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Published in final edited form as:

Title: Fibroblast activation protein-α in fibrogenic disorders and cancer: more than a prolyl-specific peptidase?

Authors: Juillerat-Jeanneret L, Tafelmeyer P, Golshayan D

Journal: Expert opinion on therapeutic targets

Year: 2017 Oct

Issue: 21

Volume: 10

Pages: 977-991

DOI: 10.1080/14728222.2017.1370455

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Fibroblast activation protein-α in fibrogenic disorders and cancer: more than a prolyl-specific peptidase?

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Key words: cancer / fibroblast activation protein -α/ FAP-α / fibrosis / inflammation / inhibitors / therapy

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Abstract

Introduction: Fibroblast activation protein-α (FAP-α) belongs to the family of prolyl-specific serine proteases. FAP-α displays both *exopeptidase* and *endopeptidase/gelatinase/collagenase* activityies. FAP-α protein and/or activity have been associated with fibrosis, inflammation and cancer, but the protein is undetectable in most normal tissues. FAP-α is selectively expressed at sites of tissue remodeling and repair and enhances tumor progression, suggesting that this protease may be a therapeutic target to treat human disorders associated with fibrotic dysregulation.

Areas covered: In this review, we summarize the mechanisms driving tissue fibrosis and describe some of the enzymes involved in fibrosis, concentrating on FAP-α. We describe its enzymatic properties, discuss the tools developed to control its activity and the problem of selectivity toward the other proteases of the family and outline its potential biological substrates. We also consider non-enzymatic functions of this protein and suggest that repression of FAP-α expression may represent therapeutic options.

Expert opinion: Questions remain regarding the biological functions of FAP-α, either dependent or independent of its enzyme activity. However, as progress is underway to develop FAP-α-specific inhibitors and therapeutic antibodies, its role in diseases associated with fibrosis is starting to emerge, ultimately leading to novel therapeutic options for inflammatory and oncologic diseases.

1. Prolyl-specific proteases and FAP-α in fibrogenic disorders

1.1. Fibrosis, fibroblasts and myofibroblasts.

Fibrosis is a non-specific terminal pathway following local inflammation and scaring. It is the hallmark of many chronic inflammatory diseases and cancer as well as a predictor of dysfunction of solid organ transplants and implanted biomaterials [1,2]. The pathogenesis of fibrosis involves an initial and repetitive tissue injury leading to abnormal tissue repair involving inflammatory and mesenchymal cells, thickening of the surrounding tissue and functional impairment. In response to tissue injury, the repair process may result in two distinct phenomena, a *normal* regenerative process, limited in time, in which injured cells are replaced by cells of the same type. In chronic *pathological* fibrotic responses, connective tissue, including *myofibroblasts*, replaces normal tissue with an uncontrolled deposition of extracellular matrix (ECM). This pathological fibrotic process finally results in the replacement of normal tissue with permanent scar tissue [3]. The mechanisms of fibrosis development depend on the underlying disease or local tissue properties and an inflammatory response to an initial injury, whatever the injury, as well as recruitment of macrophages. These cells can synthesize locally a variety of growth factors, pro-inflammatory cytokines, enzymes and ECM proteins that influence fibrogenesis. However, the mechanisms that drive fibrogenesis are different from the mechanisms inducing inflammation. Transforming growth factor-beta (TGFβ), predominantly produced by circulating monocytes and tissue macrophages as well as cancer cells, has been the most intensively studied pro-fibrogenic factor [4,5].

Myofibroblasts are fibroblast-derived cells that are present at very low frequency in normal tissues, but are activated by a variety of stimuli [5,6] (**Figure 1**) and their number increases in healing wounds and in fibrogenic disorders. Fibrosis-associated proliferating activated (myo)fibroblasts express specific molecules induced by the activation process when compared with their resting counterparts. They secrete large amounts of ECM proteins, in particular type I and type II collagens. The main feature of myofibroblasts is represented by an important contractile apparatus expressing α-smooth muscle actin (α-SMA) and promoting cell migration. Thus, activation of resident tissue fibroblasts is a key event of fibrosis development and/or progression, resulting from inappropriate

properties of these cells in response to tissue stress. The increased number of (myo)fibroblasts associated with fibrosis could originate from resident proliferating tissue fibroblasts, from circulating bone-marrow-derived fibrocytes, or be the consequence of epithelial to mesenchymal (EMT) or endothelial to mesenchymal (EndMT) cell trans-differentiation mechanisms.

In oncogenic diseases, cancer-associated fibroblasts (CAF) are recruited to the stroma of tumors and influence a variety of oncogenic processes by secreting growth factors and proteases, and producing an altered ECM [7]. In chronic inflammatory disorders, activated myofibroblasts accumulate at sites of injury and deposit excessive ECM proteins associated with impaired degradation by macrophages [8]. In cell/tissue transplantation or tissue engineering the biological response at the interface between host tissue and transplanted/implanted bio-materials can trigger a variety of adverse tissue responses such as local inflammation and fibrosis, hindering long-term functionning of the grafts or devices.

Figure 1. *Myofibroblast formation.*

Proteolytic degradation of the ECM is necessary for tissue remodeling, repair and invasion, and ultimately in the process of fibrosis. During the *normal* process of tissue remodelling, the final stages include reduced synthesis and increased proteolytic degradation of collagens, regeneration of the normal tissue cells and the vascular network, and disappearance of myofibroblasts by apoptosis. Any disruption of the proteolytic balance in *pathological* processes may compromise tissue homeostasis. Specific proteases are involved in the development, maintenance and regression of tissue fibrosis. In this review, we will concentrate on the potential role of fibroblast activation protein-α (FAP-α) in fibrogenic processes in inflammation and cancer.

1.2. Prolyl-specific peptidases in fibrogenic diseases.

As production of proline-rich ECM proteins and collagens by activated (myo)fibroblasts is a key event in the development of fibrosis, it can be hypothesized that prolyl-specific proteases are

involved in tissue fibrosis by aberrantly processing tissue-derived biologically active prolyl-containing peptides in response to tissue injury, inflammation or cancer. The prolyl residue, either in terminal position or in the core of peptides, imposes conformational constraints of the amino acid chain. Of the known human proteases, only a few prolyl-specific proteases, including the non-post-prolyl-cleaving matrix metalloproteases (MMPs) and the post-prolyl-cleaving proteases related to dipeptityl amino-peptidase IV (DPP IV) have been described [9-16], suggesting that the amino acid selectivity of prolyl-specific proteases for proline are implicated in fibrogenic processes. Alternatively, non-enzymatic functions of these proteins may also be involved (see below). The role of MMPs in fibrogenic diseases has been previously reviewed by several authors [for example, 17], to whom the readers are referred since we will not repeat here this information. We will concentrate on the prolyl-peptidases of the DPP IV family, in particular the potential interest of targeting FAP-α in fibrosis-associated disorders [16].

