective upregulation of FAP in various pathological states and its participation in their pathogenesis make it an attractive target for both diagnostic and therapeutic applications. In this review, we have summarized approaches used for molecular targeting of FAP with a focus on advances in understanding of FAP substrate specificity and the design of highly selective inhibitors. Recent discoveries in these areas have expanded the toolbox of traditional antibody-based approaches and enabled development of several FAP-targeted prodrugs and probes, synthetic alternatives to FAP antibodies [133], and PET probes that outperform currently available tracers in several respects [136–138,140].

Although a highly specific nitrile-based FAP inhibitor is currently available [116], there is room for further improvement of specific

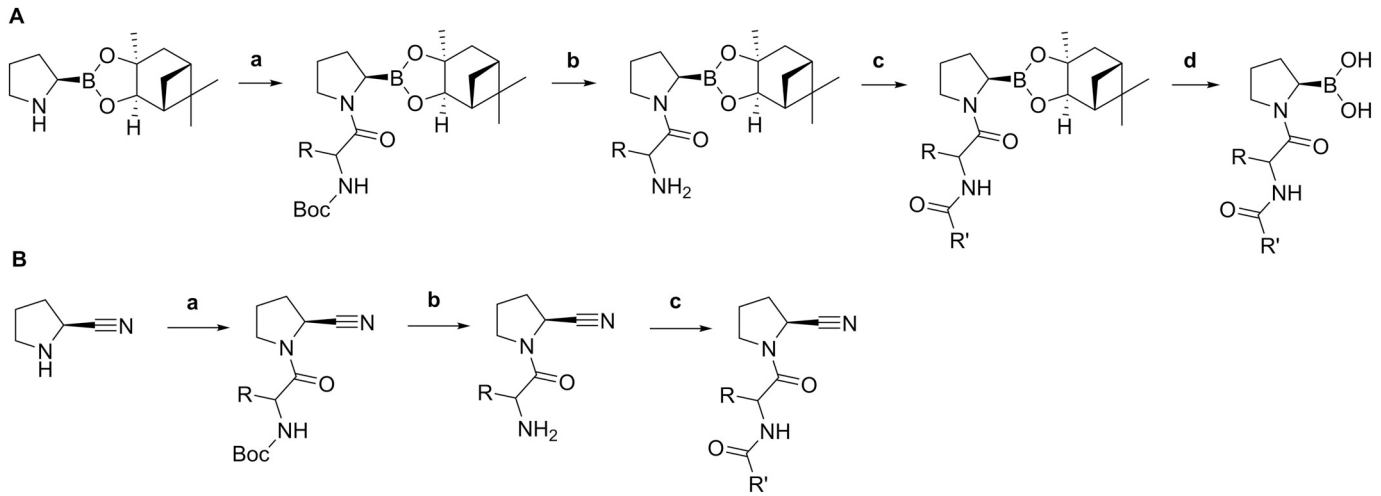


Fig. 17. General synthetic schemes for preparation of FAP-inhibiting pseudopeptides. a,c) EDC/HOBt, TBTU or HATU and appropriate carboxylic acid, b) HCl or TsOH, d) $PhB(OH)_2$.