2. The family of proline-specific peptidases (the DASH-family).

The post-prolyl-cleaving-specific peptidases encompasses several proteins: dipeptidyl peptidase (DPP) IV, quiescent cell proline dipeptidase (QPP/DPPII/DPP7), fibroblast activation protein-α (FAP-α), prolyl oligopeptidase (POP), DPP8 and DPP9, and the inactive DPP6 and DPP10. They include *exopeptidases*, such as DPP IV, FAP-α, QPP, DPP8 and DPP9, or *endopeptidases* such as POP, DPP8 and FAP-α. DPP IV and FAP-α are membrane-bound, while POP, DPP8 and DPP9 are cytoplasmic. These serine proteases exhibit similarities in their catalytic behavior [18]. The Gly-Trp-Ser-Tyr-Gly sequence around the active Ser, and the organisation of the catalytic triad are conserved between the members of the family [9-15,18]. DPP IV and FAP-α share the highest sequence homology. The exopeptidase enzymatic activity of FAP-α is comparable to that of DPP IV, but in addition FAP-α also displays endoproteolytic activity comparable to POP and gelatinase/collagenase activity (**Figure 2**). Ubiquitously-expressed enzymes in various cellular locations and with comparable overlapping enzymatic activities question the exact site, mode of action, and biological functions of these proteins in cells and tissues.

Figure 2. *Prolyl-specific peptidases: exoproteolytic (left) and endoproteolytic (right) enzymatic activities, with the preferred amino acid sequences when known*

We will first rapidly review the members of the family of serine prolyl-specific proteases other than FAP-α, then focus in more detail on FAP-α.

2.1. Post prolyl-cleaving peptidases other than FAP-α.

DPP IV/CD26 (EC 3.4.14.5.) is the representative member of the family. DPP IV is a homodimeric type II integral membrane glycoprotein able to release X-Pro or X-Ala dipeptides from *free N*-terminal sequences, allowing selectivity over prolyl-*endo*peptidases, whereas any amino acid is permitted at the X position of the substrate, branched amino acids being preferred. Each subunit comprises a *C*-terminal α/β hydrolase domain and an *N*-terminal eight-bladed β-propeller domain forming a large cavity containing the active site [19,20]. Potential substrates of DPP IV include cytokines, chemokines and growth factors carrying an *N*-terminal X-Pro- or X-Ala-motif, resulting in activation, modulation of function or initiation of degradation of the peptides. Inhibitors of DPP IV activity, the gliptin family of therapeutics, are in clinical use for type 2 diabetes [21]. The 3D structure (X-ray crystal structure) and kinetic characteristics of DPP IV have been determined [19,20,22-25]. DPP IV, known as CD26, is a marker of T-cell activation in immune and inflammatory diseases [26]. DPP IV activity has been linked to prostate, colon and lung carcinoma or glioblastoma tumor cells [27-30]. DPP IV expression, as a receptor for tumor-associated fibronectin on endothelial cells favors tumor cell adhesion and metastasis, independently of its enzymatic activity [31].

POP (PEP/PREP; EC 3.4.21.26.) is a very conserved and widely distributed post-prolyl-endopeptidase hydrolyzing peptides under 30 residues long at the carboxylic side of proline residues in the core of the chain [12-14,32,33]. POP is able to form protein-protein interactions with other cellular proteins [9,34]. The 3D structure, flexibility, substrate docking and kinetic

characteristics of POP from mamalian and non-mammalian origin have been determined [35-38]. POP shows a cylindrical structure consisting of an α/β-hydrolase domain containing the catalytic triad Ser^{554}, Asp^{641}, His^{680} as an internal cavity between the two catalytic domains and an unusual seven-bladed β-propeller non-catalytic domain. Synthetic inhibitors based on *Z*-prolyl-prolinal (ZPP) have been developed [32,37].

QPP (DPPII/DPP 7; EC 3.4.14.2) is a homodimeric glycoprotein with protease characteristics identical to DPP IV, but with no sequence homology, active at acidic pH and located in intracellular post-Golgi vesicles distinct of lysosomes. QPP is involved in protein maturation and catabolism, and in immunological disorders [39-41].

DPP8 and DPP9 (EC EC 3.4.14.) are very similar and ubiquitously expressed, soluble cytosolic enzymes of ~ 100 kDa with DPP IV-like activity but without a transmembrane domain [42-47] and exopeptidase activity. DPP8 has the same stucture as DPP IV, an N-terminal propeller domain, and a C-terminal peptidase domain. DPP8/9 inhibition can attenuate macrophage activation.

DPP6 and DPP10 are less deeply characterized. These proteins have no detectable protease activity, due to the absence of the conserved serine residue present in the catalytic domain of serine proteases. DPP6 and DPP10 regulate the expression and gating characteristics of membrane potassium channel complexes [48,49].

The role in fibrogenic disorders of DPP8, DPP9, DPP6 and DPP10, is presently not known in detail and will not be discussed in this review.

3. Fibroblast activation protein-alpha (FAP-α).

3.1. The biology and enzymology of FAP-α.

FAP-α (seprase; EC 3.4.21.B28) is an homodimeric type II integral membrane prolyl-specific serine protease belonging to the clan SC proteases and the S9B prolyl oligopeptidase subfamily [50-53]. FAP-α is most closely related to DPP IV, displaying an overall 50% similarity in sequence and a 70% similarity in the catalytic region. FAP-α exhibits a DPP IV-like fold and has a molecular weight and an enzymatic activity comparable to DPP IV, removing X-Pro-dipeptides from the free

N-terminus of peptides (exopeptidase activity); but in addition, FAP-α also displays endoprotease activity like POP, and gelatinase/collagenase activities (endopeptidase activity) like MMPs [16,54-58]. However, *N*-terminus-free substrates are cleaved by FAP-α with a 100-fold lower catalytic efficiency compared to DPP IV [59]. FAP-α activity is inhibited by general serine-protease inhibitors and boronic acid peptides [60-62]. There are at least 11 known alternative splice variants of the most frequent FAP-α primary transcript, some of them encoding isoforms devoid of enzymatic activity (the ACE-View Database, cited in [63]). FAP-α in humans is encoded by the *FAP-α* gene, located at 2q23 on chromosome 2 [64]. DPP IV and FAP-α genes are localized close to each other on chromosome 2 and they are thought to result from gene duplication. Co-expression of both enzymes is frequently observed and FAP-α can associate with DPP IV as heteromeric complexes possessing both prolyl exopeptidase and prolyl endopeptidase activities [52,55,65,66], suggesting that both molecules can reciprocally regulate each other.

FAP-α features an α/β hydrolase domain (characterized by the catalytic site sequence Gly-X-Ser-X-Gly) and an eight-bladed β-propeller domain. FAP-α is biosynthesized as an inactive 97 kDa subunit (760 amino acids, GenBank accession number U76833, 97 kDa) which needs dimerization (170 kDa) to become active. Several mutagenesis experiments have provided information about the functions of defined amino acids for either the hydrolytic or the adhesive properties of FAP-α (**Table 1**). The results demonstrated that its exo- and endo-peptidase activities and the adhesive and migratory properties could be differentiated.

Table 1. *Mutagenesis experiments performed on FAP-α.*

FAP-α aminoacid sequence predicts a 6-amino acid cytoplasmic tail, a 20-amino acid transmembrane domain and a 734-amino acid extracellular domain [50,52,53] containing the catalytic Ser^{624}, Asp^{702} and His^{734} charge relay triad located at the carboxyl terminus of each subunit (**Figure 3A**).

Figure 3. *Structure of the FAP-α protein.*

The structures of the active sites of FAP-α, POP and DPP IV are very similar [19,20,59,67,69-71], suggesting comparable enzymatic mechanisms, conformational changes and ligand binding. The crystal structure of the FAP-α homodimer (PBD ID:1Z68) shows that each monomer consists of an α/β-hydrolase domain (aa27-53 and aa493-760), and an eight blade-β-propeller domain (aa54-492) enclosing a large cavity. Both the hydrolase and propeller domains participate in the FAP-α dimerization. Two ways are available for the substrates to reach FAP-α active site: through the cavity formed between the α/β hydrolase domain and the β-propeller domain, or through a central hole formed by the blades in the β-propeller (**Figure 3B**). FAP-α has a short 6-amino acid cytoplasmic domain, suggesting that by itself the cytoplasmic domain is probably not able to transmit signals. The active catalytic triad, Ser^{624}, Asp^{702} and $His^{734,}$ is located in a small pocket of the large cavity at the interface of the α/β-hydrolase and the β-propeller domains. The proline residue of substrates is accommodated in a hydrophobic pocket composed by Tyr^{625}, Val^{650}, Trp^{653}, Tyr^{656}, Trp^{660} and Val^{705}, whereas the *N*-terminal end of substrate peptides is recognized by two glutamates (Glu^{203}-Glu^{204}) contained in an α-helix of the β-propeller domain, necessary to the exopeptidase activity; unlike the negatively charged Asp^{663} in DPP IV active site, FAP-α has a neutral Ala^{657} residue at the corresponding position, explaining its dual exo/endopeptidase activities [7,59,61,62,67]. Thus, differences in the vicinity of the Glu^{203}-Glu^{204} motif and the reduced acidity of the active site due to the presence of Ala^{657} determine the dual substrate preference of FAP-α [59,67]. A polymorphism encoding Ser^{363} to Leu in the sixth blade of the β propeller domain in FAP-α alters its tertiary structure and ablates dimerization and enzymatic activity. The mutated protein is detectable only in the endoplasmic reticulum (ER) and not at the cell surface. In the ER, Ser^{363}Leu FAP-α upregulates the chaperone BiP/GRP78, the stress response ATF6 and phospho-elF2A and was degraded by the proteasome [72], pointing to the importance of FAP-α conformation. The in vitro kinetics values (K_M) of hydrolysis of peptide substrates have been determined for the exopeptidase (dipeptidylpeptidase) and endopeptidase (prolyl endopeptidase)

activities of FAP-α, respectively (**Table 2**).

Table 2. *Kinetics constants of FAP-α on small peptides.*

FAP-α protein and/or activity has been associated with several human diseases. FAP-α protein is generally undetectable in most normal tissues whereas it is selectively expressed at sites of tissue remodeling and repair: dermal fibroblasts of fetal skin during development, granulation tissues of healing wounds, inflamed synovial tissues, activated hepatic stellate cells and myofibroblasts from cirrhotic liver, subpopulations of reactive cancer-associated stromal fibroblasts (CAFs) in epithelial cancers and some cancer cells, such as glioblastomas and glioma cells, malignant cells of bone and soft tissue sarcomas and melanomas, or some carcinomas,. Enzymatically inactive intracellular alternative splice variants of FAP-α may also be expressed under some circumstances [16,30,52,68,72-77]. FAP-α enhances tumor progression by increasing angiogenesis and ECM degradation, and by reducing the antitumor response of the immune system mediated by the STAT3-CCL2 axis [75,78,79].

3.2. FAP-α substrates and targets.

Defining the exact substrates of FAP-α is a very challenging task since potential substrates of FAP-α include small peptides and larger proteins, involving either the exopeptidase or the endopeptidase activities of the enzyme. Consequently, very few endogenous substrates of FAP-α have been formally identified. Moreover, substrate selectivity toward the other members of the DASH family is an issue; however, some sequences can differentiate the proteases of the family. In small peptides and proteins, FAP-α endopeptidase activity displays a clear, but not exclusive, preference for -Gly-Pro- sequences [80]. Many cytokines and chemokines associated with inflammation and bearing X-Pro- *N*-terminal sequences, neuropeptide Y (NPY), peptide YY (PYY), substance P and brain-type natriuretic peptide have been shown to be potential substrates of the dipeptidase activity of FAP-α, but only *in vitro* [81]. Some proteins have been demonstrated to be FAP-α substrates: FAP-α

exhibits post-proline cleaving endopeptidase activity for Ala/Ser-Gly-Pro-Ser/Asn/Ala consensus sequences, on substrates which include regulators of proteolytic or cell growth pathways such as α_2-antiplasmin, serpins, (the proteolysis of α_2-antiplasmin by FAP-α increasing its plasmin inhibitory activity) or FGF21 [73,82-86]. FGF21, a regulator of glucose and lipid homeostasis, is a substrate of circulating FAP-α in the human plasma. FGF21 has three potential Pro residues for Pro-specific proteases. At the position 2 and 4 of the *N*-terminus cleavage by DPP IV maintained FGF21 activity. At the Gly^{170}-Pro^{171} position, ten amino acids off the *C*-terminus, cleavage by FAP-α, but not POP, resulted in the loss of FGF21 activity [86]. Collagens are also recognized biological substrates of FAP-α. FAP-α degrades also gelatin, heat-denatured but not native collagen type I and IV, vitronectin, tenascin, laminin, fibronectin, fibrin or casein [87-91].

Other biological peptides can also be hypothesized to be substrates of the prolyl endopeptidase activity associated with FAP-α. Thymosin β4, a widely distributed 43-amino acid peptide critical in tissue repair and remodeling and fibroblast differentiation is overexpressed in cancer and fibrosis. The profibrotic protein thymosin β4 *N*-terminus contains a four-amino acid sequence, Ac-Ser-Asp-Lys-Pro- (Ac-SDKP-), which was shown to be released from thymosin β4 by prolylendopeptidases [92]. Thymosin β4 and its degradation product Ac-SDKP are able to reduce inflammation and fibrosis [93-96]. Matrikines, peptide fragments derived from the ECM, are neutrophil chemoattractants and regulators of endothelial permeability, and thus may also be of relevance in tissue repair. The sequential action of the MMP-8, MMP-9 and prolyl endopeptidases is critical in the generation from collagen of the tripeptide Pro-Gly-Pro (PGP) and acetylated-PGP matrikines, [97,98]. Roflumilast, an anti-inflammatory agent inhibiting phosphodiesterase-4, was shown to reduce pulmonary inflammation by decreasing prolyl endopeptidase activity and the generation of Ac-PGP and PGP [99].