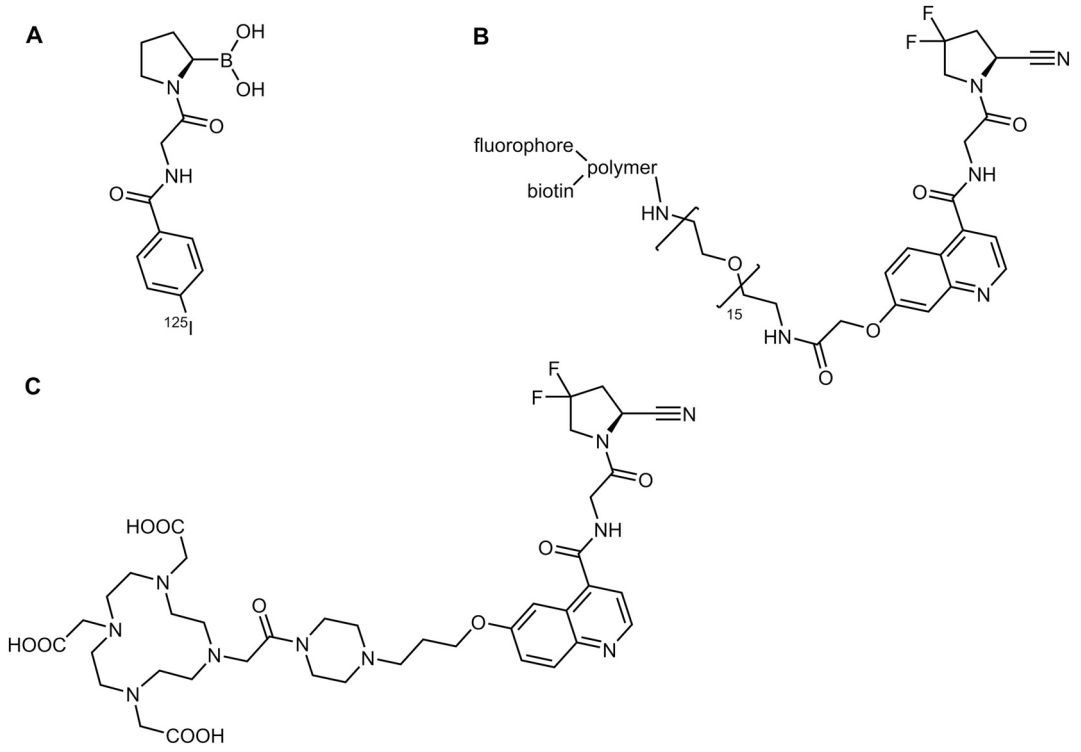


Fig. 18. Structures of inhibitor-based FAP-targeted imaging probes. A) Meletta et al. [132], B) Dvořáková et al. [133], C) Lindner et al. [137].

inhibitor binding using as-yet unexplored parts of the substrate binding cleft probed by pseudopeptide inhibitors and substrates. Several warheads other than nitrile and boronic acid are known to be useful for serine protease inhibition. Based on warhead structure and reactivity, the inhibitor behavior could be tuned for specific applications. Moreover, some warheads could offer inhibitor extension into the less-explored P1' area. This would open a new space for FAP SAR studies and potentially facilitate discovery of new structural scaffolds leading to inhibitors with improved inhibition potency and selectivity.

FAP inhibitor-based probes can address several problems related to the use of antibodies, including limited stability, high production costs, difficulty of chemical modification, and potential induction of neutralizing antibodies *in vivo*. Importantly, the high conservation of the FAP active site across various species facilitates use of inhibitor-based probes in preclinical models. Another potential advantage of inhibitor-based probes for therapeutic applications is their ability to inhibit FAP enzymatic activity. Although inhibition of FAP enzymatic activity by itself does not seem to be sufficient to block tumor progression, it may be beneficial in combination with selective delivery of radionuclides [141] or highly toxic drugs into the tumor microenvironment.

The clinical utility of various potential FAP diagnostic and therapeutic applications reported in the literature needs to be tested in larger independent studies. Visualization of FAP in the tumor microenvironment may be a useful alternative to FDG PET/CT, especially in organs with high physiological glucose uptake such as the brain. Due to its low background in normal tissues, whole body PET/CT may prove useful in screening for clinically silent metastatic spread in cancer patients [142]. It also may be useful in guiding the selection of suitable candidates for FAP-targeting cancer treatment strategies. Based on preclinical evidence, FAP-targeted therapies are more effective when combined with other anticancer treatments. Due to their association with immunosuppression, ablation of FAP-expressing cells seems to be particularly attractive in combination with immunostimulatory therapies such as anticancer vaccines or checkpoint inhibitors.

In summary, understanding the pathophysiological functions of FAP, together with a detailed knowledge of its molecular structure, might lead to the development of tools applicable to clinical practice.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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