FAP-α activity has also been used to activate functionalized chemotherapeutic prodrugs for other targets [100]. FAP-α activity was imaged in tissues *in vivo* by a Lys-Gly-Pro-Gly-Pro-Asn-Gln-Cys-prodrug of a near infrared fluorescent probe [101]. CAFs were specifically targeted for photodynamic therapy using a photosensitizer coupled to the peptide TSGPNQEQK representing

the cleavage site of FAP-α on α_2-antiplasmin [102]. The enzymatic activity of FAP-α was used to more selectively target a Thr-Ser-Pro-Arg-Ser-prodrug of the proteasome inhibitor bortezomib [103]. A Z-Gly-Pro-doxorubicin prodrug was shown to target FAP-α-expressing cells and to selectively release doxorubicin, being less cardiotoxic than free doxorubicin [104]. Nanomicelles loaded with the FAP-α substrate Z-Gly-Pro-doxorubicin prodrug were developed for selective delivery of doxorubicin to tumors [105]. The cytotoxins thapsigargin and melittin were also modified as FAP-α-activated prodrugs [106,107]. We have previously shown that double harmonic nanoparticles whose surface was functionalized with a covalent inhibitor of prolyl endopeptidases could specifically label cells expressing this activity [108].

3.3. FAP-α inhibitors and antagonist: preclinical and clinical trials targeting FAP-α in cancer and fibrotic diseases.

FAP-α in cancer. Tumors are heterogeneous populations of cells, which include the tumor cells, inflammatory and immune cells, endothelial cells and pericytes and cancer-associated (myo)fibroblasts (CAFs) expressing α-SMA and FAP-α, as a membrane-associated proteolytically active and easily accessible form. FAP-α is expressed in particular in the stroma directly surrounding epithelial cancers (**Figure 4**), but also in melanoma and sarcoma, in healing wounds as well as in chronic inflammation and fibrotic conditions [7,50,54,109-1178] but not in normal tissues or benign or premalignant tumors.

Figure 4. *FAP-α protein expression determined by immunohistochemistry (brown precipitate) in human normal colon tissue (left) and cancer (right). Both samples have been processed and evaluated according to standard diagnostic procedures and are from the same patient.*

Depending on the particular cancers and conditions, FAP-α can act as a tumor promoter or suppressor, dependent or independent of its enzymatic activity [72,77,113]. For example, FAP-α expression in stromal cells of breast and lung cancers may also be possibly associated with longer

survival [110,117]. FAP-α has been involved in cancer progression by interfering with the functions of the cancer microenvironment, the cancer stroma [74,83,113,114,119-122]. The proteolytic activity of FAP-α is pro-fibrogenic but the protein itself is a regulator of cell apoptosis, adhesion and migration, independently of its enzymatic activity [68,83,121,122,123]. FAP-α participates in malignant melanoma cell invasion of the ECM by interacting with β1-integrins in a collagen-dependent manner [60,124-127] and promotes glioma cell invasion through the brain parenchyma by degrading the proteoglycan brevican [30,63]. FAP-α also interacts with other cell membrane proteins, including urokinase-type plasminogen activator receptor (uPAR) localizing this receptor to the invadopodia that are associated with degradation of the ECM in cancer invasiveness and metastasis [82,124,126,128,129]. Splice transcript variants of FAP-α encoding different isoforms have been found, some of them devoided of enzymatic activity and of cell surface expression [77,72]. A variant of FAP-α encoding a truncated isoform (239 amino acids, 27 kDa) missing part of the cytoplasmic, transmembrane and membrane proximal domains but overlapping the carboxy-terminal catalytic region has been identified in human melanoma cells [53]. In carcinogenesis, FAP-α expression is up-regulated during tumor stem cell differentiation by TGF-β, FGFR1, 12-O-tetradecanoyl phorbol-13-acetate and retinoids [51]. However, interactions with the other members of the family must be considered. Both FAP-α and POP are overexpressed in cancer, regulating tissue remodeling, angiogenesis and immune tolerance. DPP IV and FAP-α have been inversely involved in the progression of carcinomas and melanomas. DPP IV is down-regulated during the neoplasic transformation of melanocytes, coincident with an increase in their invasive potential [131], growth factor independence [128,129], and interaction with endothelial cells [31,63,130-134]. FAP-α and DPP IV are frequently co-expressed and can form heterodimers that enable fibroblasts to migrate [135,136]; so that in terms of invasiveness, both enzymes seem to cooperate. Preclinical studies have evaluated the therapeutic interest of FAP-α with some promising results: FAP-α-expressing cells suppressed antitumor immunity in an experimental model of pancreas cancer; in a transgenic mouse model of lung carcinomas, the ablation of FAP-α caused necrosis of both cancer and stromal cells; in other murine models of lung and colon carcinoma FAP-α

inhibition slowed the growth of the tumors; FAP-α expression was also shown to improve the uptake of chemotherapeutics in multiresistant cancers [137-139].

Altogether, FAP-α inhibition is generally considered a potential therapeutic target for oncologic diseases. Three general approaches to inhibit/antagonize FAP-α have been used experimentally and in a very limited number of preclinical and clinical trials: (i) synthetic small molecule inhibitors of FAP-α activity, (ii) anti-FAP-α antibodies and immunotherapeutics and (iii) genetic deletion of the FAP-α protein. These combined approaches allowed the emerging recognition of the role of FAP-α protein in cancer progression. As CAFs expressing high levels of FAP-α can indirectly inhibit tumor growth by controlling the functions of the tumor stroma, several attempts have targeted this population of tumor-associated cells.

Synthetic inhibitors of FAP-α. As stated previously, the active site and enzymatic mechanism of FAP-α are very similar to those of the other members of the DPP IV family of serine proteases, in particular DPP IV itself and POP. Most known FAP-α inhibitors often resemble the dipeptide cleavage products, with a boroproline at the P1 site; however, many of these inhibitors also inhibit DPP IV, DPP-II, DPP8 and/or DPP9. Thus, synthetic small molecule inhibitors developed for members of the family, in particular DPP IV and POP, may be considered as potential inhibitors of FAP-α exopeptidase and endopeptidase activities. The cross-inhibitory properties for FAP-α of inhibitors developed for POP, DPP8/9 or DPP IV [21,32,61,69,99,140-157] was generally not evaluated in detail, with some exceptions (**Table 3**). Thus, the challenge has been in identifying inhibitors that are selective for FAP-α over both the dipeptidyl peptidases with which it shares exopeptidase specificity, and prolyl oligoendopeptidases, with which it shares e ndopeptidase specificity.

The design of families of small organic synthetic inhibitors of FAP-α, either covalent or non-covalent inhibitors, has been initially based on previously developed DPP IV and POP inhibitors. Then selectivity was further refined and the molecules evaluated in experimental and preclinical biomedical models. Many DPP IV inhibitors are known and several are marketed as drugs [21]. Whereas selectivity toward the strict exopeptidase DPP IV, which requires a free *N*-terminal amino

acid, has been achieved, selectivity toward the other prolyl-specific endoproteases gelatinases of the family, in particular POP, is much more difficult to obtain (**Figure 5**). Thus, the issue of POP selectivity is not always achieved and unfortunately not always evaluated in published results.

Figure 5. *FAP-α and POP amino acid sequence specificities and consequences for developing specific inhibitors.*

The lead molecule in the development of POP inhibitors was Z-Pro-prolinal. The structural characteristics of the inhibitor-POP complex were determined [151]. In POP the presence of hydrophobic residues, such as Cys^{255} and Trp^{595}, forms a non-polar environment for the indole ring of inhibitors [88], providing a window to develop selective inhibitors able to discriminate between the two enzymes. Whereas POP inhibitors selective toward FAP-α, have been prepared using molecular modeling [32,148], very few inhibitors with selectivity for FAP-α versus POP have been developed [61,62,143,147,149,150,152,153].

Table 3. *Synthetic inhibitors of FAP-α.*

Boronic acid derivatives are reversible covalent inhibitors of serine proteases, therefore most, but not all, FAP-α inhibitors contain a boronic acid group. The most studied small molecule inhibitor is the dual DPP IV/FAP-α inhibitor PT-100/talabostat (**5**). PT-100 (**5**) inhibits 90% of DPP IV-like exopeptidase activity, but only 20% of the FAP-α endopeptidase activity forming a high affinity complex between the active site Ser and the boron [154,155]. PT-100 (**5**) was safe in patients and upregulated cytokine expression, an effect that was mediated by the inhibition of FAP-α, not by DPP IV [156]. PT-100 (**5**) was not directly cytotoxic *in vitro* but caused regression of tumors *in vivo* involving tumor-specific cytotoxic T lymphocytes (CTLs), and production of cytokines and chemokines promoting T-cell effector functions [157]. In some preclinical models, PT-100 (**5**) decreased the growth of tumors [138,157], but not in all models [112,113]. Combining the dual

DPP IV/FAP-α inhibitor PT-100 (**5**) with oxaliplatin enhanced the efficacy of chemotherapy by modifying the tumor microenvironment [158]. The administration of PT-100 (**5**) demonstrated clinical response in a phase II trial of stage IV melanoma patients [159-161], but further research is presently suspended. A PT-100 (**5**) analog, PT-630 (**6**), a dual FAP-α/DPP IV inhibitor that does not permeate cell membrane, was shown to decrease experimental lung and colon tumor growth. FAP-α enzymatic activity was inhibited, but not FAP-α protein expression in the tumor extracts [117]. Starting from a FAP-α/POP non-specific lead, rounds of refinements produced a FAP-α-specific, ARI-3009 (**1**) inhibitor [149], but presently no pre-clinical or clinical data have been reported. The binding of the cyclic amide boronic acid-based inhibitor (**2**) developed by Tran and co-workers [69] in the active sites of FAP-α, DPPIV and POP was explored by docking studies. Interaction with specific amino acids in the three enzymes was demonstrated, blockade of the *N*-terminus of the inhibitors providing selectivity for FAP-α and POP versus DPP IV. The phenyl ring of the inhibitor is located partially outside of the active pocket and was not directly involved in the binding. We took advantage of this observation to achieve chemical modification using copper-free click chemistry of this phenyl ring to prepare nanoparticles presenting a prolyl endopeptidase inhibitor at their surface to label human cells [107]. To identify peptide motifs for designing FAP-α-selective inhibitors, dipeptide substrate libraries of the chemical structures P2-Pro1 and acetyl (Ac)-P2-Pro1 were used, where P2 was varied [61,62]. The results designed Ac-Gly-boroPro (**3**) as FAP-α lead inhibitor design, selective versus DPP IV, DPP-7, DPP-8, DPP-9 and POP. The pseudopeptide acetyl-Arg-2-(2-(2-aminoethoxy)ethoxy)acetic acid-(D)Ala-(L)boroPro (**13**), a dual FAP-α/POP inhibitor, reduced tumor growth more than a monospecific POP inhibitor [108]. Optimization of a nonselective DPP IV inhibitor led to the discovery of a class of substituted 4-carboxymethylpyroglutamic acid diamides (**4**) as carbonitrile-based FAP-α inhibitors, selective versus DPP-IV, DPP-II, DPP8, and DPP9 [145]. Unfortunately, selectivity toward POP was not reported. A series of carbonitrile inhibitors, *N*-acetylated-Gly-(2-cyano)pyrrolidines (**14,15**), where the acyl and lipophilic cyanopyrrolidine substituents were systematically varied, provided compounds with selectivity for FAP-α versus the other members of the family but no biological

evaluation was reported [143]. Starting from the FDA-approved xanthine-based DPP IV inhibitor linagliptin [21], which also displays significant FAP-α potency, substitutions on *N*1, *N*7 and *C*8 of the xanthine scaffold were investigated, allowing the identification of the first selective xanthine-based FAP-α inhibitors (**7**) with low micromolar potency [152]. A series of FAP-α inhibitors based on a *N*-4-quinolinoyl-Gly-(2S)-cyanoPro (**8**) scaffold has demonstrated low nanomolar FAP-α inhibition combined with high selectivity with respect to both the dipeptidyl peptidases and POP. Pharmacokinetic evaluation of selected inhibitors in rats demonstrated high oral bioavailability, plasma half-life and the potential to selective complete FAP-α inhibition *in vivo* [150,153]. From a series of diphenylphosphonates, modest selectivity for FAP-α versus DPP IV could be achieved with the Gly-Pro^{P}(OPh)$_2$ derivative (**9**), but anti-invasive properties were demonstrated only *in vitro* [147].

Antibody-based antagonists and immunotherapeutics. Anti-FAP-α antibodies were shown to very selectively target tumors [162] and were safe [115]. In a preclinical murine xenograft model of human HT-29 colon cancer cells, treatment with anti-FAP-α antisera inhibiting dipeptidyl peptidase activity attenuated tumor growth [163]. The humanized monoclonal anti-FAP-α antibody F19/sibrotuzumab requires the tertiary structure of the protein for binding but does not inhibit FAP-α activity and is not directly cytotoxic. Several phases I and a limited phase II clinical trials have been conducted using F19/sibrotuzumab. The treatment was well tolerated in patients with advanced FAP-α-expressing cancers as it specifically accumulated in tumors and not in normal tissues, but no clinical response was observed [115,162,164]. The expression of the cell surface FAP-α was depleted in CAFs with an anti-FAP-α internalizing antibody, suppressing the invasive and pro-tumorigenic properties of these cells [165], showing that the expression of the protein at the cell surface is necessary for cancer progression and that FAP-α eradication is necessary for blocking the pro-tumorigenic role of CAFs. Anti-FAP-α antibodies conjugated to cytotoxic/cytostatic chemotherapeutics have been also evaluated in clinical trials [117]. The tubulin inhibitor mertansine/DM1/FAP-α-antibody-drug conjugate inhibited tumor growth and induced tumor

regression in a preclinical model [166]. An immunotoxin combining an anti-FAP-α antibody with the *Pseudomonas* exotoxin 38 (PE38), which prevents protein synthesis in cells was developed to specifically deplete CAFs. In experimental breast cancer or established experimental melanoma, treatment decreased tumor burden, likely mediated by modulation of the immune tumor microenvironment. [167,168] The indoleamine-2,3-dioxygenase (IDO, a potent immunoregulatory mediator) inhibitor, 1-methyl-tryptophane was conjugated to a FAP-α fragment (aa245-467) to produce a tumor vaccine and boost the anti-tumor immune response by IDO inhibition following dissociation from the FAP-α fragment [169]. Targeting tumor infiltrating T cells with a bispecific mGITRL-FAP-α-fusion protein could induce tumor rejection while minimizing systemic autoimmune side effects [170]. Targeting tumor stroma with chimeric antigen receptor engineered T-cells (CAR-T cells) expressing FAP-α was also successfully developed for cancer immunotherapy with some efficacy, however, they induced significant cachexia and lethal bone toxicities in murine tumor models [171-173].

Genetic deletion of the protein. The expression of FAP-α was depleted from CAFs surface using siRNA or shRNA technologies [115,117,165,174]. FAP-α knockdown reduced FAP-α expression, inhibited tumor growth, promoted collagen accumulation and suppressed angiogenesis and the invasive properties of these cells, showing that the expression of the protein at the cell surface is necessary for cancer progression and that FAP-α eradication is mandatory for blocking the pro-tumorigenic role of CAFs.

Inhibition/antagonism of FAP-α in fibrogenic and inflammatory diseases. Fibrosis is the final pathway of many progressive chronic diseases [2]. Presently, only very few drugs have been clinically approved as antifibrotic therapeutics. The evaluation of the inhibition/antagonism of FAP-α has been much less studied in fibrogenic and inflammatory diseases than in oncologic diseases. The mediators of fibrosis include inflammatory mediators, cytokines and chemokines, reactive oxygen/nitrogen species, several proteases, the endothelin and angiotensin systems and growth

factors, in particular TGFβ and PDGFs and their cognate receptors, associated to the accumulation of ECM proteins, in particular type I and type III collagens, produced by myofibroblasts. This ultimately destroys the structure of an organ leading to loss of function. Renal fibrosis (glomerulosclerosis and interstitial fibrosis with tubular atrophy) is the final consequence of all chronic kidney diseases and a result of immune and non-immune injuries after kidney transplantation leading to chronic allograft dysfunction [1]. Diabetic nephropathy is associated with increased expression of DPP IV on endothelial and tubular epithelial cells. The dual DPP IV/ FAP-α inhibitor linagliptin has incretin-independent anti-fibrotic effects in diabetic nephropathy by inhibiting interaction of DPP IV with the integrin β1 [94]. FAP-α was shown to be specifically overexpressed by profibrogenic stimuli in myofibroblasts of intestinal strictures in Crohn's disease [175]. Iterestingly, in the fibrotic lung FAP-α increased collagen catabolism and clearance in concert with MMPs, showing protective effects [90].

4. Conclusion

The cell surface serine protease FAP-α participates in ECM degradation and is involved in many cellular processes, including tissue remodeling, fibrosis, wound healing, inflammation and tumor progression. FAP-α exhibits post-proline cleaving exopeptidase and gelatinase/collagenase endopeptidase activities and some biological substrates of FAP-α have been determined. Both active and enzymatically inactive FAP-α proteins have biological functions. FAP-α has been involved in many stages of oncogenesis, from initiation to progression and metastasis and the strong correlation between CAFs and FAP-α expression suggest that it is a relevant target for cancer diagnosis and treatment. Studies aiming at blocking FAP-α functions in cancer have included targeting FAP-α-expressing cells by inhibiting FAP-α enzymatic activity, developing anti-cancer vaccines and genetic deletion of FAP-α. The information obtained indicated that FAP-α expression in cancer-associated stromal cells was likely a more relevant candidate for therapeutic intervention than in the cancer cells themselves. The effects of inhibitors of FAP-α activity or deletion/ablation of the protein in experimental cancer models suggested that the enzymatic activity ***and*** the presence

of the protein in the cancer stroma were critical for FAP-α tumor growth promoting activity. The initial clinical trials using either anti-FAP-α antibodies or not very selective synthetic inhibitors were up to now disappointing, but showed that interfering with FAP-α does not induce side-effects.

5. Expert opinion

FAP-α expression is associated with cancer, wound healing, tissue remodeling and chronic inflammation. Activated subpopulations of (myo)fibroblasts and CAFs specifically express FAP-α, according to poorly understood mechanisms and during very defined stages of fibrogenic processes in the progression of the pathologies, suggesting a complex level of regulation. The mechanisms regulating FAP-α expression are not yet understood and will need to be defined. Thus, it will be necessary to better understand the basic biology of FAP-α not only in pathological situations like cancer and inflammation but also in normal situations such as tissue remodelling in healing to better define the therapeutic options in targeting this protein. It will also be necessary to identify the relevant biological substrates of FAP-α and to understand the mechanisms inducing/repressing FAP-α expression. The expression of FAP-α may be beneficial in some situations, like wound healing or some cancers, but it is generally detrimental in most cancers. FAP-α displays dual prolyl-specific exopeptidase and endopeptidase activities, as well as non-enzymatic functions likely dependent on its association with other cellular proteins. Therefore, for therapeutic purposes it will be of utmost importance to study the best options, either inhibiting the two peptidase activities or only one of them, or abrogating the expression of the protein. A few synthetic inhibitors able to discriminate between FAP-α activity and the other members of the family have now been designed. In the few clinical trials for cancer performed up to now, antagonizing FAP-α seemed to induce positive outcome, but the selectivity of the synthetic inhibitors versus the other members of the family is an unresolved issue since it appears that the different proteins of the family may have opposing pathological functions. Thus, preclinical and clinical trials may now be undertaken to evaluate the effect of FAP-α selectivity, either as monotherapy or combined therapies which need to be defined. FAP-α has been shown to directly interact with other cellular proteins, such protein-

protein interactions may offer new therapeutic opportunities to prevent the accumulation of proliferating fibroblasts and myofibroblasts and the development of disease-associated fibrosis. To date, few investigations on the effects of FAP-α inhibitors on fibroblast functions have been conducted and it is presently not known whether inhibition induces physiological changes on the long term in fibroblast functions. Finally, we believe that using FAP-α-specific prodrugs for delivering cytotoxic agents to FAP-α-expressing cells and tissues might be of greater therapeutic value in the future than the inhibition of the protease activity itself.

Article highlight box

- fibroblast activation protein-α (FAP-α) belongs to the prolyl-specific family of serine proteases,
- FAP-α displays exopeptidase and endopeptidase activities shared by the same active site triad,
- FAP-α is specifically expressed by cells and tissues under stress, but not during homeostasis,
- FAP-α is a therapeutic target for stress-associated fibrogenic disorders,
- for therapeutic purposes both inhibiting FAP-α activity and abrogating FAP-α protein expression seem to be necessary,
- the design and therapeutic evaluation of inhibitors specific for FAP-α versus the other members of the family is mandatory before its true therapeutic value as a target can be ascertained.

Declaration of interest

The authors declare no conflicts of interest and have no other relevant affiliations or financial involvement than those disclosed. No external funding was associated with this study.

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Tables.

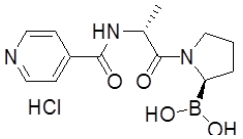
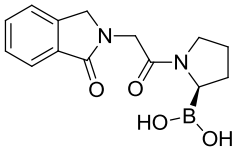
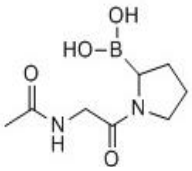
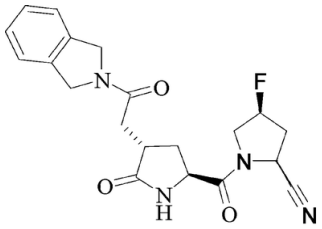
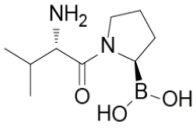
Table 1. *Mutagenesis experiments performed on FAP-α.*

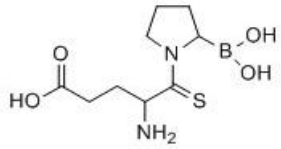
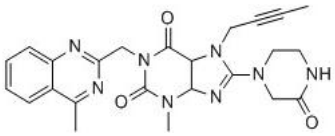
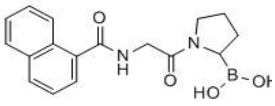
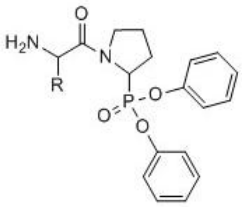
aa	*mutated to*	*consequence*	*references*
R^{123}	A, M, E	dipeptidyl and endopeptidase activities reduced	67
E^{203}	A, D or Q	dipeptidyl and endopeptidase activities reduced	67,68
E^{204}	A, D or Q	when associated, no inhibition of cell adhesion, migration and invasion	
S^{624}	A	dipeptidyl and gelatinase activities reduced, no inhibition of cell adhesion, migration and invasion	16,68
S^{656}	A	dipeptidyl and gelatinase activities reduced, no inhibition of cell adhesion, migration and invasion	67
N^{704}	A	dipeptidyl and endopeptidase activities reduced	67
A^{657}	F or V	dipeptidyl and gelatinase activities reduced	
	D or N	endopeptidase activity inhibited, dipeptidase activity increased	
	Q, S or T	endopeptidase activity inhibited, dipeptidase activityunchanged	67

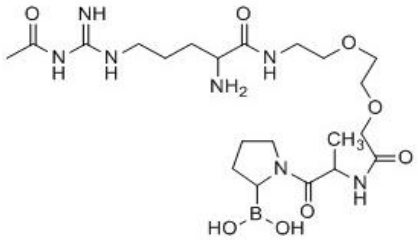
Table 2. *Kinetics parameters of FAP-α on small peptides.*

peptide sequence	***K_M***	***references***
exopeptidase activity		
Gly-Pro-	0.25 mM	16,61
Ala-Pro-	0.24-0.46 mM	67
Lys-Pro-	0,90 mM	16
Ile-Pro-	0.10 mM	61
Phe-Pro	0.24 mM	61
endopeptidase activity		
Ac-Gly-Pro	0.33 mM	61
Thr-Ser-Gly-Pro-Asn-Gln	1.3 µM	67
Ala-Ser-Gly-Pro-Asn-Gln,	2.2 µM	62
Thr-Ala-Gly-Pro-Asn-Gln	0.7 µM	62
Thr-Ser-Gly-Pro-Ser-Gln	1.9 µM	62
Thr-Ser-Gly-Pro-Asn-Ser	2.2 µM	62
Ala-Ser-Gly-Pro-Ser-Ser	4.3 µM	62
Arg-Gly-Thr-Ser-Gly-Pro-Asn-Gln-Glu-Gln-Glu (antiplasmin-cleaved sequence)	29 µM	73

Table 3. *Synthetic inhibitors of FAP-α.*

name	***structure***	***IC_{50}/K_i /selectivity***	***references***
1 ARI3009		selective vs POP	149
2 cyclic amide boronic acid		K_i FAP-α = 7.5 nM K_i POP = 2.8 nM K_i DPP IV = 22'780 nM	69
3 *N*-acetyl-Gly-boroPro		K_i FAP-α 23 nM K_i POP 211nM K_i DPP IV 377 nM	61,62
4 4-carboxy-methyl-pyroglutamic acid diamide		IC_{50} FAP-α 20 nM IC_{50} DPP IV > 50 µM selective vs DPP-II, DPP8, and DPP9	145
5 PT-100 Val-boroPro talabostat		K_i FAP-α 6.2 nM K_i DPP IV 22 nM K_i POP 980 nM	154,155

6 PT-630 Glu-boroPro- thioxamide		K_i FAP-α 5 nM; IC_{50} FAP-α 23 nM K_i DPP IV 3 nM; IC_{50} DPP IV 3 nM	118
7 xanthine-based linagliptin-derived		selective vs DPP IV	152
8 *N*-4-quinolinoyl- Gly-(2S)-cyanoPro		IC_{50} FAP-α 1.8 nM selective (>100) vs DPP IV and POP	150,153
9 Gly-Pro^{P}(OPh)$_2$ *R=Gly*		K_i DPP IV 59.4 nM K_i FAP-α 3.27 µM	147
10 Tyr-Pro^{P}(OPh)$_2$ *R=Tyr* **11** Pro-Pro^{P}(OPh)$_2$ *R=Pro* **12** Val-Pro^{P}(OPh)$_2$ *R=Val*		K_i DPP IV 14.2 µM K_i FAP-α 2.59 µM K_i DPP IV 42.8 µM K_i FAP-α 4.60 µM K_i DPP IV 3.13 µM K_i FAP-α 3.63 µM	

13 *N*-acetyl-Arg-2-(2-(2-aminoethoxy)ethoxy) acetic acid-(D)Ala-(L)boroPro (M83)		dual FAP-α/POP inhibitor K_i FAP-α 5.7 nM K_i POP 7.3 nM	109

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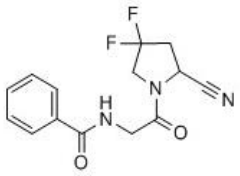
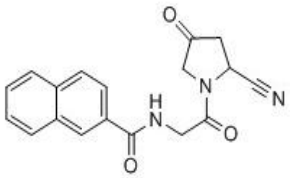
14 *N*-benzoyl-Gly-(2-cyano)pyrrolidine		selective vs DPP IV,DPPII,DPP9 IC_{50} FAP-α 0.85 μM IC_{50} POP > 10 μM	143
15 *N*-naphthoyl-Gly-(2-cyano)pyrrolidine		IC_{50} FAP-α 6.7 μM IC_{50} POP > 100 μM	

Legends for Figures

Figure 1: *Myofibroblast formation*.

Activated proliferating myofibroblasts develop from resting precursors, either resident tissue fibroblasts, or circulating fibrocytes, or following cell (epithelial or endothelial) transdifferentiation, perturbating tissue homeostasis. This process is mediated by the secretion of cell-derived activating factors, including cytokines, proteases, extracellular matrix proteins, and in particular TGFβ. The expression of specific molecules and the secretion of proteases by myofibroblasts drives the development of fibrogenic processes and indirectly influences a multitude of other cell types.

Figure 2. Prolyl-specific peptidases: exoproteolytic (left) and endoproteolytic (right) enzymatic activities, with the preferred amino acid sequences when known.

Figure 3. Structure of the FAP-α protein.

A. Schematic structure of the amino acid sequence of FAP-α. Yellow: cytoplasic motif; green: transmembrane domain; pink: α/β hydrolase domains; grey: β-propeller domain; red arrows: catalytic Ser/Asp/His charge relay triad; blue arrows: substrate binding motif.;

B. Overall 3D structure of the FAP-α dimer. Active site residues Ser^{624}, Asp^{702} and His^{734} are located at the interface of the two monomers in the α/β-hydrolase domain of each monomer and the β-propeller domain; [PDB 1z68].

Figure 4. FAP-α protein expression (brown precipitate) in human normal colon tissue (left) and cancer (right) determined by immunohistochemistry.

Figure 5. FAP-α and POP aminoacid sequences specificities and consequences for developing specific inhibitors, left, exopeptidase activity, and right, endopeptidase activity (adapted from [61,62]).

Figures

Figure 1.

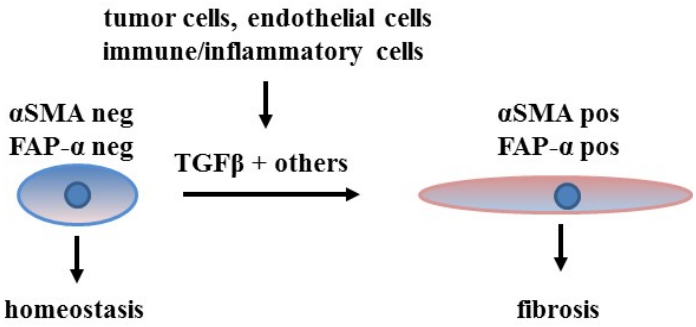


Figure 2.

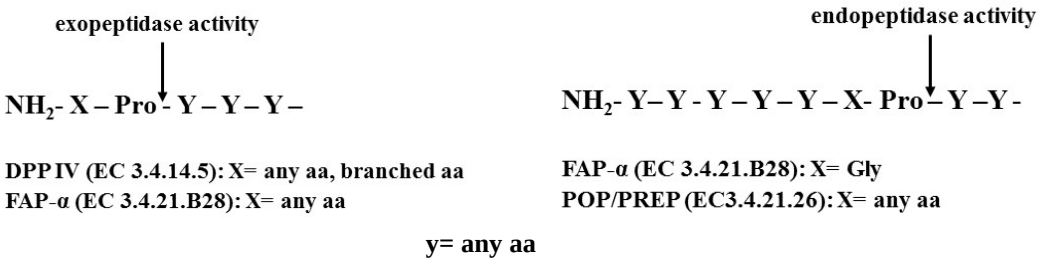
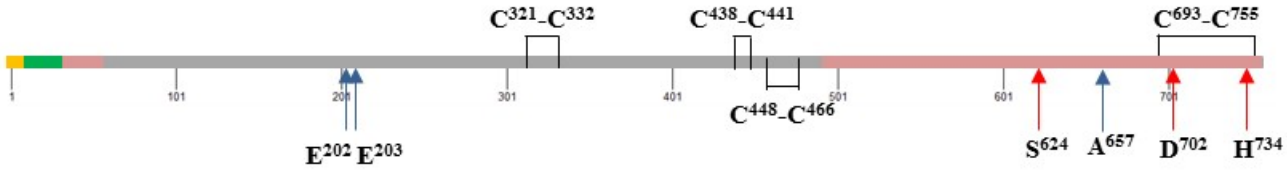

exopeptidase activity
NH2- X – Pro- Y – Y – Y –
DPP IV (EC 3.4.14.5): X= any aa, branched aa
FAP-α (EC 3.4.21.B28): X= any aa
endopeptidase activity
NH2- Y– Y - Y – Y – Y – X- Pro– Y –Y -
FAP-α (EC 3.4.21.B28): X= Gly
POP/PREP (EC3.4.21.26): X= any aa
y= any aa

Figure 3.

A.


C321-C332
C438-C441
C693-C755
1
101
201
301
401
501
601
701
C448-C466
E202 E203
S624
A657
D702
H734

B.

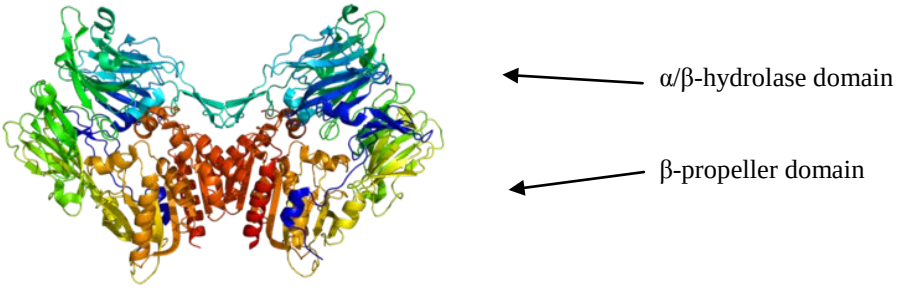

α/β-hydrolase domain
β-propeller domain

Figure 4.

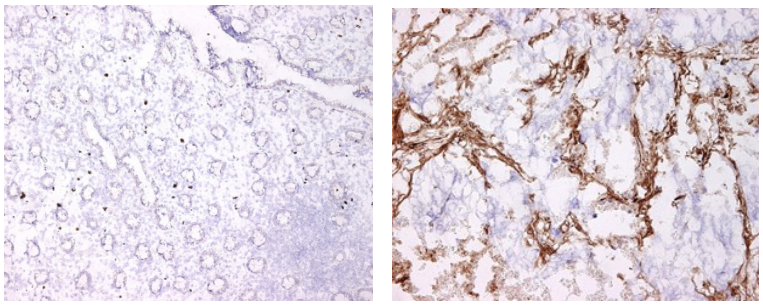
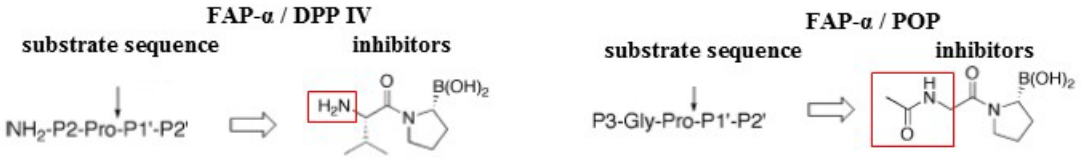


Figure 5.


FAP-α / DPP IV
substrate sequence
inhibitors
NH2-P2-Pro-P1'-P2'
H2N
O
N
B(OH)2
FAP-α / POP
substrate sequence
inhibitors
P3-Gly-Pro-P1'-P2'
H
N
O
O
N
B(OH)2